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Development of an integrated fermentation and formulation approach for the endophytic bacterium *Kosakonia radicincitans* DSM16656^{T} as a novel plant biostimulant

"Desarrollo integrado de los procesos de fermentación y formulación de la bacteria endófita *Kosakonia radicincitans* como biofertilizante de última generación"

Fredy Mauricio Cruz Barrera

Universidad Nacional de Colombia

Facultad de Ingeniería, Ingeniería Química y Ambiental

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Fredy Mauricio Cruz Barrera

Thesis submitted in partial fulfillment of the requirements for the degree of:

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Advisor:

Ph.D. Martha Isabel Gómez Álvarez

Co-advisor (a):

Ph.D., M.Sc, Chemical Engineer Juan Carlos Serrato Bermúdez

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Resumen

Los endófitos bacterianos que promueven el crecimiento de las plantas o PGPBE se consideran el futuro de los bioestimulantes en plantas, con base en su innegable ventaja sobre los microorganismos epífitos que se aplican comúnmente para mejorar la nutrición y protección. Sin embargo, la investigación actual de estos microorganismos asociados a las plantas tiene deficiencias en relación con la influencia de las estrategias de cultivo y de pre-formulación con la subsecuente capacidad de colonización de plantas. Además, todavía no se ha abordado la influencia de las condiciones de cultivo y el estrés osmótico en PGPBE sobre la capacidad de solubilización de fósforo. Esta investigación doctoral tuvo como objetivo mejorar la capacidad de la cepa PGPBE *Kosakonia radicincitans* DSM 16656^T para colonizar plantas, solubilizar fosfato y favorecer la supervivencia al secado durante la formulación. La estrategia incluye el pre-acondicionamiento de las células con osmo-adaptación, acumulación de hidroxiectoina y posterior encapsulación de las células utilizando pectina amidada. Los resultados demostraron que los fenotipos halotolerantes de las bacterias proporcionan una notable influencia positiva en la supervivencia al secado y aumentan aproximadamente en 3 veces la capacidad de colonización de la raíz, lo que respalda los efectos de la promoción del crecimiento en plantas de rábano hasta de 41,1% en la materia fresca. Es así como, el pre-acondicionamiento condujo a un aumento del 23,1% en la capacidad de solubilización de fosfato *in vitro* al afectar positivamente la producción de fosfatasas ácidas. Más allá de la acumulación de hydroxiectoina, este trabajo proporciona evidencia que apoya la noción de que la hydroxiectoina puede producir cambios significativos en el metaboloma endógeno de *K. radicincitans* DSM 16656^T durante la fase de crecimiento exponencial en salinidades altas. Los cambios metabólicos incluyen alteraciones en el ciclo del ácido tricarboxílico, novo-síntesis de metabolitos intracelulares específicos como manitol, mio-inositol y trehalosa, y cambios en los aminoácidos como L-leucina, L-asparagina, L-serina, L-metionina y L-prolina. El significativo cambio de 11,07 veces de L-aspartato sugiere un potencial proteoma ácido en salinidades altas, lo que amplía el conocimiento de las bacterias endófitas bajo estrés salino. Además, las bacterias pre-osmoadaptadas formuladas en capsulas secas de pectina amidada aumentaron en 10 veces la capacidad endofítica y la eficacia como promotor del crecimiento de plantas de rábano bajo condiciones de invernadero. También, los agregados bacterianos formados durante la activación de las capsulas juegan un papel esencial en las etapas tempranas de colonización. Estos hallazgos ubican la respuesta metabólica al estrés salino y la acumulación de hidroxiectoina por *K. radicincitans* DSM 16656^T en un contexto fisiológico, allanando el camino hacia la interacción entre el fenotipo celular asociado con la tolerancia al estrés salino y la capacidad de supervivencia de los endófitos Gram-negativos. Por último, esta investigación proporciona información para comprender el éxito de los endófitos bacterianos pre-acondicionados e inmovilizados, asi como sus mecanismos durante los primeros establecimientos de colonización de plantas, alentando la integración de las estrategias de fermentación y formulación para explotar las bacterias endofíticas.

Palabras clave: Endófitos bacterianos; encapsulamiento, pre-acondicionamiento, solutos compatibles; capsula de pectina amidada.

Abstract

Plant growth-promoting bacterial endophytes or PGPBE are considered the future of plants growth bio-stimulators, based on their undeniable advantageous over epiphytic microorganism commonly applied to enhance host plant nutrition and protection. However, current research of these plant-associated microorganisms has deficiencies concerning cultivation and pre-formulation strategies on further bioactivity as an endophyte. Besides, the repercussion of cultivation conditions and osmotic stress in PGPBE on P-solubilizing ability has not been yet addressed. Through the strategy by pre-conditioning of cells with osmoadaptation, hydroxyectoine accumulation and a further encapsulation of cells in amidated pectin beads, this doctoral research aimed at enhancing the capacity of the PGPBE *Kosakonia radicincitans* DSM16656^T strain to colonize plant seedlings, solubilize phosphate and support the drying survival during formulation processing. Here, it was demonstrated that halotolerant phenotypes of bacteria provide a remarkable positive influence on drying survival and about 3-fold increased the plant endosphere colonization capacity, supporting growth promotion effects in radish plants up to 41.1 % in the fresh matter. Interestingly, the pre-conditioning lead to an increase of 23.3 % in the *in vitro* phosphate solubilization ability by positively affecting acid phosphatases production. Beyond the accumulation of hydroxyectoine, this work provides evidence supporting the notion that hydroxyectoine can produce significant changes in the endogenous metabolome of *K. radicincitans* DSM16656^T during the exponential growth phase at high salinities. Metabolome changes include alterations on tricarboxylic acid cycle, *novo*-synthesis of specific intracellular metabolites such as mannitol, *myo*-inositol and trehalose, and up-regulation on amino acids such as L-leucine, L-asparagine, L-serine, L-methionine and L-proline. The significant 11.07-fold change of L-aspartate suggests a potential acidic proteome at high salinities, extending the knowledge of traits for salt-stressed bacterial endophytes. Likewise, pre-osmoadapted bacteria formulated in amidated hydrogel pectin dried beads, 10-fold increased the ability to multiply endophytically and the efficacy as a plant growth promoter in radish plants. GFP-tagged bacteria approach revealed that bacterial aggregates formed during the re-hydration of beads play an essential role in early colonization stages. Overall, these findings place the metabolic salt stress response and the hydroxyectoine accumulation by *K. radicincitans* DSM16656^T into a physiological context, paving the way into the interaction between the cellular phenotype associated with salt stress tolerance, and drying survival capacity of Gram-negative endophytes. This investigation provides insights for understanding the success of pre-conditioned and immobilized bacterial endophytes, also their mechanisms during early plant colonization establishments, encouraging the integration of fermentation and formulation strategies for exploiting endophytic bacteria.

Keywords: Bacterial endophytes; encapsulation; pre-conditioning; compatible solutes; amidated pectin bead.

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Introduction

Microbial diversity is suggested as an essential aspect for plant and human health. Plant biostimulants offer one way to directly counteract global concerns for ensuring sustainable and eco-friendly food supply. Within the frame of global population increment issues, and environmental concerns associated with chemical-based products in agriculture, the interest in plant biostimulants has arisen. Inside the plant biostimulants, the plant growth-promoting bacterial endophytes (PGPBEs) have played a crucial role in scientific motivation for establishing them as a real alternative.

The stimulation of plant growth and yield improvements by PGPBEs have been reported on laboratory, greenhouse and field level in several plant species (Garima and Nath 2015; Lally *et al.* 2017; Verma *et al*. 2018). A well-studied PGPBE is *Kosakonia radicincitans* DSM16656^{T} (syn. *Enterobacter radicincitans*) (Brady *et al.* 2013b), which endophytic preferences in plant tissue have been demonstrated (Kampfer *et al*. 2005b). This bacteria can stimulate the growth in a range of plant host and nutrient uptake (Scholz-Seidel and Ruppel, 1992; Schilling *et al*. 1998; Ruppel *et al*. 2006; Berger *et al*. 2015). Although these high-adapted bacteria strains pose such remarkable properties, the majority of scientific efforts have used fresh cultivated cells before evidence biological benefits, without a formulation approach and phenomena associated during plant interaction (Berger *et al.* 2015; Garima and Nath, 2015; Berger *et al.* 2017).

In brief, the main restrictions for marketing these bio-stimulants based on endophytic bacteria are the requirements for high technical expertise in the active ingredient mass production, and the formulation-drying issues, for determining the best delivery systems (Ryan *et al.* 2008; Bashan *et al*. 2014). Additionally, it is becoming essential to understand

the relationship of these both bottlenecks with the persistence, chemotactic and plant colonization ability (Lodewyckx *et al.* 2002).

Relative few studies have focused on the culture media design in the world of bacterial endophytes (Nour *et al.* 2012; Youssef *et al.* 2016), and most of them aiming only at the plant-associated bacteria isolation (Eevers *et al.* 2015). In particular, the mass production of *K. radicincitans* DSM16656^T by submerged fermentation and the effects on the culture media and scaled mass production process has not been widely addressed, so there are more questions than answers. Thus, the best possible scenario is to develop a liquid culture medium allowing efficient economic microorganism production, that can provide stable and more extended biological activity prior to formulation-application procedures.

The increasing interest in the bacteria endophytes exploitation towards the inoculants developments requires the cultivation strategies and delivery systems approaches integration. Most of the PGPBEs as *K. radicincitans* DSM16656^T are Gram-negative bacteria, which downstream processing operations produce lethal effects on cell viability. Thus, formulation-drying processes provide challenges that limit the adoption of these endophytes in agriculture. Few studies regarding formulation-drying processes of Gram-negative bacteria endophytes are available in the literature. Despite the lack of information, reviews on bacteria with agriculture interest on biocontrol and biofertilization (Bonaterra *et al*. 2007; Cabrefiga *et al.* 2014), suggest that the osmoadaptation is a potential alternative for protecting cells against drying processing.

Among formulation strategies for Gram-negative PGPBs, the encapsulating cells in biopolymeric materials is emerging as a promising alternative. The immobilization focuses mainly on increasing their tolerance against biotic and abiotic factors, such as antagonist or dryness (Vemmer and Patel, 2013; Krell, Jakobs-Schoenwandt, Persicke *et al.* 2018; Krell, Jakobs-Schoenwandt, Vidal *et al.* 2018). In particular, the encapsulation of Gram-negative PGPBEs has used alginate as a polymeric material (Lally *et al*. 2017; Aziz *et al.* 2018; Grobelak *et al.* 2018). However, the alginate-systems benefits are contradictory, and the study of other natural polymers is required.

Pectin as structural plant-wall polysaccharide component may mimic natural conditions that support endophytic behavior. The pectin-based systems for endophytes have been rarely studied (Krell, Jakobs-Schoenwandt, Vidal *et al.* 2018); nonetheless, the potential exploitation of pectin as PGPBEs carrier remains unexplored. Altogether, encapsulation in pectin-based beads is proposed as a platform for applying bacterial endophytes.

For ensuring drying survival, osmoadaptation and compatible solutes have been used as strategies for protecting bacteria in the food industry (Beales, 2004). However, this approach has not been studied on integrating cultivation-formulation processing in PGPBEs. Besides, pre-conditioning and its influence on endophytic biological signature such as phosphorous solubilization are unexplored. Noteworthy, metabolic networks modifications in endophytic bacteria comprise a black box. So, the metabolic profiling alterations upon osmotic stress and compatible solutes supply are unknown for Gram-negative bacterial endophytes and knowledge regarding the potential benefits of pre-conditioning are lacking. Summarizing, the bacterial endophytes anhydrobiotic engineering and formulation techniques are demanding fields of studies for ensuring the adoption of these beneficial microorganisms (Garcia, 2011; Santoyo *et al.* 2016).

The research work proposes as the central hypothesis that integrating the culture medium design, including pre-conditioning of cells and the formulation by encapsulation of *K. radicincitans* DSM16656^{T}, the endophyte can improve its resistance for drying, maintaining its ability to promote plant growth (Radish as plant model), supporting the endophytic ability and phosphorus solubilization.

Pre-conditioning step includes the use of osmoadaptation and the accumulation of compatible solutes. It is hypothesized, that providing exogenous compatible solutes during the adaptation at high salinities in culture media may synergistically influence the phosphatase enzymes of *K. radicincitans* DSM16656^{T}, enhancing its physiological machinery for phosphate solubilization and subsequent plant colonization activity. Besides, cells drying survival could improve by using this approach. The primary assumption is based

on the pre-conditioning by osmotic stress that may protect bacterial cells by shifting metabolic profiling, providing the accumulation or synthesis of advantageous metabolites. Likewise, through encapsulation in polymeric materials such as amidated pectins, the protection of cells and the endophytic establishment could increase.

Objectives of this research

This doctoral research aimed at extending the knowledge for Gram-negative bacterial endophytes, regarding the pre-conditioning-formulation relationship processes with further plant establishment.

The three main objectives are:

- To identify the parameters in the fermentation and pre-conditioning processes for the endophyte *K. radicincitans* DSM16656^T, which provides the increase of biomass yield and the resistance to the desiccation.
- To develop a formulation based on the endophyte *K. radicincitans* DSM16656^T that protects the active principle from the drying process and maintains the biostimulant capacity as an endophyte.
- To reveal the metabolic profiling response of *K. radicincitans* DSM16656^T to osmotic stress and compatible solutes supply that support drying survival and endophytic performance.

The investigation with *K. radicincitans* DSM16656^T will comprise three main phases: the fermentation, the formulation and the efficacy test under controlled conditions.

Synopsis of the chapters

The document comprises seven chapters. In Chapter 1 is addressing state of the art for bacterial endophytes, and the challenges for their adoption, including the cultivation and delivery systems development. Chapter 2 discusses the assessments carried out to define culture media, phenotypic fingerprint, and to elucidate the sources of the most favorable nutrients for the *K. radicincitans* DSM16656^T cells proliferation. In Chapter 3, it is provided evidence regarding that halotolerant bacterial phenotypes increase the root-colonizing

capability, the phosphate-solubilizing ability, and the endophytic establishment. To circumvent deleterious effects of drying, Chapter 4, focusses on the pre-formulation research aiming at providing desiccation tolerance to the endophyte by an osmoadaptation and compatible solute inclusion procedure. The formulation prototype selection and the efficacy as delivery systems of the endophyte in a radish plant system are discussed in Chapter 5. To understand why the pre-conditioning of cells as an anhydrobiotic engineering strategy provides benefits on drying survival, and colonization capabilities, a metabolomics approach is presented in Chapter 6. Finally, Chapter 7 deals with the conclusions and recommendations that emerge from the research.

The participants in this project comprise competent institutions such as Leibnitz IGZ and Bielefeld Applied Sciences University in Germany, as well as Agrosavia and Universidad Nacional de Colombia, which guarantee a uniquely strong consortium to address the key issues.

1. CHAPTER 1. REVIEW: PLANT GROWTH PROMOTING BACTERIAL ENDOPHYTES, GENERAL BIOTECHNOLOGICAL PROSPECTION

1.1 Increasing the plant performance in agriculture

World agriculture in the 21st century will face three major challenges: how to feed a growing world population, how to contribute to reducing the prevalence of rural poverty, and how to decrease the negatives effects on natural resources. Concerns arise for increasing crop yields, due to limited suitable soils and water resources available for crop production, the cost of chemical fertilizer inputs, and the decline of crop yields globally, climate change, and the arising of environmental protection security. Nutrient efficient plants will play an essential role in solving these issues in modern agriculture (Fageria *et al.* 2008; Webb and Buratini 2016). Human activity is having a notorious global impact on the ecosystems, fuelled by a rising world population that could reach 10 billion by 2056. In this regard, during the last decades, much research has been focused on making further improvements and the development of innovative strategies. Among these approaches, the green farming and bio products-based inoculants address a demanding contribution for increasing eco-friendly agriculture performance.

Plant-associated microorganisms fulfill essential functions for plant growth and health. These microbes improve nutrient acquisition, hormonal stimulation, and plant defense mechanism. Microbial inoculants based on these beneficial microbes, comprise a milestone for enhancing agricultural biotechnology. The annual growth rate by 10-12% market for microbial inoculants, convert this topic into a worldwide trend (Berg, 2009; Calvo *et al.* 2014). Regarding their modes of action, these bio-based products can be biofertilizers, biopesticides, phytostimulators and plant biostimulants.

A plant biostimulant is any substance and/or microorganism whose function upon application to the plants or rhizosphere is to stimulate nutrient efficiency acquisition, tolerance to abiotic stress, and crop quality traits (Calvo *et al.* 2014). In general, the future success of biostimulants requires interdisciplinary research on mass production, formulation, signaling with the plant-environment in combination with education and business management. Altogether, the biostimulant development offers a promising and environmental alternative to cope with the growing concerns in modern agriculture. Hence, new trends have arisen on these plant biostimulants based on exploiting a type of plant-associated microorganism called

endophytes (Ryan *et al.* 2008; Santoyo *et al.* 2016; Khaksar *et al.* 2017; Le Cocq et al. 2017; Mei *et al.* 2017).

Endophytes are bacteria (including actinomycete) or fungal microorganism which spends the whole o part of its life cycle colonizing inter and/or intra-cellular healthy tissue of host plant. They can be isolated from surface-disinfested plant tissue or extracted from inside the plant, commonly causing no apparent symptoms of the disease (Hallmann, Quadt-Hallmann *et al.* 1997; Tan and Zou, 2001; Santoyo *et al*. 2016).

1.2 Bacterial endophytes: General considerations

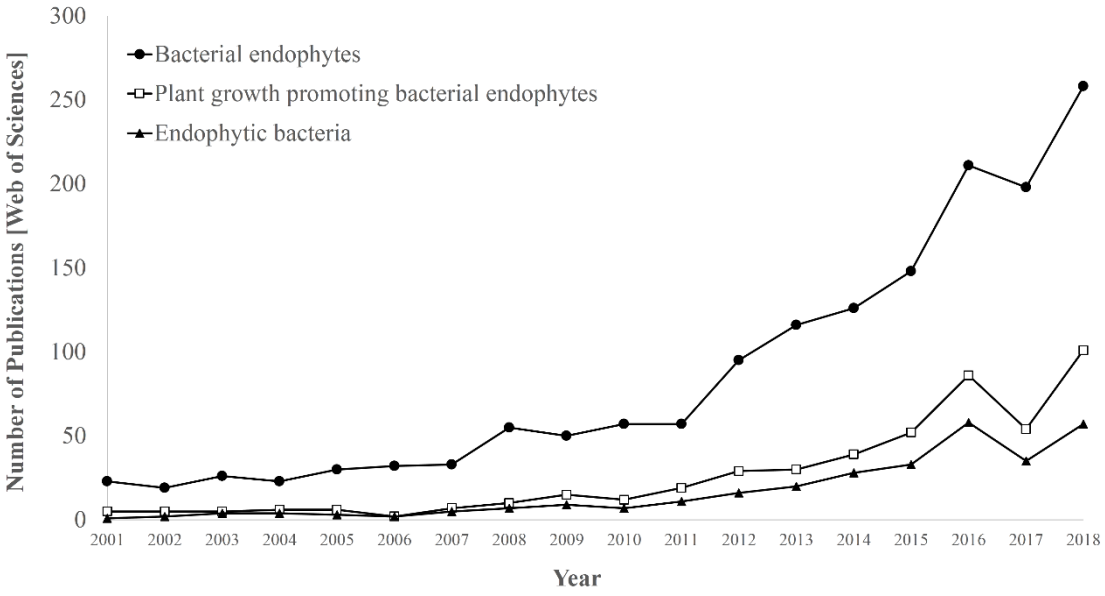
Plant growth-promoting bacteria or PGPBs have been described in the last decades by their favorable effects on plants biostimulation (Compant *et al*. 2010; Santoyo *et al.* 2016). PGPBs can directly facilitate the growth of plants by nitrogen fixation, the production of phytohormones and siderophores, the solubilization of phosphorus or indirectly by preventing the incidence of plant pathogens or by improving stress resistance (Glick and Bashan, 1997).

Within the PGPBs, we can distinguish two large groups, the rhizobacteria which live near to the roots, and the endophytic bacteria, with the ability to colonize plant tissues, and even live as free cells in the soil (Lacava and Azevedo, 2013). Historically, endophytic bacteria have considered being virulent plant pathogens, but during the last two decades, discoveries demonstrated several beneficial effects on host plants. For both types of PGPBs, the mechanisms of growth promotion in plants are practically the same. Nevertheless, the significant difference that endophytic PGPBs present in comparison to rhizobacteria is once they established within the tissues and organs, they do not affect by soil variables (Salt stress, pH, water content and competitiveness with another microorganism, etc.) which on the contrary can influence the rhizobacteria directly.

Altogether, the scientific interest has undoubtedly turned towards the endophytic bacteria, as demonstrated with the increment of publications year by year regarding these topics (Figure 1. 1), related to their physiological characteristics and the symbolic relationship with the

plant, being potential active ingredients for the new generation of plant biostimulants (Santoyo *et al.* 2016).

Figure 1-1: Evolution of the number of scientific publications during the last two decades regarding bacterial endophytes and related keywords [source: Web of Sciences]



Among the plant biostimulants exist the biofertilizers. According to (Vessey, 2003) a biofertilizer is a substance which contains living microorganisms which, when applied to seed, plant surfaces, or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant. Currently, the demand for microbial biofertilizers is increasing worldwide owing to a higher degree of environmental awareness, the increasing number of laws protecting the environment, and the ever-expanding demand for eco-friendly products (Garcia-Fraile *et al.* 2015). The market for biofertilizers is expected to reach 1.88 billion dollars by 2020, with a growth rate of 14%. Nevertheless, the largest share in the market focuses on genera such as *Rhizobium* sp., *Azotobacter* sp. *Azospirillum* sp. and phosphorus-solubilizing bacteria, mainly Gram-positive that can form spores (Garcia-Fraile *et al.* 2015) (Table 1.1). In this way, the gap that exists for Gram-negative usage and endophytic bacteria is considerable, but at the same time promising in the context of adopting biofertilizers.

In particular, the plant growth-promoting bacteria endophytes (PGPBEs) promote the growth of plants through nitrogen fixation (Reis *et al.* 2001; Sevilla *et al.* 2001; Reinhold-Hurek and Hurek, 2011), the solubilization of phosphorus (Verma *et al*. 2001; Wakelin *et al.* 2004), the siderophores production (Verma *et al*. 2011; Rungin *et al*. 2012) and by preventing infections secreting antibacterial and antifungal agents (Rosenblueth and Martinez-Romero, 2006). Besides, by establishing the systemic resistance of the plant and the production of phytohormones such as indole acetic acid and cytokinins (Pirttila *et al.* 2004; Compant *et al*. 2010). Noteworthy, endophytic bacteria can improve the ability of plants to remove environmental pollution (Doty, 2008).

Unfortunately, in many cases the PGPBEs fail to induce the desired effects when applied in the field, due to the competition with other microorganisms in the rhizosphere and subsequent deficiency in the colonization of the plant, this being a critical step to guarantee the beneficial effects (Lugtenberg *et al*. 2001; Santoyo *et al.* 2016).

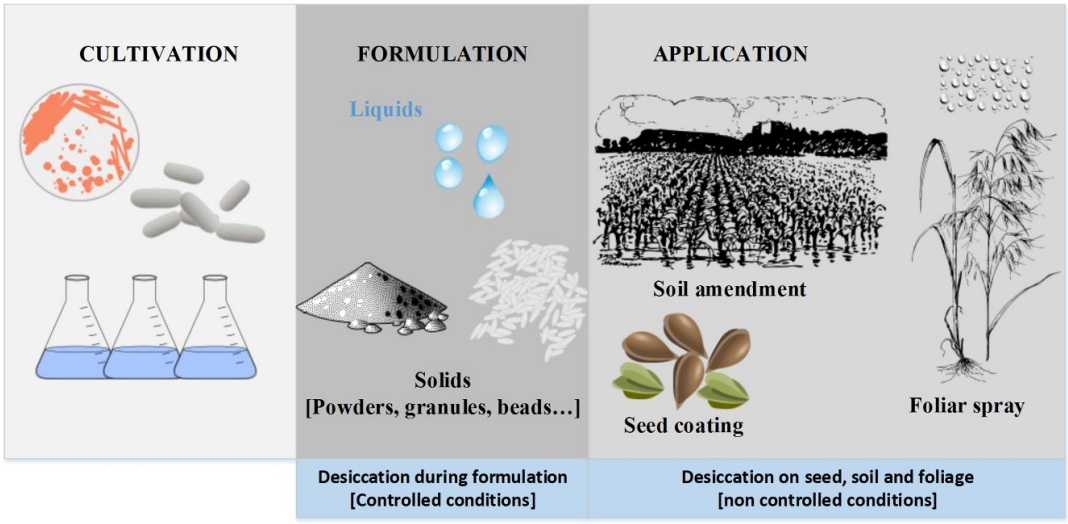
It has been reported that endophytic bacteria are more specialized than rhizobacteria, being more effective in their interaction with the host plant (Ali *et al*. 2014). Unlike endophytic seed bacteria that are already established since germination (Hardoim *et al.* 2012), endophytic PGPBs use different mechanisms to enter endoplant habitat. Mainly through wounds in the roots, lateral fractures or intact epidermis, caused naturally by the growth of the plant (Sprent and de Faria, 1988; Santoyo *et al*. 2016). Endophytes appear to actively penetrate plant tissues using hydrolytic enzymes like cellulase and pectinase (Hallmann, Quadt-Hallmann *et al.* 1997). Another important mechanism is facilitating by root tissue fractures, along with the drainage of metabolites, causing active sites of attraction for the rhizobacteria (Hallmann, Quadt-Hallmann *et al.* 1997; Kandel *et al*. 2017). Other entry sites for endophytic PGPBs include damaged stomata particularly in the leaves or young stems (James *et al.* 2001), lenticels present in the periderm of stems and roots (Ruiza *et al*. 2011), as well as during the radicles germination (Adams and Kloepper, 2002).

Table 1-1: Examples of commercially available biofertilizer products based on plant growth-promoting bacteria-PGPBs (Garcia-Fraile *et al*. 2015)

Product	Company	Bacterial strain
Cell-Tech®	Novozymes	Rhizobia
Nitragin Gold®	Novozymes	Rhizobia
TagTeam®	Novozymes	Rhizobia + *Penicillium bilaii*
Nodulator®	BASF Canada Inc.	*Bradyrhizobium japonicum*
Vitasoil®	Symborg	PGPB consortia
Biomix®	Biomax	PGPB consortia
Nitrofix®	Labiofam S.A	*Azospirillum* sp
Azotobacterin®	JSC	*Azospirillum brasilense B-4485*
EVL coating®	EVL Inc.	PGPB consortia
Life®	Biomax	PGPB consortia
Biozink®	Biomax	PGPB consortia

In general, the main restrictions for the commercialization of biofertilizers based on endophytic bacteria worldwide are related to the requirements of the technical experience in the mass production of the active principle, its efficient formulation and the maintenance of biological activity, with a view to developing the best delivery system (Lacava and Azevedo, 2013; Santoyo *et al*. 2016). Typical liquid and solid formulation for PGPBEs are applied using technics such foliar spray, seed coatings, slurries or soil amendment (Figure 1.2) (Bashan *et al*. 2014; Santoyo *et al.* 2016). Nevertheless, it is increasingly important to determine the influence of biotic and abiotic factors on the mass production of endophytes, including desiccation stresses during formulation and further application (Figure 1.2). Besides, it is essential the understanding of their relationship with chemotaxis and plant colonization (Kandel *et al*. 2017).

Figure 1-2: Schematic representation of the main processes involved during the mass production, formulation and application of biofertilizers based on PGPBs or PGPBEs.



1.3 Bacterial endophytes as plant growth promoters

Estimations suggest that the planet contains about 300,000 species of plants, which the vast majority contain a hyperdiverse of endophytes (Smith *et al*. 2008). Among these plant-associated microorganisms, bacterial endophytes can promote plant-growth by either direct or indirect mechanisms. Direct influence on plant development occurs when a bacterium either facilitates the uptake of essential nutrients or modulates the hormones within the plant. Essential nutrients include nitrogen, phosphorus and iron. In the case of hormones, PGPBE can synthesize phytohormones such as auxin, cytokinin and gibberellin. Some endophytic bacteria can modulate ethylene levels by synthesizing the enzyme, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, that cleaves the compound ACC, the precursor of ethylene in higher plants (Bacon and White, 2016; Santoyo *et al.* 2016).

Indirect mechanism of plant growth promotion takes place when a PGPBE decreases the deleterious effects on plants following infection with a phytopathogen. This commonly occurs by the antagonistic abilities that some PGPBEs have, including the enzymes production, secretion of antibiotics, bioactive substances and the induction of systemic resistance in plants, inhibiting the pathogen (Sessitsch *et al*. 2004; Brader *et al*. 2014). Table

1.2 summarizes a selection of studies related to bacterial endophytes (PGPBE) and their influence on the performance of different crops.

Table 1-2*:* Selection of **s**tudies regarding the efficacy of plant growth-promoting bacterial endophytes (PGPBE)

PGPBE	PGP mechanisms	Crop	Reference
Enterobacter sp. strain 638	Carbon source utilization	Poplar trees	(Taghavi *et al*. 2009)
K. pneumonia 342	Nitrogen-fixing	Maize	(Riggs *et al*. 2001)
Pantoea agglomerans	P-solubilizing	Peanut	(Taurian *et al*. 2010)
Pseudomonas sp	Organic acid releasing	Peas	(Oteino *et al.* 2015)
B. subtilis NA-108	Indole-3-Acetic acid (IAA) producing	Strawberry	(Pereira *et al*. 2012)
Pseudomonas sp	Indole-3-Acetic acid (IAA) producing	Sugarcane	(Pirhadi *et al.* 2018)
B. subtilis DS-178	Siderophore production-Zinc solubilisation	Wheat	(Singh *et al*. 2018)
Ensifer sp. CA-14	Nitrogen-fixing	Leguminous Ormosia macrocalyx	(Hernandez-Hernandez *et al*. 2018)
Burkholderia sp.	Indole-3-Acetic acid (IAA) producing	Blueberry	(Ortiz-Galeana *et al*. 2018)
Bacillus sp.	Cellular redox balance, Proteins expression	Apple	(Tamosiune *et al.* 2018)
Serratia grimesii BXF1	ACC deaminase	Bean	(Tavares *et al.* 2018)
Streptomyces spp.	Induction of plant defense	Sorghum, wheat	(Patel *et al.* 2018)
Bacillus thuringiensis NUU3	Increment of proline contents	Chickpea	(Egamberdieva *et al*. 2017)

1.3.1 *Kosakonia radicincitans* DSM16656^T

The recently named bacterium *Kosakonia radicincitans* DSM16656^T (Brady *et al.* 2013a), formerly *Pantoea agglomerans,* is an endophytic bacterium belonging to the *Enterobacteriaceae* family. *Kosakonia* in honor of the taxonomist Y. Kosako and the epithet *radicincitans* for *radix–icis* roots and *incitants* means plant growth-promotion (Brady *et al.* 2013a).

Up to date, four genomes of *K. radicincitans* strains have been completed and published: *K. radicincitans* DSM16656^T from wheat [GCA_000280495.2] (Witzel *et al.* 2012); *K. radicincitans* GXGL-4^a from maize [GCA_001887675.1]; *K. radicincitans* UMEnt01/12 from banana [GCA_000691205.1] (Suhaimi *et al.* 2014) and *K. radicincitans* YD4 from Yerba mate [GCA_000877295.1] (Bergottini *et al.* 2015). The scrutiny of these genomes has clarified the results found in inoculated crops upon their utilization. Hence, *K. radicincitans* have genes involving on nitrogen-fixing encoding for two different nitrogenases (Ekandjo *et al.* 2018), the organic phosphate mineralization, siderophore production, auxin efflux carrier and dissimilation of nitrite and nitrate (Witzel *et al.* 2012; Bergottini *et al.* 2015).

K. radicincitans DSM16656^T strain is a facultative isolate that presents motility with short spherical rods, with a length of 0.8-1.2 µm and width of 1.0-1.6 µm (Kampfer *et al.* 2005a). On one hand, this Gram-negative bacterium can increase the growth and yield of different crops such as wheat (*Triticum aestivum*), maize (*Zea mays*), tomato (*Solanum lycopersicum*) and radish (*Raphanus sativus*), among others (Remus *et al.* 2000; Krey *et al.* 2011; Berger *et al.* 2015; Berger *et al.* 2018). On the other hand, it can colonize phyllospheres and rhizospheres, and survive in plants internal tissues (Remus *et al.* 2000). *K. radicincitans* DSM16656^T can fix nitrogen (Ruppel and Merbach, 1995; Ekandjo *et al.* 2018), solubilize tricalcium phosphate (Schilling *et al.* 1998), produce phytohormones such as auxins, indole-acetic acid (IAA), and cytokinins such as N6-isopentyladenosine and N6-isopentyladenine (Scholz-Seidel and Ruppel, 1992). This endophytic bacterium can mediate anti-herbivore defense (Brock *et al.* 2018) and glucosinolate profile in *Arabidopsis thaliana* (Brock *et al.* 2013), alters the plant secondary metabolite composition in cruciferous vegetables (Schreiner *et al.* 2009) and even product quality in tomato (Berger *et al.* 2017). These features provide

to this bacterium endophyte a remarkable bioprospection for applying it as an active ingredient in biofertilizers or bio-stimulators in crop production systems.

The Leibnitz Institute for ornamental crops (IGZ) in Germany has extensive studies with the endophytic strain *K. radicincitans* DSM16656^T. However, as with other endophytes, their results are neither repetitive nor reproducible when the microorganism is cultivated (Santoyo *et al*. 2016). In addition, despite the potential use of *K. radicincitans* DSM16656^T as a commercial bio-stimulator, this Gram-negative bacterium presents a low resistance for drying and their benefits have only demonstrated mainly with fresh cultivated cells (Berger *et al.* 2018). Hence, aiming at developing a formulation based on this promising bacterium, it is necessary to overcome aspects related to the formulation-drying process and the stability during storage, which are highly demanding in order to maintain the viability and the biological benefits of the bacteria.

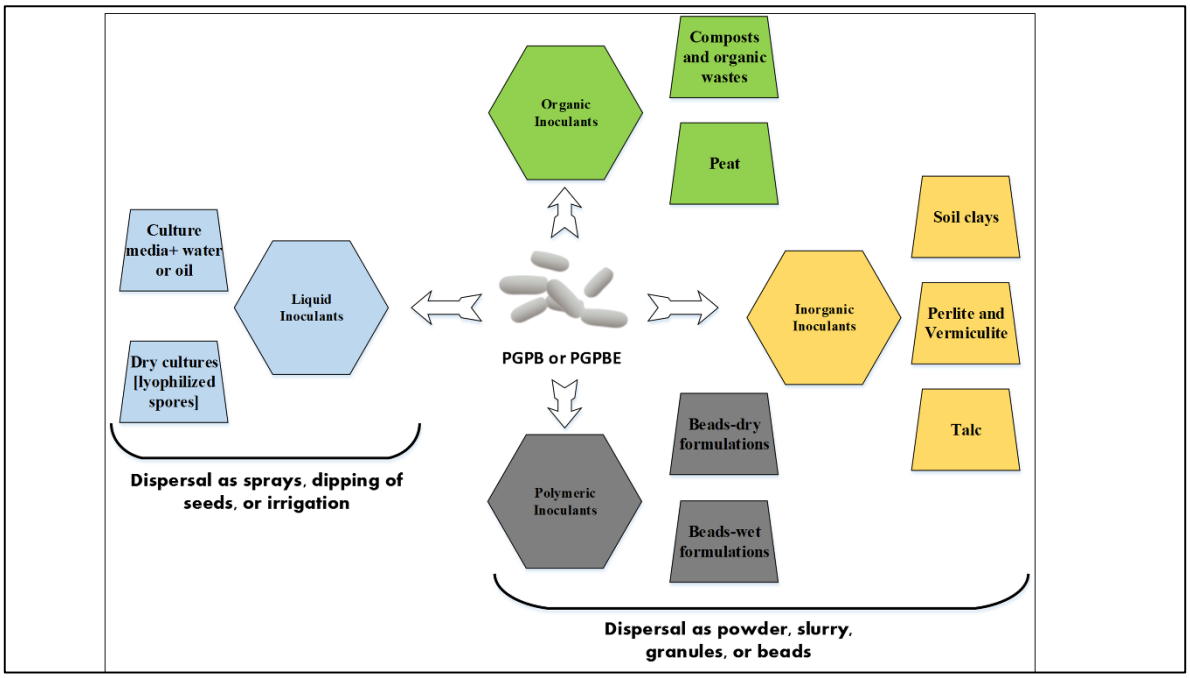
1.4 The role of formulation in the utilization of endophytic bacteria

It is frequent that outcomes obtained at laboratory or glasshouse conditions are not easily transferred to the field, particularly when dealing with Gram-negative, non-sporulating bacterial endophytes. Lacking spores-forming ability, they are more susceptible to deleterious factors occurring during processing and field application (Santoyo *et al.* 2016; Mei *et al.* 2017). Therefore, endophytic Gram-negative bacteria require convenient, protective formulations to support their efficiency at the target site and to assist the practical use by farmers (Bashan *et al.* 2014; Berninger *et al*. 2018).

Effects caused by downstream processes, such as formulation and drying, are among the most damaging factors for the viability of endophytic PGPBs. Drying is used for granulates and powders, and is necessary prior to the storage of the final product. In general, the formulates based on endophytes, including PGPBs, seeks to increase the establishment of microorganisms in the soil and within the plant, trying to increase their beneficial effects by favouring the penetration and colonization of the host plant tissue, reducing costs through a decrease of the doses and the application frequencies (Lacava and Azevedo, 2013; Kandel *et al*. 2017). Historically, biofertilizers distinguish five types of formulation such as the use of

carriers (e.g., peat, talc, activated carbon), granulates, liquid formulations, dry inoculants (e.g., soil, powders) and cell immobilization (Herrmann and Lesueur, 2013; Mahanty *et al.* 2017). Figure 1.3 is summing up the type of formulations and delivery strategies for bacterial inoculants in agriculture.

Figure 1-3: Overview of application techniques for bacterial inoculants in agriculture



Source: Adapted from Berninger *et al.* 2018

The application techniques for the inoculants are closely related to the culture and formulation strategy. The understanding of the relationships centers the efforts in this field into the endophyte-plant-rhizosphere triangle, to generate the best formulation strategy and mode of delivery for the endophyte. A large number of parameters that can vary in the formulation process and that can also significantly influence their viability and biological activity makes it difficult to identify the most critical factors. Nevertheless, essential parameters that intervene in the formulation process are considered: the growing conditions, the composition of the immobilization matrix and the drying process (Herrmann and Lesueur, 2013; Lacava and Azevedo, 2013; Mahanty *et al.* 2017).

Generally, the formulation and drying processes generate deleterious effects on microorganisms, which include stress due to sudden changes in temperature, effects caused by cutting stress, oxidative stress due to contact with air, osmotic pressure, decrease in intracellular pH, capillarity forces and intracellular solute concentration triggered by water loss (Fu and Chen, 2011). Interestingly, along with the increment of publications related to endophytic bacteria, the scientific concern on how to formulate these microorganisms has increased during the last two decades (Figure 1.4). This challenging area is attracting widespread interest; nevertheless, the number of publications is still low (Table 1.3), and the majority of these studies have been maintained under industrial secret. Hence, the formulated-based on bacterial endophytes is considered a relatively unexplored issue.

In the field of the formulation of biofertilizers, several knowledge gaps continue limiting their positioning as a sustainable alternative to chemical fertilizers. According to Herrmann & Lesueur (2013), with a revision since 1980, less than 1% of research articles on rhizobacteria and PGPBs are related to formulation aspects of inoculants. On the other hand, compared to the classic PGPBs, fewer results are found for technical studies of formulation and drying for endophytic bacteria (PGPBEs) as an active ingredient, this using keywords such as "endophytes", "Gram Negative bacteria", "drying", " formulation "and" PGPB "in robust databases such as Web of Science.

Figure 1-4: Evolution of the number of scientific publications during the last two decades regarding the formulation of bacterial endophytes [source: Web of Sciences]

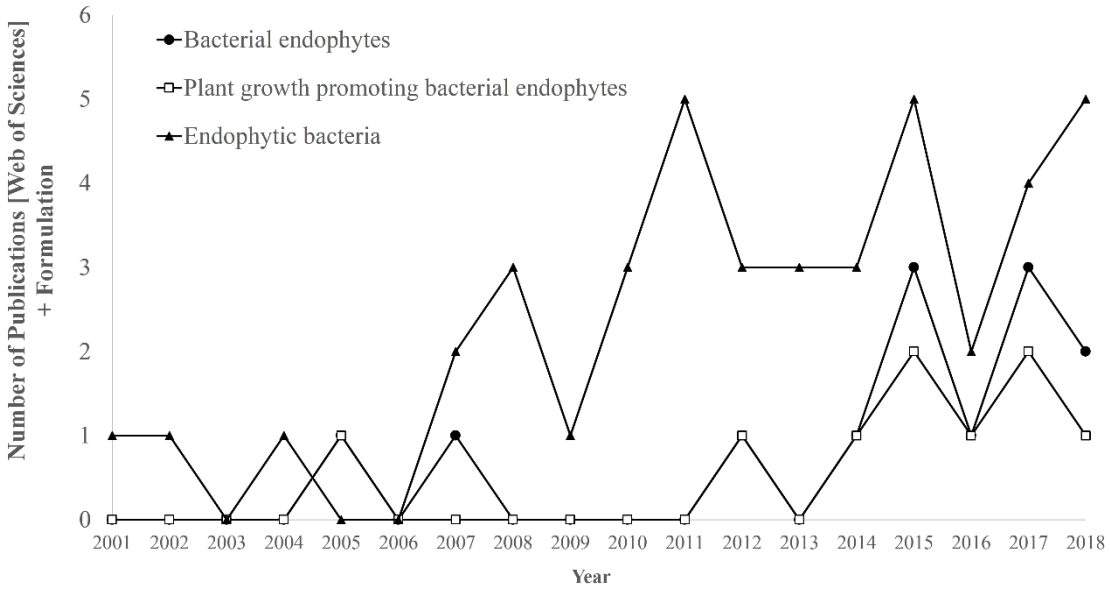


Table 1-3: Selection of **s**tudies regarding the formulation of plant growth-promoting bacterial endophytes

PGPBE	PGP mechanisms	Crop	Type of formulation	Reference
Bacillus pumilus	Nitrogen fixation, P-solubilizing	Potato	Pellet (granule)	(Ortuno *et al.* 2018)
Bacillus sp**.**	Induction systemic resistance	Cotton	Talc-based	(Rajendran *et al*. 2007)
Paraburkholderia phytofirmans PsJN	Nitrogen fixation, P-solubilizing	Maize	(Spray) Talc-based + xanthan + Geloil	(Bejarano *et al*. 2017)
Bacillus cereus CE3	Induction systemic resistance	Chestnut	Wetting powder Diatomite + sodium lignin sulfonate	(Cheng *et al.* 2015)

Bacillus subtilis Tu-100	Suppression of pathogens	Oilseed rape	Pellet	(Hu *et al.* 2011)
Herbaspirillum seropedicae HRC54	Nitrogen fixation	Sugarcane	Foliar spray+ humic acid	(da Silva *et al.* 2017)
Kosakonia radicincitans	Nitrogen fixation, P-solubilizing	Maize	Spray formulation	(Berger *et al.* 2018)
Bacillus spp.	Induction systemic resistance	Banana	Talc-$CaCO_3$-CMC (drench application)	(Harish *et al.* 2008)
Burkholderia sp. UPMB3	-	Storage study	Dry Vermiculite-based	(Bazilah *et al.* 2011)
Paraburkholderia phytofirmans PJN	-	Storage study	Zeolite-based granular	(Berninger *et al.* 2017)

1.4.1 Polymeric inoculants

The advancement reached in formulation development has led to new strategies, including the entrapment and immobilization of microorganisms that seem particularly promising (John *et al.* 2011). This approach involves the attachment or the entrapment of cells into a matrix. Techniques for immobilization included adsorption on surfaces, flocculation, covalent bonding materials, cross-linking, and encapsulation with polymers (Cassidy *et al*. 1996). Among these approaches, encapsulation is considered the most advantageous technique for elaborating carriers for microorganisms (Herrmann and Lesueur, 2013). Thus, encapsulated-living cells are protected in a nutritive shell against mechanical and stress from the surroundings such as temperature, pH, organic solvents, predators, and others (John *et al*. 2011; Bashan *et al.* 2014). Once capsules are located into the soil, soil microbiome actively degrade them, facilitating that the encapsulated cells be gradually released towards the soil, usually at rates according to seed germination times or the emergence of seedlings (Bashan *et al.* 2002; Bashan *et al.* 2014). A wide variety of biological cores are encapsulated-

compatible, including bacteria (spore-forming and non-sporulating), fungal spores, hyphal segments, yeast cells, mycorrhizas, and plant extracts among others (Malusa *et al*. 2012).

Different techniques are used to encapsulate bacterial cells, including physical processes such as spray drying, extrusion or fluidized drying; chemical processes such as co-crystallization, molecular inclusion or interfacial polymerization. Finally, physicochemical processes, such as coacervation, liposomes and gelation/inverse gelation (Schoebitz, Lopez *et al.* 2013). Table 1.4 summarises the main advantages and disadvantages of these methods.

Table 1-4: Comparison of physical-chemical encapsulation techniques for microbial inoculants

	Spray drying	**Fluidized bed**	**Extrusion**	**Ionic gelation**	**Inverse gelation**	**Oil-entrapped emulsion**	**Liposomes**
Core	Beads	Capsules	Either	Beads	Capsules	Beads	Capsules
≤ 500 µm	Yes	No	No	No	No	No	Yes
Water Soluble	Yes	Yes	Yes	No	No	No	No
Uniform	No	Yes	Yes	Yes	Yes	Yes	Yes
Time for process	10-30 s	10 min-1 h	10 min-1 h	1-2 h	1-2 h	1-2 h	Minutes
Cost/ Advantage	Very low/ fast	Low/flexible	Medium/ biocompatible	Medium/ biocompatible	Medium/ biocompatible	Medium/ biocompatible	Very high/ biocompatible
Disadvantages	Dust and high Temp.	Coating fine particles	Diffusion and release	Few wall materials/ process control	Few wall materials/ process control	Few wall materials/ process control	Few wall materials, very thin walls/process control
Useful for	Thermal resistance cells	Irregular particles and uniform walls	Enzymes and vitamins	Cells, enzymes, suspensions	Cells, enzymes and suspensions	Cells, enzymes and suspensions	Capsules ≤ 2 µm

Source: (Schoebitz, Lopez *et al.* 2013)

The polymers that are suitable for encapsulation include natural-origin (polysaccharides, protein materials), the synthetics (polyacrylamide, polyurethane) and homo-, hetero-, or co-polymers (Herrmann and Lesueur, 2013). Alginate is the most commonly used polymers for encapsulating cells and prepared inoculants (Table 1.5). Alginate is a natural, biodegradable and nontoxic polymer which forms a 3D porous gel when mixed with multivalent cations (Ca^{2+})(Bashan *et al.* 2002). Capsules or beads containing the microorganism are formed through the dispersion of cells suspension into the polymer matrix and then simply dropping it in the cationic solution. During the cross-linking reaction and encapsulation procedure, nutrients, fillers and additives can be included, to either extend shelf life, improve functioning or inoculation efficacy (Malusa *et al.* 2012). Afterward, capsules are dried to ensure easy handling and further packaging. To dry these capsules, different technologies have been applied, such as spray drying, extrusion, emulsion, coacervation, solvent extraction/evaporation and thermal gelation (Vemmer and Patel, 2013).

Beyond the cell protection provided by the encapsulating material, many advantages of entrapping cells have been described over free cells, under different conditions, especially storage and field applications. Entrapment processes are mainly not stressful to cells, are made with biodegradable and nontoxic carriers, and are carried out under aseptic environments minimizing contamination (Cassidy *et al.* 1996). Regarding that bead are highly concentrated, their volume is advantageous, limiting space for storage, transportation and handling (John *et al.* 2011). The capsules or beads in a range of 10-100 μm (microencapsulation) offer direct contact with seeds, while macroencapsulation (larger size, millimeters to centimeters) requires the released cells to move through the soil toward the plants (John *et al.* 2011). Several studies have demonstrated the benefits of cells entrapment cells in terms of shelf life and supporting biological activity even at room temperature.

In comparison to free cells, encapsulation of PGPB cells can enhance P-solubilizing efficiency (Young *et al.* 2006; Minaxi, 2011), activity of ACC deaminase (Reed and Glick, 2005), rhizosphere colonization (Trivedi and Pandey, 2008) and indole-3-acetic acid (IAA) production (Meza *et al.* 2015) among others.

Although the encapsulation as formulation strategy has shown a high prospection, most of the reported investigations were at laboratory level, and few commercial bacterial products are available in the market. The high production cost and advanced technical handling needed, appeared to be the explanation of the low adoption. Hence, new approaches have to remain affordable, compatible with current application technologies providing flexibility to farmers (Herrmann and Lesueur, 2013). In particular, for bacterial endophytes, the challenges are higher, since there are not existing commercial products based on these microorganisms. Thereby, research efforts have to address these issues supporting the replacement of chemical fertilizers and the positioning of biological inoculants.

Table 1-5: A sample of polymeric inoculants based on plant growth-promoting bacteria to increase plant performance

Formulation	Additives or treatment	Bacteria used	Plant species or substrate	Reference
Alginate	None	*Azospirillum brasilense*	Tomato	(Bashan *et al.* 2002)
Alginate	None	*Pseudomonas asplenii*	Canola	(Reed and Glick, 2005)
Alginate	None	*Azospirillum brasilense*	Chlorella vulgaris	(de-Bashan *et al.* 2008)
Alginate	None	*Methylobacterium oryzae/ Azospirillum brasilense*	Tomato	(Joe *et al.* 2014)
Alginate	Kaolin, starch, talc	*Streptomyces* sp.	Tomato	(Sabaratnam and Traquair, 2002)
Alginate	Potato starch	*P. fluorescens/Serratia* sp.	Wheat	(Schoebitz, Ceballos *et al*. 2013)
Chitosan	None	Several PGPBs	Cucumber	(Murphy *et al*. 2003)

CMC/corn starch	MgO/ZnO	*Rhizobia*	Cowpea, sugarcane	(Fernandes *et al.* 2009)
Alginate	Humic acid	*Bacillus subtilis*	Lettuce	(Young *et al.* 2006)

Due to the growth of scientific interest in the development of formulations for endophytes and taking into account state of the art in this issue for biofertilizers (Herrmann and Lesueur, 2013), the formulation of *K. radicincitans* DSM16656^T by encapsulation is proposed for application in soil. This formulation alternative is potentially the most beneficial and with great applicability for PGPR endophytes, that is how capsules would improve the planting of the formulation and its inclusion into both organic and mechanized agriculture.

1.5 Prospectings and challenges of bacterial endophytes

At the global level, there are considerable challenges for the implementation of endophytes as new alternatives for PGPBs in applied biotechnology to agriculture (Ryan *et al.* 2008; Kandel *et al.* 2017). This fact explains how the number of publications on endophytes has increased dramatically in recent years; however, not many publications focus on the techniques of industrial application for them (Brader *et al.* 2014; Santoyo *et al.* 2016). Accordingly, scientific efforts have arisen in order to solve the problems of endophytes in biotechnology and agriculture. For instance the European cooperation project BestPass: Boosting plant-endophyte stability, compatibility and performance across scales, into the 'Marie Skłodowska-Curie Innovative Training Network ', 2015-2020, which aims the environmental and economic exploitation of this type of microorganisms, through the development of new endophytic inoculants (bacteria and fungi) and the biotechnological processes involved. Thus, for the development of endophytic PGPBs bioproducts, is not only essential to select promising microorganisms responsible for the promotion of plant growth, but also integrate solutions from a technological point of view, to design processes that include the solution of problems related to the loss of viability, maintaining or increasing their biological activity and improving storage stability, among others. Nevertheless, the current progress in technology-related microbial science, plant-pathogen interactions,

genomics, and genetic engineering, would also support the optimization of protocols for using biofertilizers. Although the utilization of biofertilizer is blooming with great acceleration, still, the technology is nascent but evolving (Mahanty *et al*. 2017).

Issues related to biofertilizers adoption included the scaling up of the process from laboratory to the field, which includes massive production of strain, formulation, storage, and shipping. Moreover, it is necessary to study the persistence of biofertilizers in soil environment under stressful conditions. Another aspect to take into account is the education of farmers about the long-term benefit of using biofertilizers instead of chemicals. In addition, the misconception that only acts as precursors of diseases (Glick, 2012; Mahanty *et al.* 2017). Notably, scientists have to prove to the public and regulatory authorities that both non-transformed bacterial strains and genetically engineered strains do no present hazard or risk to humans. Thus, laws and regulation should be strict enough to accomplish this statement.

Undoubtedly, scientific interest in endophytes and their wide variety of applications has been growing in recent years (Glick, 2012; Santoyo *et al.* 2016; Kandel *et al.* 2017). Many PGPB microorganisms have been isolated and marketed as promising biofertilizers; however, its effects on crop yields have fluctuated from one crop to another, from one place to another and from one season to another. This issue is mainly caused by the survival rate and the stability of the biological activity of the inoculum introduced in the seeds, in the roots and/or in the soil (Khalid *et al.* 2004). In many cases, PGPBs do not induce the desired effects when applied in the field in massive scales, due to the insufficient colonization of the rhizosphere and/or plant tissue, being the most important aspects required for the expression of the beneficial effects (Lugtenberg *et al.* 2001).

1.5.1 Mass production of bacterial endophytes

As mentioned before, the biological activity of endophytic PGPBs is affected by their massive production (submerged fermentation) and the subsequent formulation process (drying) (Santoyo *et al.* 2016). The biological activity includes the behavior of chemotaxis (motility), the production of phytohormones, and mainly the ability to improve the intake of nutrients by the plant, either nitrogen or phosphorus. Considering the production aspects, it is essential to close the gap between the production methods of an active ingredient

(endogenous PGPB) and its sustainable application as an agent of growth promotion in plants. Besides, deepen the understanding of the relationship between formulation strategy, its components, biological activity, and stability of the active ingredient.

Regarding the mass production of endophytes, it has been an everlasting concern. Generally, artificial culture media are often unable to mimic the biotic and abiotic endogenous conditions required for the growth of this type of microorganisms, which cannot adapt immediately to these changes in conditions (Pham and Kim, 2012). However, during the last 25 years, advances in cultivation techniques have been achieved, especially for endophytic fungi (Lohse *et al.* 2015), including modified culture media are also included (Alain and Querellou, 2009), using changes in growth conditions (Janssen *et al.* 2002) and co-culture and dilution of media (Kamagata and Tamaki, 2005; Vartoukian *et al*. 2010). Recently, another approach includes simulated natural environments using root extracts as culture media (Nour *et al.* 2012; Youssef *et al*. 2016). These studies have contributed significantly to the understanding of the production of endophytic microorganisms. Recent studies have indicated that there is considerable variation in the biological activity of endophytic bacteria with the culture medium composition (Yi *et al.* 2015).

Culture media of plant origin have become a sustainable alternative for the cultivation of endophytic PGPBs, with times of duplication comparable to those achieved with chemically defined media (Nour *et al.* 2012). Nevertheless, the composition of this type of extracts is very heterogeneous, since it changes according to the season and the way of obtaining it; in this way, further research is recommended to extend the answer to why culture media based on plant tissue extracts increase the level of rhizobacteria propagation for industrial applications (Youssef *et al.* 2016). Finally, it is important to point out that *K. radicincitans* DSM16656^{T} has been commonly produced for fresh applications using the standard nutrient solution I (Merck, Cat No: 1.07882, Darmstadt, Germany) (Berger *et al.* 2013; Berger *et al.* 2015), which imply a broad and challenging field of research for the design of the culture broth.

Notably, the mass production of the endophytic bacterium *K. radicincitans* DSM16656^T has been done by submerged fermentation, using relative expensive culture media (Berger *et al.* 2013). Nevertheless, the best scenario for its scaling has as a starting point the development of a culture medium for its mass production, which allows the efficient production of the bacterium, maintaining or improving its biological activity during downstream operations such as formulation.

1.5.2 Drying as the main issue during the formulation step

During the process of formulating microorganisms as active ingredients of biofertilizers, the damages caused by heat transfer phenomena and by dehydration into the cells are two critical factors to consider (Fu and Chen, 2011; Herrmann and Lesueur, 2013). Excessive heat mainly affects macromolecules and higher-order structures such as proteins and nucleic acids (Ghandi *et al.* 2012). On the other hand, stress due to dehydration mainly affects the cytoplasmic membrane, changing its fluidity and lipid peroxidation (Teixeira *et al.* 1996). Generally, drying processes used for biological formulations involved both deleterious effects, therefore drying stress-avoidance strategies are demanding. Approaches including spray drying (Schuck *et al.* 2013), fluidized bed drying (Schoebitz, Lopez *et al.* 2013; Berninger *et al.* 2018), and lyophilization (Carvalho *et al.* 2002) among others have dealt with these issues. It is important to mention that the formulation by spray drying is the most adopted at an industrial level, mainly with Gram-positive bacteria, such as *Lactobacillus* sp, *Streptococcus* sp, *Enterococcus* sp. and *Bacillus* sp (Schuck *et al*. 2013). In contrast, studies of dry formulations for Gram-negative bacteria are not conventional, and most of them have not been successful and are difficult to compare (Fu and Chen, 2011) (Table 1.6).

It is defined that tolerance to thermal and mechanical stress is higher in Gram-positive bacteria, followed by yeasts, with Gram-negative bacteria being the most sensitive to these factors, possibly due to their thin cell wall structure. Thus, the bioprospection of inoculants based on Gram-negative bacteria such as *K. radicincitans* DSM16656^T requires technical solutions related to the excessive loss of viability caused by the general formulation process.

The use of formulation agents with protective characteristics has been widely described as essential for the survival of bacteria during dehydration operations; however, their

effectiveness is closely related to the bacteria to be protected, and a particular study is required for each active ingredient. These agents can act on the membrane, replacing the water or modifying the glass transition temperature (Fu and Chen, 2011) (Table 1.6). Hence, there is limited knowledge about the drying method necessary for biofertilizers based on gram-negative PGPBEs, especially when it is needed to scale the formulation process and the cost of production has to be profitable.

The great prospection of PGPBEs as active ingredients in new plant biostimulant inoculants has no limit. Although its obvious physiological advantages compared to existing alternatives, its application and technological development are deficient. With the existence of these technical gaps, a great opportunity arises to generate scientific knowledge, wherefrom the vision of chemical engineering of bioprocesses could overcome the limitations for obtaining the new generation of biofertilizers/biostimulants.

Alternatives for protecting bacterial endophytes have been reported, including normally the use of drying protectants embedded into the formulation (Lactose, skimmed milk, etc.). Besides, the osmoadaptation as a pre-conditioned mechanism for strengthening cells before drying, and the inclusion of osmolytes such as trehalose, glycine, and betaine.

A mini-review of drying survival studies is given in Table 1.6.

Table 1-6: Research studies focusing on enhancing drying survival in non-sporulating bacteria, with agricultural interest.

Bacterial agent	Protectants	Other parameters	Drying	Strategy	References
Azospirillum brasilense	Trehalose	Encapsulation in alginate beads	AD	Cell flocculation, stationary phase	(Schoebitz *et al.* 2012)
Pantoea agglomerans	Sucrose	Cell load, rehydration media	FD	Rehydration in 1% NFSM, 10% sucrose	(Costa *et al.* 2000)
Enterobacter sp**.**	Skimmed milk	Consortia formulation	FD	Consortium with *Pseudomonas* sp.	(Barra *et al.* 2016)
Paraburkholderia phytofirmans PsJN	Trehalose, sucrose galactose, CMC gelatin	Storage temperature, inorganic carrier	AD, FD	Air-drying, skimmed milk, storage 4C	(Berninger *et al.* 2017)
Pseudomonas aeruginosa	EPS	Inorganic carrier	AD	EPS as protectant	(Tewari and Arora, 2014)
Pseudomonas fluorescens	Lactose	Growth conditions	FD	Harvesting after 16 h , mild heat shock at 38C	(Bisutti *et al*. 2015)
Pseudomonas fluorescens	Glycine betaine	Osmoadaptation by NaCl	VD	Osmoadaptation (0.7 M NaCl)	(Bonaterra *et al*. 2007)
Pseudomonas fluorescens	Glycine bataine	Osmoadaptation by NaCl, nutrient addition	AD	Osmoadaptation (0.7 M NaCl and 0.1 mM glycine betaine)	(Cabrefiga *et al*. 2011)
Pseudomonas fluorescens	Lactose, skimmed milk, sucrose, starch, trehalose	Osmoadaptation	FD	Osmoadaptation with lactose as protectant	(Cabrefiga *et al.* 2014)

AD, air-drying; SP, spray-drying; VD, Vacuum-drying, FD, Freeze-drying

To strengthen *K. radicincitans* DSM16656^T cells before the formulation operation, the use of pre-conditioning from the fermentation process is proposed as a strategy. There are options such as manipulation of culture conditions, pre-adaptation of cells through thermal, oxidative or osmotic shocks (Santivarangkna *et al.* 2007; Broeckx *et al.* 2016). These mechanisms could trigger intracellular and extracellular protection agents such as compatible solutes or the activation of chaperones proteins (Hsp33, DnaK, GLoE) repairing other proteins and membrane protectors (Winter *et al*. 2005; Jiao *et al.* 2015)). It is essential to mention that *K. radicincitans* DSM16656^T would have the ability to produce some Hsp proteins according to the description of their genome (NCBI Accession PRJNA16 1109). Besides, *K. radicincitans* could accumulate compatible solutes upon osmotic stress during pre-conditioning that could support the drying resistance and subsequent endophytic establishment. The existence of these agents before the formulation operations could substantially improve stress tolerance in general, resulting in higher viability, biological activity and storage stability.

2. CHAPTER 2. CULTIVATION: SCREENING OF NUTRIENTS AND CULTURE MEDIA DESIGN FOR *Kosakonia radicincitans* DSM16656^{T}

2.1 Introduction

The design of culture medium continues being one of the most investigated areas in the production of microorganisms or metabolites on a large scale, offering increasing challenges (Singh, Haque *et al.* 2017). Before 1970, culture media design was carried out using classic methods, usually expensive and time-demanding, with a high number of experiments. However, with the advent of modern mathematical and statistical techniques, it has become more dynamic, efficient, economical and robust. Thus, to design a production culture media, fermentation conditions and appropriate media components (e.g. carbon, nitrogen, macro and microelements) must be identified and optimized in consequence.

Increasing the active ingredient productivity reduces the total cost of the production and development of microbial-based products. In general, higher productivity can be achieved by improving the microorganism or by optimizing the process parameters. With the development of a culture medium, the research aims to have the best conditions for growth or replication of the active ingredient, whether bacteria, fungus, virus or protozoa. In small scales, the active ingredient of biopesticides is frequently produced using defined culture media with standard substances in their content (Kennedy and Krouse, 1999; Singh, Haque *et al.* 2017). Nonetheless, when the product needs to be taken to another scale, economic problems emerge that make its implementation in bioproducts difficult. As a first approximation, the culture media focuses on the simple fact of producing biomass, cells or the agent of interest. For endophytes, with the focus on the integration of bioproduct development, the culture media design must also guarantee the stability of biological activity, ensuring or enhancing its metabolic signature involved in their plant-associated lifestyle identity.

During the design and optimization of culture media, several strategies can be used, independently of the fermentation technique: submerge fermentation (smF) or solid fermentation (SSF). It includes the evaluation of one factor at a time or the manipulation of several of them simultaneously. In the first method, the greatest limitation encountered is the difficulty for estimating the interactions of the factors (Kennedy and Krouse, 1999; Singh, Haque *et al.* 2017). Within this strategy, there are three approaches: removing one

component of the culture media at a time, reviewing the effect it has on the production of biomass or metabolite, and supplementation, which is typically used when is required to know the effect of various carbon or nitrogen supplements. The latter been used mainly to evaluate the effect on metabolite production or anti-fungal activity (Tripathi *et al*. 2004; Singh *et al.* 2012). Besides, within this strategy, there is the replacement technique, which uses the carbon or nitrogen source that shows positive effects in supplementation experiments as the only source.

The multivariable strategy of more than one factor at a time uses statistical methods such as the design of experiments (DOE) for the optimization. Regarding fundamental theories of experimental design, showed that changing more than one factor or variable in the media is more efficient than changing each of them independently (Fisher, 1992). Most DOEs allow preliminary selection between two to ten factors in a limited number of experiments. With these methods, several factors or media components are compared simultaneously, and the effects are ranked. Compared to the strategy of one factor at a time, with the use of DOE, fewer experiments and materials are used, and the interactions between the components can be determined (Adinarayana and Ellaiah, 2002; Gundogdu *et al.* 2016). Within the experimental design, exists the Plackett-Burman design (PBD), which was introduced by R. L Plackett and J.P Burman in 1946, as a solution to determine the main effects in any process. This design is a two-level model where it is assumed that the interactions of the factors are not significant. Then, it is possible to evaluate "n" variables in "n + 1" experiments.

Among endophytes, a distinction is made between obligate endophytes (Croes *et al.* 2013) and facultative endophytes (Kamnev *et al.* 2005), where the first is expected to be the hardest to cultivate (Eevers *et al*. 2015). Relative few studies have focused on the culture media design in the world for bacterial endophytes (Nour *et al*. 2012; Youssef *et al.* 2016). Different media are reported in the literature, depending on the species and research goal. In particular, for the isolation of plant-associated bacteria, a distinction exists between complex, rich media that contain high amounts of somewhat undetermined nutrients and minimal media that contain lower yet precise amounts of nutrients (Eevers *et al*. 2015).

Although the plant-based culture media for endophytes has shown promising yields (Nour *et al.* 2012), considering their heterogeneity, it becomes difficult to use these approaches to delve the cultivation-integration aimed in this research. Hence, the study on the culture media design looks for a defined media. Then, the reduction of complex components was limited as necessary.

In general, rich media produces a favorable environment for endophytes to grow, an in consequence, are preferable for plant-associated bacteria proliferation (Eevers *et al*. 2015). That is the case of *Rhizobium* sp or *Azospirillum* sp, which have been produced through media such as TYG, BTB-1, nutritive medium, LB medium, among others, which contain components such as yeast extract or tryptone (Bashan *et al.* 2011). Moreover, endophytic bacteria such as *Brevundimonas sp. Sphingomonas sp. Agrobacterium tumefaciens* were produced in MS media (sucrose, tryptone), *Enterobacter sp. Pseudomonas sp* in TSA media (Soytone, tryptone, yeast extract) (Eevers *et al.* 2015) and *Azospirillum brasilense* C16 (glutamate, yeast extract) (Moreno-Galván *et al.* 2012), maintaining the endophytic identity. Based on a literature review, the design of a rich defined media was selected, which might mimic the *in planta* conditions better than a minimal defined media, and therefore might allow a more straightforward adaptation for endophytes (Alain and Querellou, 2009; Eevers *et al.* 2015).

Beyond the rich culture media design, and considering the available literature regarding culture media in Gram-bacteria endophytes, there are knowledge gaps in terms of assaying the influence of amino acids and metal ions on kinetic growth performance. Therefore, it was proposed to screening these growth factors to delve the issues.

2.1.1 Effects of osmotic stress and compatible solutes on *K. radicincitans* DSM16656^T

Since osmoadaptation and use of osmolytes within the cultivation is a potential strategy to strength bacterial cells (Chapter 1, Table 1.6), the evaluation of these stressors is demanding for *K. radicincitans* DSM16656^T. By a high-throughput microfermentation system, here was proposed to determine the influence of osmotic stress and compatible solutes on bacterium

kinetic growth. Thereby, upon the culture media design, the sensitivity analysis of salt-amended media and compatible solutes uptake were carried out.

Regarding the reported benefits on Gram-negative bacteria under osmotic stress, the compatible solutes ectoine, hydroxyectoine, proline, betaine, glutamine, glycine, and glutamate were selected for analysis (Table 2.5).

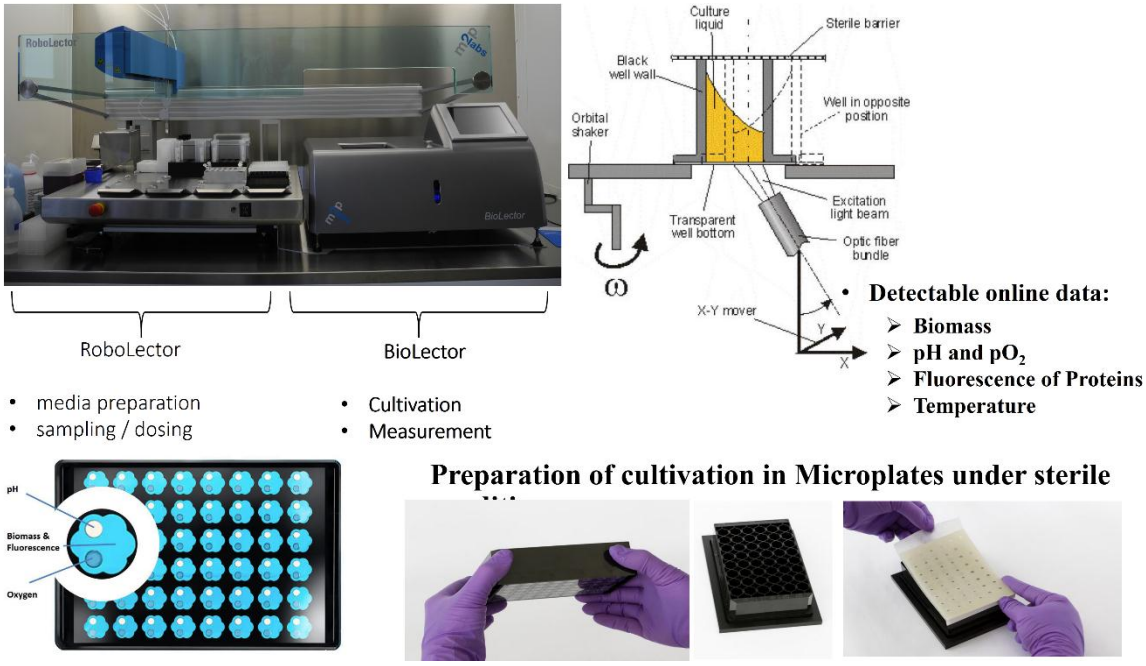
2.1.2 Microfermentation approach

Microfermentation uses mini-bioreactors for cultivating microorganisms with economic value, which have gained increasing acceptance in industry and academic research. Critical applications of microfermentation include the screening of media compositions and bioprocess development, optimization and validation (Huber *et al.* 2009; Kensy *et al.* 2009). This approach involved monitoring growth kinetic as a prerequisite for understanding the whole process. In particular, with microfermentation techniques such as BioLector® platform, essential online information can be optically obtained on biomass formation, pH-evolution and dissolved oxygen tension (DOT) in media (Figure 2.1). Numerous studies had been carried out experimentally proving the value of this effective device (Buchenauer *et al*. 2009; Funke *et al.* 2009; Funke *et al.* 2010). Thus, high-throughput cultivations can accelerate and intensify research and development in the field of systems biology as well as modeling and bioprocess optimization, reducing human manual error and workload.

To obtain a suitable culture media for this endophyte, this research initially screened media from literature, in order to select the principal C-source and N-source. Having the key criteria of cost for preparing, it was followed by the design and optimization of media. As the main biological response variables, biomass yield and final concentration at 24 h (CFU mL^{-1}) were used.

Summarizing, the result obtained from the different approaches, including microplate test (Biolog GEN III fingerprint), the selection of literature's media, Plackett-Burman strategy and microfermentation experiments, were used as criteria for design the final culture media for *K. radicincitans* $DSM16656^{T}$.

Figure 2-1 Features of Biolector® and microplates preparation for screening culture media and conditions during microfermentation studies of *K. radicincitans* DSM16656^T.



2.2 Materials and Methods

2.2.1 Phenotypic fingerprint of *K. radicincitans* DSM16656^T: GEN III microplate

K. radicincitans strain DSM 16656^T was characterized phenotypically using the Biolog GEN III MicroPlate^{TM} system (Biolog system, Biolog Inc., Hayward, CA). The Biolog GEN III assay utilizes a variety of carbon and nitrogen sources, and was carried out accordingly to manufacturer instructions. Briefly, bacterial cells from overnight bacterial cultures grown on nutrient broth media (Merck, Darmstadt, Germany) were suspended in Biolog inoculation fluid at the recommended concentration, and 100 µL of the suspension was added to each well of the GEN III MicroPlate and incubated at 30°C for 48 h.

The Biolog plate contained 96 total wells, 71 with different carbon sources, including osmotic stressors assays, and the positive (maximum growth) and negative (no carbon source/growth) control. When bacteria cells respire, they cause a reduction of the

tetrazolium indicator dye in each well, which results in the development of a purple colour. Metabolic reactions were read using a Biolog plate reader set to incubate the plate at 30°C for 48 hours (Cytation 3, Biotek, Winooski, VT, USA). The plate reader measured the absorbance of each well every hour at a wavelength of 590 nm.

The kinetic growth rate was calculated at exponential phase, as the linear slope in the logarithmic plot of OD vs time:

$$\mu = \frac{Ln\ OD_2 - LnOD_1}{(t_2 - t_1)}$$

OD represents the optical density in the time point 1 and 2 after 5 h and 10 h respectively on the fitted line (Multiskan FC microplate photometer, Thermo Scientific, USA).

2.2.2 Culture media screening

To look into the ability of *K. radicincitans* DSM16656^T to grow in different media a screening phase was conducted. An examination of six culture media available in literature was performed (Moreno-Galván *et al.* 2012; Eevers *et al.* 2015). The fermentation was carried out in 250 mL flasks equipped with baffles. The incubation conditions were 100 mL of culture media (initial concentration at 1.0x10^5 CFU mL^{-1}) in a rotary incubator at 30°C, 190 rpm for 24 hours (IKA KS 4000 IC Control, Staufen, Germany). For each culture media, 5 biological replicates were used. Cell concentration (CFU mL^{-1}) in nutrient broth (Merck, Darmstadt, Germany) at 24 h of cultivation were utilized as response variables. Details of media composition including the standard nutrient broth for *K. radicincitans* DSM16656^T are given in Table 2.1.

Table 2-1: Culture media evaluated in the screening phase for *K. radicincitans* DSM16656^T

Component (g L^{-1})	Standard	M1	M2	M3	M4	M5	M6
Peptone without casein	15	13.5				6.75	6.75
Yeast extract	3	7	2.92			3.5	3.5
Monosodium glutamate			28.33				
Na_2HPO_4					3		
NH_4Cl					6		
Glucose	1						
Glycerol		14.9				14.9	45
Lactose					8		
Starch				10			
NaCl	6	2.5		2	5	10	10
K_2HPO_4		2.3		2	1	2.3	2.3
KH_2PO_4		1.5				1.5	1.5
$MgSO_4\,H_2O$		0.14			1	0.14	0.14
$K_2HPO_43H_2O$			1.34				
$FeCl_3$			0.02				
$CaCO_3$				0.02			
Casein				0.3			
$FeSO_47H_2O$				0.01			
KNO_3				2			
$MgSO_47H_2O$			0.5	0.05			
pH	7 ± 0.2	7 ± 0.2	7 ± 0.2	7 ± 0.2	7 ± 0.2	7 ± 0.2	7 ± 0.2

2.2.3 Culture media design

Upon screening of culture media and Biolog GEN III assays, advantageous components such as glycerol and yeast extract were selected. Details are given in results (2.3.1-2.3.2). In this section, a Plackett-Burman design was used to determine the main effects of 7 factors,

including main C-source, N-source, NaCl (potential osmotic stressor), K-source, Mg-source and the more sensitive microelements Fe^{+3} and Mn^{+2} (see section 2.3.4) on cell concentration yields. Factor levels were selected according to the concentration of reported culture media (Table 2.1) and the range of concentrations in plant root juices (Table 2.4). Table 2.2 and 2.3 provide information on factors and experimental design matrix.

Table 2-2: Factors and levels used in Plackett-Burman design

Factor (g L^{-1})	**-1**	**+1**
Glycerol	**15**	**45**
Yeast extract	1	8
NaCl	10	40
K_2HPO_4	0.5	4
$MgSO_4$	0.5	1.5
*Fe/Mn ratio	1.1	3.3
Initial pH	4	7

*Fe/Mn ratio was adjusted varying Fe^{+3} concentration from 0.4 ppm to 2.4 ppm maintaining constant Mn^{+} at 0.7 ppm (see also 2.2.4, Table 2.4).

Table 2-3: Matrix of experiments tested in Plackett-Burman design (g L^{-1})

Factor	**X1**	**X2**	**X3**	**X4**	**X5**	**X6**	**X7**
Run	**Glycerol**	**Y. extract**	**NaCl**	**K_2HPO_4**	**$MgSO_4$**	**Fe/Mn**	**pH**
1	15	1	10	0.5	0.5	1.1	4
2	45	8	10	4	0.5	1.1	4
3	15	8	10	0.5	0.5	3.3	7
4	15	8	40	4	0.5	3.3	7
5	45	8	40	0.5	1.5	3.3	4
6	15	1	40	4	1.5	1.1	7
7	45	8	10	4	1.5	1.1	7
8	45	1	40	0.5	0.5	1.1	7
9	15	8	40	0.5	1.5	1.1	4

10	45	1	10	0.5	1.5	3.3	7
11	15	1	10	4	1.5	3.3	4
12	45	1	40	4	0.5	3.3	4

Once the main effects of components were determined, the influence of glycerol as the main C-source on cell concentration and biomass yield was carried out. Hence, glycerol was adjusted to 1.5, 2.0, 3.0 and 4 % maintaining constant the other components and biomass harvesting was settled at 24 h.

2.2.4 Microfermentation procedure

By microfermentation technics in Biolector® system and considering glycerol as main C-source, the influence of selected amino acids and minerals on *K. radicincitans* DSM16656^T kinetic performance was determined. Assays were conducted in a defined basal media (g L^{-1}): Glycerol (15); NH_4SO_4 (4), NaCl (10); Mg SO_47H_2O (0.5); K_2HPO_4 (2.74); KH_2PO_4 (1.31).

Microelements effect on kinetic growth behavior

Metal-ions Fe^{+3}, Mn^{+2}, Zn^{+2} and Cu^{+2} influence on kinetic growth of endophyte were assayed. Here, the ions concentrations were adjusted considering the range of contents according to crude plant juices trying to mimicking natural conditions (Nour *et al.* 2012) (Table 2.4). Firstly, a mixture of microelements was prepared and further a higher concentration of each microelement within this solution was assayed.

$ZnSO_4$, $FeSO_47H_2O$, $MnSO_4H_2O$ and $CuSO_4$ were added to basal media to adjust the following two concentration levels for each metal [-, +]. Zn [0.4, 2.0 ppm]; Fe^{+3} [0.2, 4.0 ppm]; Mn [0.7, 4.0 ppm]; Cu [0.4, 2.0 ppm].

Amino acids effect on kinetic growth behavior

Basal media was supplemented with $ZnSO_4$ (0.001 g L^{-1}; Zn^+ [0.4 ppm]); $FeSO_47H_2O$ (0.001 g L^{-1}; Fe^{3+} [0.2 ppm]); $MnSO_4H_2O$ (0.002 g L^{-1}; Mn^+ [0.7 ppm]) and $CuSO_4$ (0.001

g L^{-1}; [0.4 ppm]). Further, amino acid media was composed by metal-ions supplemented basal media + glutamic acid, L- alanine, L-leucine, proline, glycine, histidine at 0.2 mM. To evaluate the effect of each amino acid, the amino acid media was adjusted to a higher concentration at 1 mM.

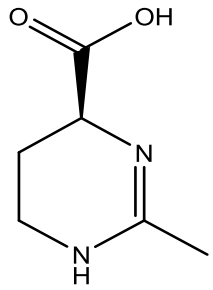
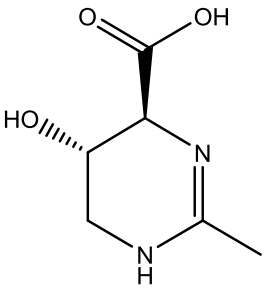
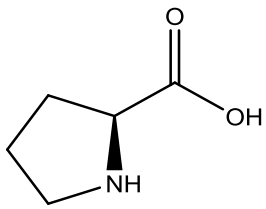
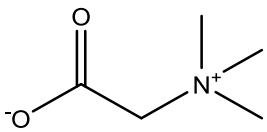
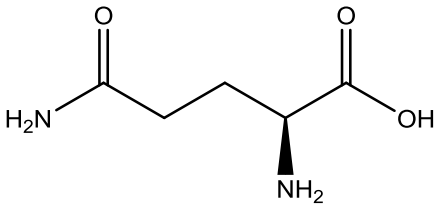
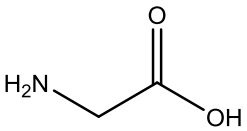
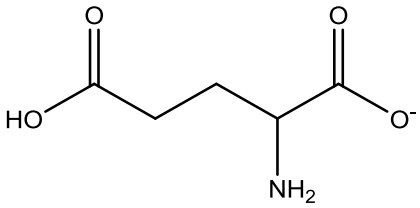
Table 2-4: Elemental composition of culture media evaluated in comparison to plant juices for culturing diazotrophic rhizobacteria (Nour *et al*. 2012).

			Merck media	**M1**	**M2**	**M3**	**M4**	**M5**	**M6**
	MIN	**MAX**							
C (%)-g/L	33.4	71.3	2.2	8	10.29	3.92	3.37	6.91	18.68
N (%)-g/L	0.65	0.96	1.41	2.06	2.67	0.03	1.57	1.03	1.03
C/N	54	84	1.56	3.89	3.85	130.77	2.14	6.72	18.15
MACRO (ppm)									
Ca^{++}	22	1636	0	0	0	8.01	0	0,00	0,00
Mg^{++}	174	653	0	24.59	49.31	4.93	175.63	13.81	13.81
K^{+}	640	2028	0	673.19	459.08	1347.81	861.77	1463.11	1463.11
Na^{+}	782	4715	2359.34	983.06	0	786.45	3908.64	3932.24	3932.24
P			0	266.68	181.86	455.17	1991.72	750.28	750.28
$HCO3^{-}$	1336	447	0	0	0	12.13	0	0,00	0,00
Cl^{-}	2847	9834	3639.63	1516.51	13.11	1213.21	4358.51	1516,51	1516,51
$SO4^{-}$	124	2784	0	97.18	194.87	22.94	694.13	97,18	97,18
$PO4^{-3}$			0	817.77	557.67	1395.8	6107.64	817.77	817.77
MICRO (ppm)									
Zn^{+}	0.297	1.617	0	0	0	0	0	0	0
Fe^{+}	0.039	3.967	0	0	6.885	2.01	0	0	0
Mn	0.644	1.157	0	0	0	0	0	0	0
Cu^{+}	0.14	0.96	0	0	0	0	0	0	0

2.2.5 Effects of osmotic stress and compatible solutes on *K. radicincitans* DSM16656^T cultivations

The effect of compatible solutes on the growth of *K. radicincitans* DSM16656^T under osmotic stress was evaluated. Assays were conducted in the BioLector® micro-fermentation system with 48-well BH microplates with 1 ml culture media (R3) at 1200 rpm and 30°C. A mixture of compatible solutes (1mM) was added to the culture medium and served as a comparative parameter to evaluate the independent effect of each osmolyte. As independent treatments, each of the compatible solutes in the media was evaluated at 1mM: Ectoine, hydroxyectoine, betaine, proline, glutamine, glutamate, L- glycine and trehalose (Table 2.4). Osmotic stress was settled at 4% NaCl.

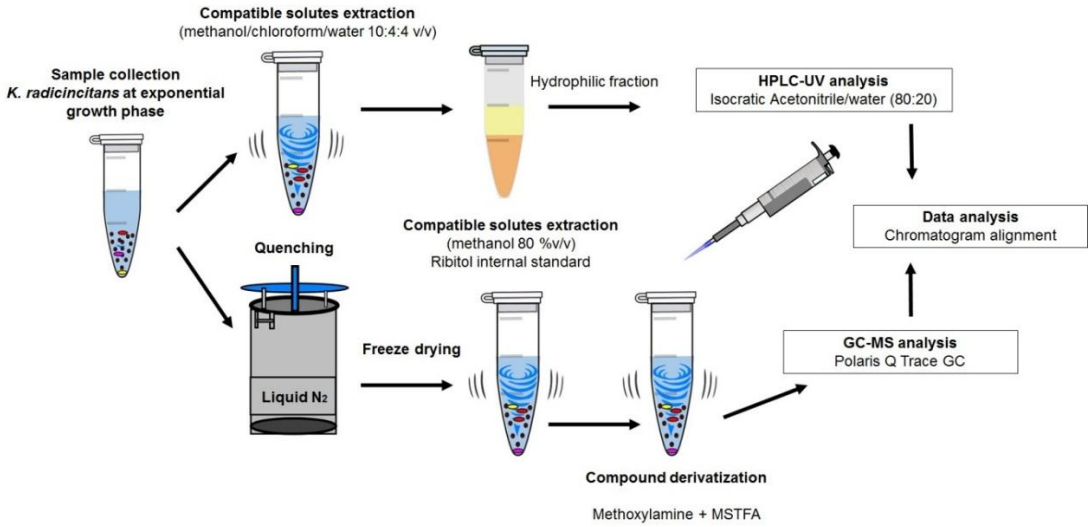
Table 2-5: Chemical structures of compatible solutes assessed

Ectoine	**Hydroxyectoine**	**Proline**	**Betaine**
			
Glutamine	**Glycine**	**Glutamate**	
			

In order to confirm the intracellular uptake of the compatible solute a HPLC and GC-MS approach was conducted. HPLC -UV techniques determined the intracellular accumulation of compatible solutes such as ectoine, and hydroxyectoine during cultivation. The detection of the amino acid proline included a derivatization step (Methoxylamine + MSTFA) and

further GC-MS evaluation (Figure 2.2). Details of GC-MS procedures are given in Chapter 6.

Figure 2-2 Schematic representation for the sample preparation prior to the identification and the quantification of compatible solutes *in K. radicincitans* DSM16656^T cells.



2.2.6 Determination of ectoine and hydroxyectoine accumulation

The intra and extracellular concentrations of ectoine and hydroxyectoine were carried out by previously reported methods (Teixido *et al.* 2005). Briefly, samples of *K. radicincitans* DSM16656^T cells grown in DM 4% NaCl plus either ectoine or hydroxyectoine [1 mM] at 190 rpm, 30°C were centrifuged for 10 min at 10000 rpm and 20°C (Mikro HT 200R, Hettich GmbH & Co. KG, Tuttlingen, Germany). The supernatant fraction served for further high-performance liquid chromatography (HPLC) analysis. The bacterial pellets were re-suspended in HPLC grade water and centrifuged to discard residues of culture medium. Subsequently, approx. 50 µL of concentrated biomass was extracted for quantitative evaluations with 570 µL of an extraction solution (methanol/chloroform/water 10:4:4, v/v) by intense shaking for 5 min followed by the inclusion of equal volumes (170 µL) of chloroform and water (Kunte *et al*. 1993). After shanking for10 min, the phase separation was ensured by centrifugation (5 min at 10000 rpm). The hydrophilic top layer containing compatible solutes was recovered. Ectoine and hydroxyectoine quantification was achieved by HPLC using an EC 150/4.6 NUCLEODUR® 100-5 NH_2-RP column and a UV-detector

at 215 nm, at a flow rate of 1 mL min^{-1} at 30°C accompanied by a column heater and using a solvent gradient established between eluents A and B (80% ACN in HPLC water) (see Figure 2.2). The peak areas were calculated and compared with calibration curves created with standards of each solute [0.1-1 mM] (Appendix 1). Results were expressed as *µmol* compatible solute g^{-1} (dry weight *K. radicincitans* cells). All results are the mean of three replicates bacterial samples per stress condition and incubation time

2.3 Results

2.3.1 Phenotypic fingerprint of *K. radicincitans* DSM16656^T: GEN III microplate

At the end of the 24 h of the incubation period, *K. radicincitans* DSM16656^T strain had differential utilization of the carbon and nitrogen source (Figure 2.3, 2.4, 2.5). As expected, there was no visible growth in any of the negative control wells and no significant difference in the absorbance readings of the positive control well. Osmotic stressors assays resulted in positive absorbance at 24 h of 1.807, 1.564 and 0.586 for 1%, 4% and 8% NaCl respectively. Regarding OD_{600} and estimated μ_{max} parameters, the carbon sources utilized more efficiently were D-mannitol, D-trehalose, D-maltose and glycerol. Interestingly, polyols are advantageous C-sources for the bacterium, in comparison to sugars such as glucose and galactose. Citric acid, mucic acid, acetic acid, and gluconic acid seem to be another potential nutrient source for the endophyte. Conversely, D-malic acid, quinic acid, and butyric acid are not beneficial C-source for *K. radicincitans* DSM16656^T (Figure 2.4). These carbon sources are found naturally in plants, available in-store forms such as sucrose, raffinose, whereas arabinose, xylose, mannose, glucose, and galactose exist in association with the plant cell walls components (Yang *et al.* 2017).

Considering the assimilation of N-sources, amino acids such as L-alanine, proline, L-serine, and L-aspartic acid provide to the endophyte notorious benefits that can suggest the affinity of the bacteria to these substances. Histidine and arginine are not potential N-sources for the endophytic bacteria (Figure 2.5). The raw data absorbance values for triplicate samples from the 24 h time point and specific growth rate (µ) were averaged (Appendix 2).

Figure 2-3 Comparative analyses of *K. radicincitans* DSM16656^{T} growth in the Biolog GEN III microplate assay with different C-sources.

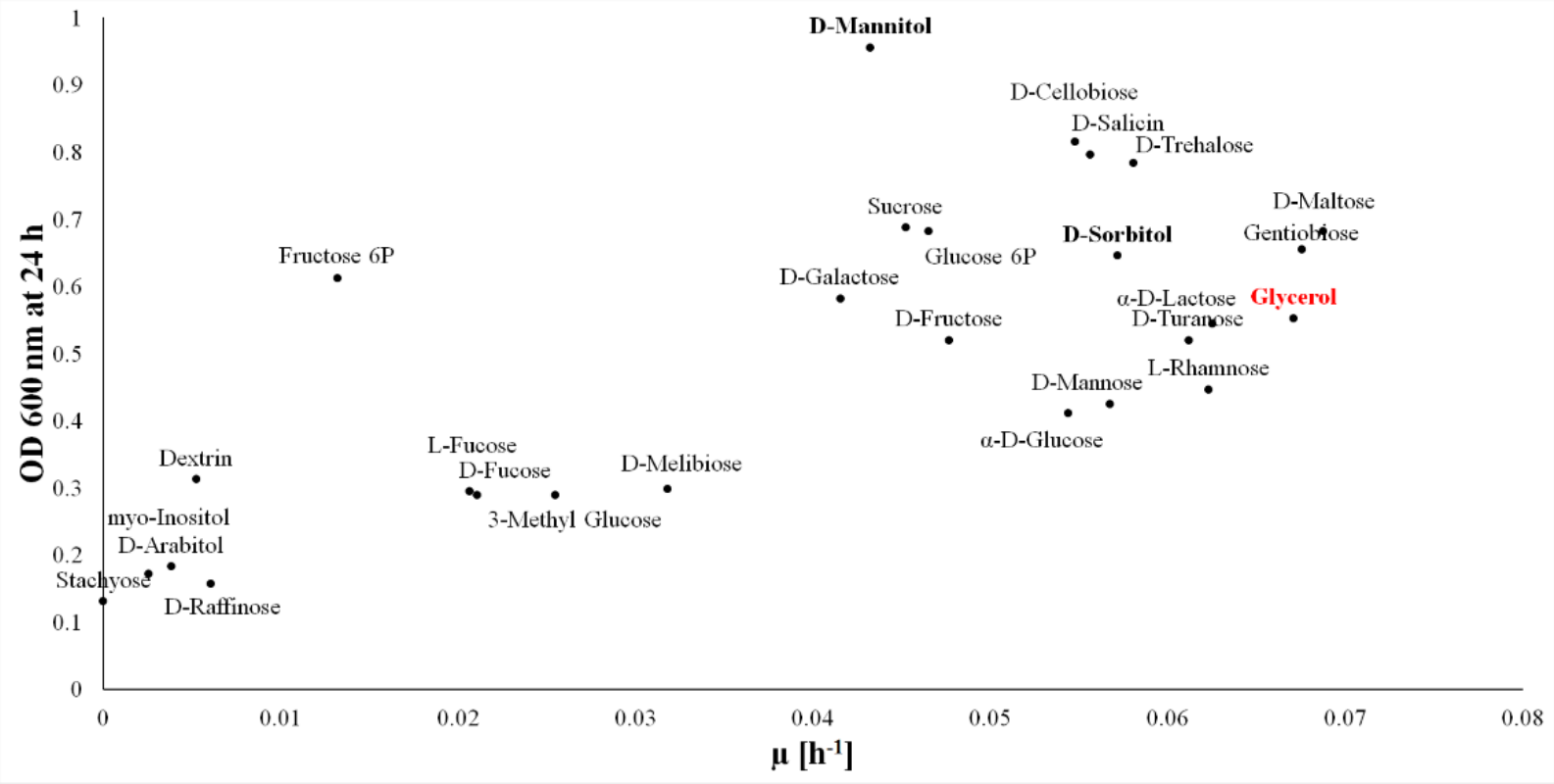


Figure 2-4 Comparative analyses of *K. radicincitans* DSM16656^T growth in Biolog GEN III microplate assay with different organic acids as Carbon-sources.

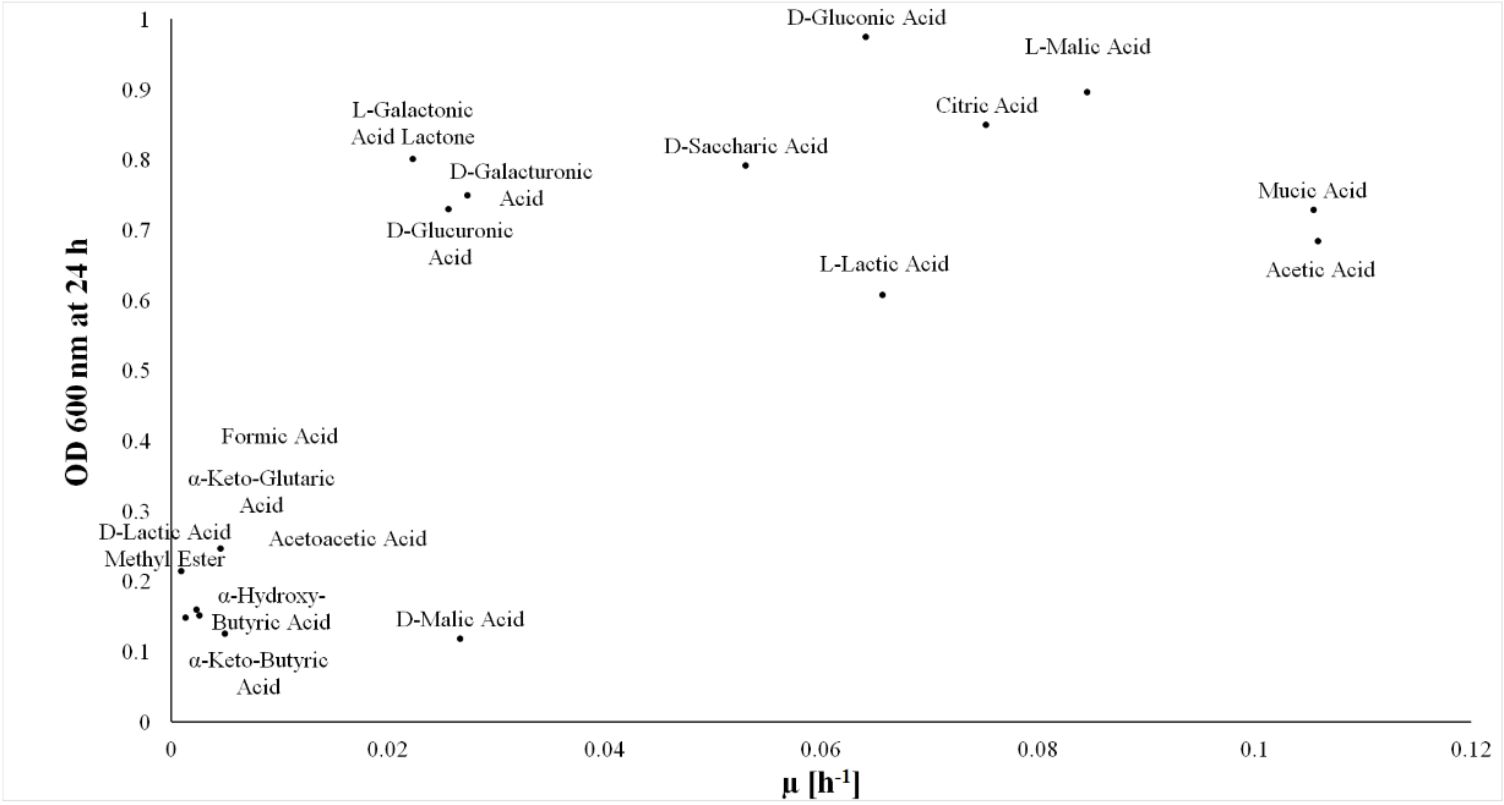
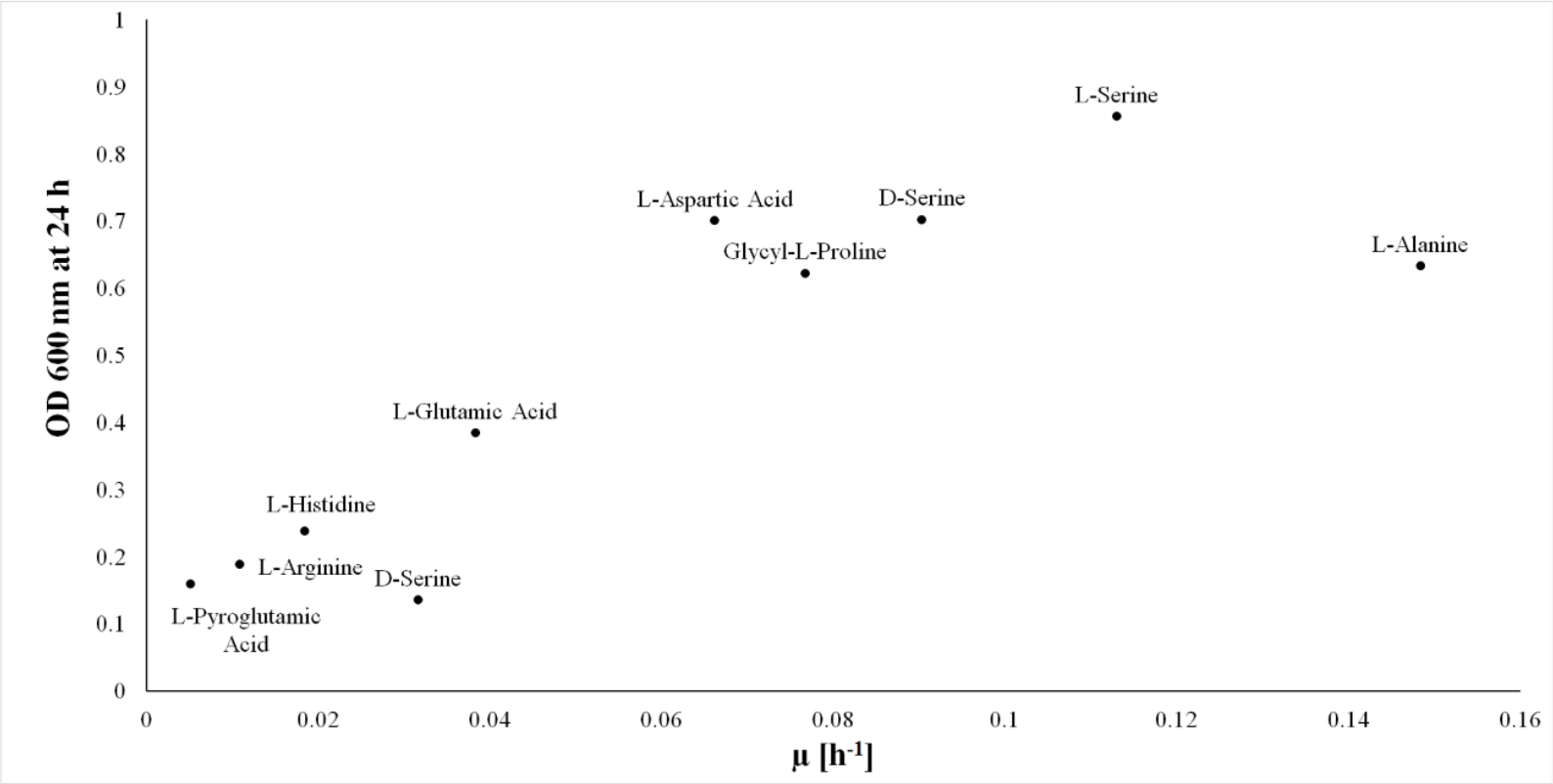


Figure 2-5 Comparative analyses of *K. radicincitans* DSM16656^{T} growth in the Biolog GEN III microplate assay with different amino acids as Nitrogen-sources.

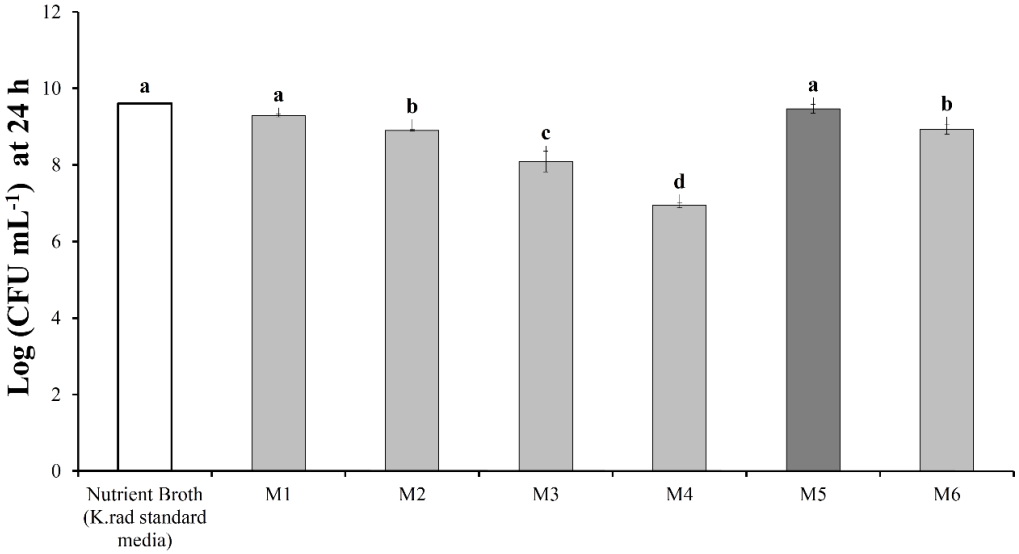


The phenotypic assays in Biolog were similar to the obtained with other *Enterobacteriaceae* endophytes, which hydrolyzed a wide range of carbon sources and were slightly halophyte for proliferating at 4% NaCl (Mahlangu and Serepa-Diamini, 2018). The wide carbon utilization could facilitate bacterium entrance and hence, its survival inside plant host forming colonies thereafter (Yang *et al.* 2017).

2.3.2 Culture media screening

The assessment of six different culture media elucidated the media M5 as advantageous for producing viable cells of the endophyte (Figure 2.6). M5 was selected over M1 for further analysis because the less amount of peptone and yeast extract required. Thereby, viability (CFU mL^{-1}) in M5 after 24 h was significantly different in comparison to the standard nutrient broth use for *K. radicincitans* $DSM16656^T$ ($p<0.05$), with cells concentrations higher than 2.0 x 10^{10} CFU mL^{-1}. Culture media M3 and M4 based on starch and lactose respectively showed the lowest yields. Aiming to eliminate the use of peptone, and decreasing the costs of the culture media by leaving only the yeast extract as a nitrogen source, a Plackett-Burman design was planned.

Figure 2-6 Cells concentrations (CFU mL^{-1}) in different culture broth after 24 h of fermentation of *K. radicincitans* $DSM16656^T$. Different letters indicate significant differences according to Tukey post hoc test, ($n >4$; $p<0.05$).

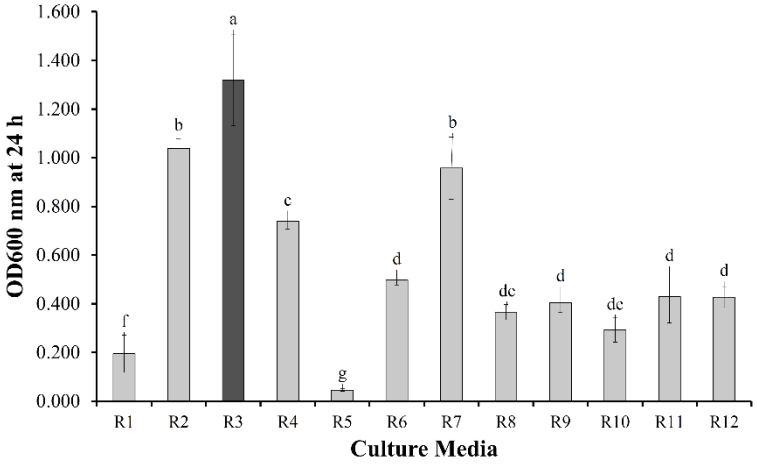


2.3.3 Culture media design

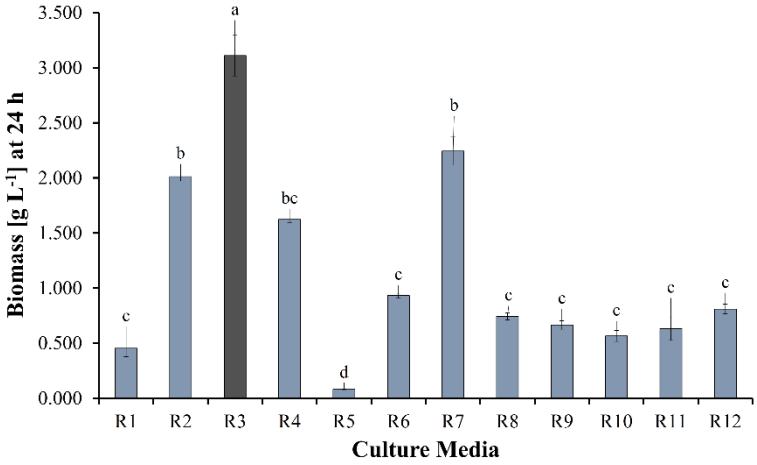
Considering culture M5 and having the glycerol as the main carbon source, 7 factors with 12 different media were tested using a Plackett-Burman design. Factors and their levels details are given in Tables 2.3 and 2.4. Screening demonstrated the treatment R3 with the highest values of OD_{600} at 24 h is significantly different from the other treatments ($F_{11, 48}$ = 370.7, p <0.001, α = 0.05) (Figure 2.7 A). Similarly, with this media (R3), dry biomass (g L^{-1}) yield reached the highest, values over 3.5 g L^{-1} ($F_{11, 48}$ = 26.88, p <0.001, α = 0.05) (Figure 2.7 B). Pareto diagram analysis clarifies that the concentration of yeast extract, K_2HPO_4 and the initial pH have positive and significant effects. With a negative and significant effect, the concentration of NaCl and $MgSO_4$ were established (Figure 2.7 C).

Interestingly, glycerol concentration had not a significant effect under the evaluated conditions. In Figure 2.8, sole glycerol effect was elucidated in media R3, which confirmed the Pareto results. Hence, before 24 h the production of biomass and viability (CFU mL^{-1}) at 1.5 % and at 4% of glycerol are statistically the same, according to repeated measures ANOVA ($p < 0.05$) (Figure 2.8, Table 2.6). Considering a biomass chemical formula ($CH_{1.78}$ $N_{0.24}$ $O_{0.33}$) with elemental composition of carbon (48.7%) and nitrogen (13.9%) (Peacock and Richardson, 2012), at 1.5 % glycerol the highest estimated yields at 30 h were found at 0.497 g C in biomass/g C in glycerol and at 0.918 g N in biomass/ g N in yeast extract.

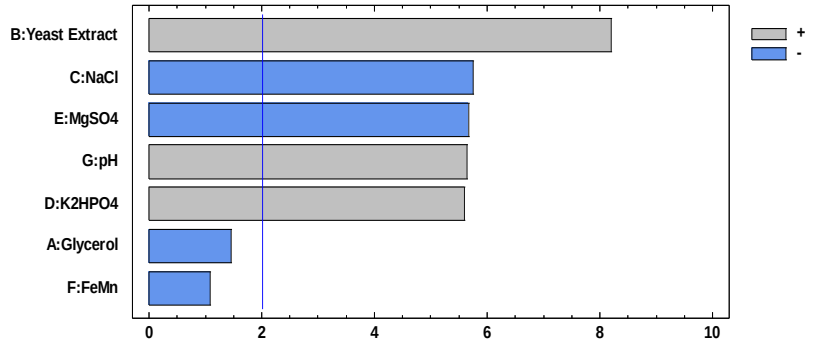
Figure 2-7 A. Optical density OD_{600} and **B.** Production of dry biomass (g L^{-1}) after 24 hours of fermentation of *K. radicincitans* DSM16656^T in the proposed culture media with the Plackett-Burman design. **C.** Pareto diagram for optical density and **D.** Production of dry biomass



A



B


B:Yeast Extract
C:NaCl
E:MgSO4
G:pH
D:K2HPO4
A:Glycerol
F:FeMn
0
2
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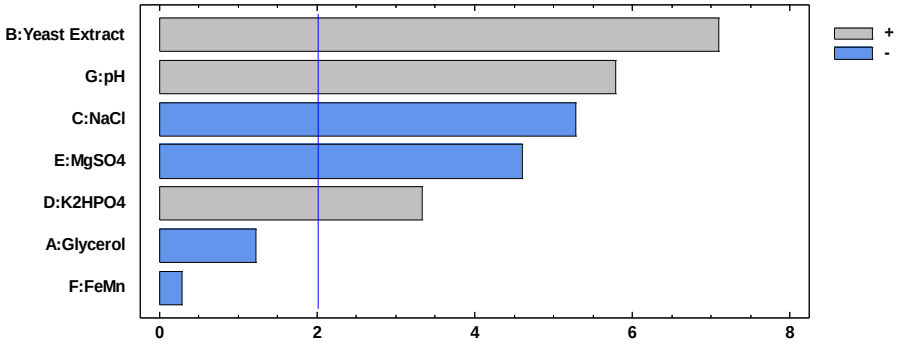
10
+
-

C


B:Yeast Extract
G:pH
C:NaCl
E:MgSO4
D:K2HPO4
A:Glycerol
F:FeMn
0
2
4
6
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D

Figure 2-8 Effect of glycerol concentration as C-source on kinetic growth of *K. radicincitans* DSM16656^{T}. Different letter indicate significant differences according to RM-ANOVA test ($p < 0.05$).

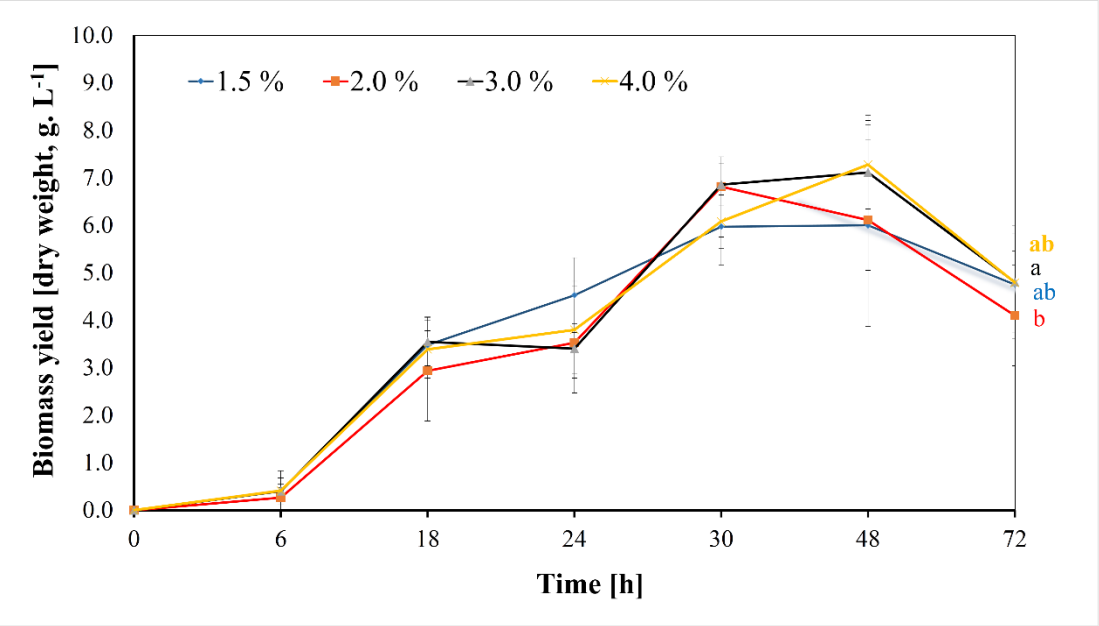


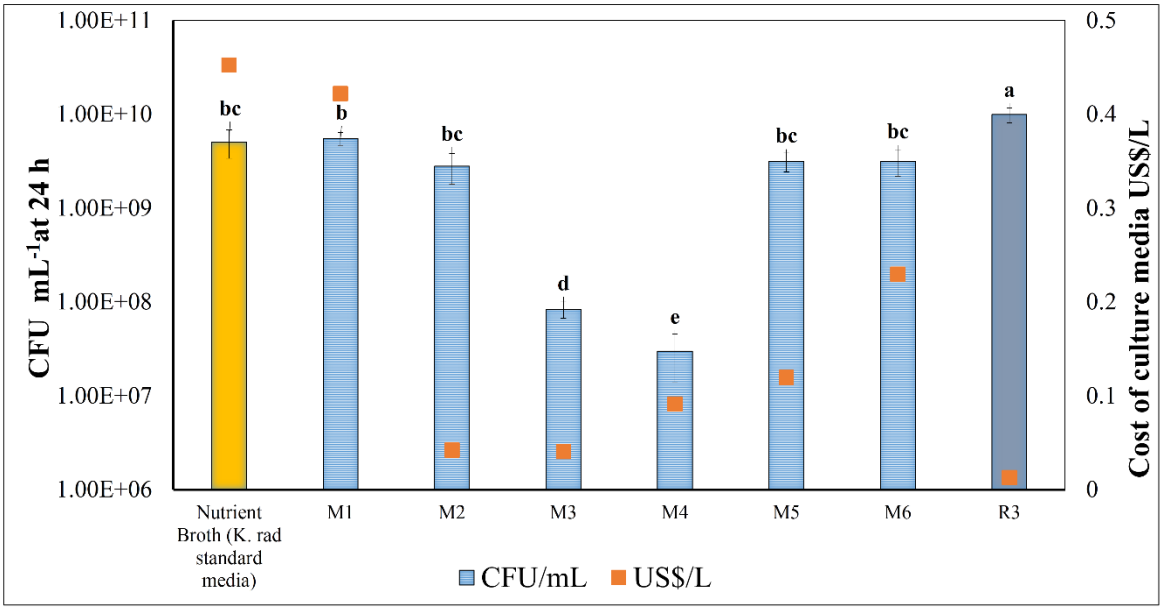
Table 2-6: Concentration of cells at 24 h and at 48 h. Different letters indicate significant differences between treatments (24 h, $F_{3, 19}=0.66$; $p=0.5914$), (48 h, $F_{3,19}=0.35$; $p=0.793$) ($n=5$, $p < 0.05$).

Glycerol [g L^{-1}]	[CFU mL^{-1}] at 24 h	[CFU mL^{-1}] at 48 h
15	2.03 ± 0.85 x10^{10} a	1.98 ± 0.19 x10^{10} a
20	2.09 ± 0.91 x10^{10} a	2.97 ± 0.19 x10^{10} a
30	1.78 ± 0.42 x10^{10} a	2.73 ± 0.73 x10^{10} a
40	1.57 ± 0.33 x10^{10} a	2.39 ± 0.30 x10^{10} a

Figure 2.9 illustrates the viability and cost of preparation of the 6 media evaluated in the screening section, the nutrient broth standard and the final R3 media from Plackett-Burman design. Results suggest that the media R3 is notably cheaper in comparison to the other

media. Thus, the elimination of peptone, the inclusion of yeast extract and the use of glycerol at 1.5 % decrease the cost of preparing 1 liter of media. Hence, R3 media permits the production of *K. radicincitans* DSM16656^T over 10^{11} CFU mL^{-1} after 24 h with a cost at 0.0128 US$ L^{-1}.

Figure 2-9 Comparative compilation of culture media, regarding viability (CFU mL^{-1}) at 24 h and cost of broth preparation (US$ L^{-1})



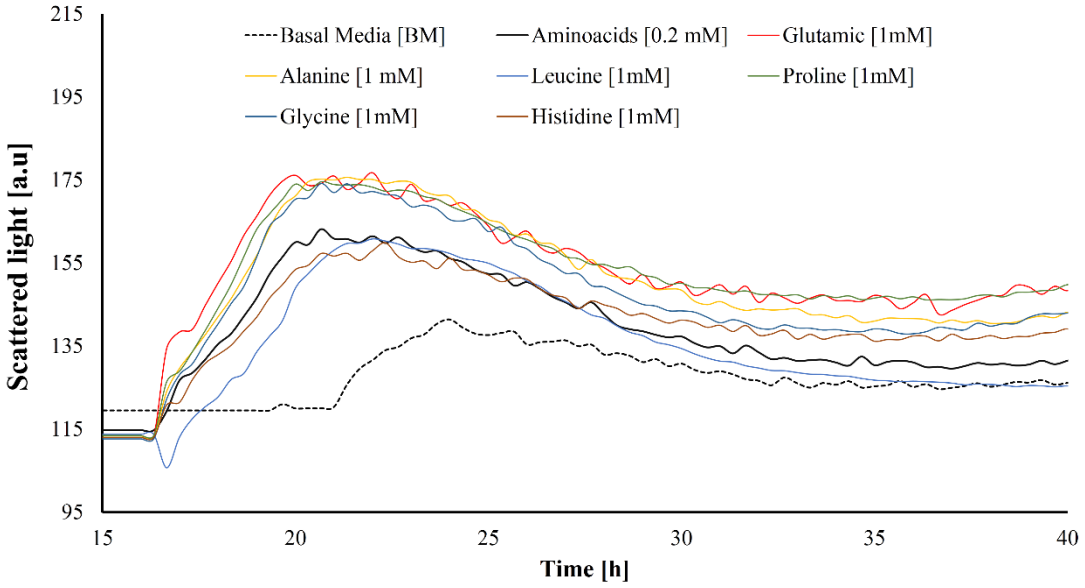
Further studies demonstrated the benefits of buffering the media R3 by the addition of KH_2PO_4 (1.31 g L^{-1}) and K_2HPO_4 (2.74 g L^{-1}), which extended the limit of growing from 3.76 ± 0.22 g L^{-1} to 4.53 ± 0.96 g L^{-1} of biomass yields at 24 h of cultivation (n=4). Culture media price increased slightly to 0.014 US$ L^{-1}.

2.3.4 Microfermentation approach

To look into the influence of amino acids as well as macro and micro minerals on kinetic growth of *K. radicincitans* DSM16656^T, a microfermentation technique was conducted. To avoid the interference of yeast extracts trace components on main effects, a basal media was used (g L^{-1}): Glycerol (15); NH_4SO_4 (4), NaCl (10); Mg SO_47H_2O (0.5); K_2HPO_4 (2.74); KH_2PO_4 (1.31); $ZnSO_4$ (0.001); $FeSO_47H_2O$ (0.001); $MnSO_4H_2O$ (0.002); $CuSO_4$ (0.001).

Figure 2.10 permits to elucidate that the amino acids mixture at 0.2 mM as a supplement of basal media reduce the time to start proliferation from 21 h to 16.6 h. Interestingly, glutamic acid, proline, alanine and glycine at a higher concentration of 1 mM facilitated the kinetic growth and respiration of bacterial cells. Conversely, histidine and L-leucine at 1 mM have a harm kinetic performance, since the lag phase required is longer in comparison to the mixture of amino acids [0.2 mM] used as a control. BioLector® scattered light signals demonstrated the importance of amino acids in culture media for enhancing kinetic growth.

Figure 2-10 Effect of selected amino acids [0.2 – 1.0 mM] on kinetic growth of *K. radicincitans* DSM16656^{T} Biolector® approach.



Finally, the supplementation of R3 media with amino acids at 1 mM (L-alanine, L-proline and glutamic acid) allows obtaining concentration higher than 2.0x10^{11} CFU mL^{-1} at 24 h of cultivation. Thus, the final concentration in supplemented R3 media was ca 32-fold higher in comparison to the standard nutrient broth media with 6.1 ± 2.1 x10^{9} CFU mL^{-1}. Inclusion of amino acids solution increase slightly R3 amended media at 0.0176 US$ L^{-1}.

Amino acids screening results for DSM16656^{T} are in agreement with the nutritional profile abundance in plant juices-based cultures, to cultivate rhizospheric microorganisms, which predominant amino acids were (µg L^{-1}): glutamic (470), alanine (250), proline (200) and arginine (360) (Nour *et al*. 2012). Another study supporting the found affinity was presented by Youssef *et al* (2016), who determined glutamic, alanine and proline as essential amino acids in a plant-based culture for isolating bacterial endophytes. Unlikely, histidine was found in the lowest concentration.

Figure 2.11 showed the response of *K. radicincitans* DSM16656^{T} to microelements such as Fe^{+3}, Mn^{+2}, Zn^{+2}, and Cu^{+2}, when they were included in basal media. In this research, the range of concentrations for each metal-ion was adjusted according to plant juices composition used for the isolation and proliferation of bacterial endophytes (Table 2.4). Biomass yield increases significantly with the inclusion of microelements at a low level [-], and it is constant at a high level of concentration [+].

The effect of each microelement on bacteria growth by microfermentation in BioLector® demonstrated that from 0.2 to 0.7 ppm, the four elements have a positive effect on kinetic performance, since the scattered light signal showed bacteria activity after their inclusion in basal media (Figure 2.12). Results suggest that *K. radicincitans* DSM16656^{T} cells are tolerant to both Zn^{+2} and Fe^{+3} at the concentration in media from 2.0 to 4.0 ppm. In contrast, at 4 ppm and 2 ppm for Mn^{+2} and Cu^{+2} respectively, these two elements affect the kinetic growth of the endophyte significantly (Figure 2.12).

Toxicity of Cu^{+2} at 0.5 mM was correlated to the less diverse community of rhizobacteria, indicating a low tolerance to this metal-ion (Sharaff *et al.* 2017). Conversely, according to a recent study (Brigido *et al.* 2019), is rarely found a bacterial endophyte with Mn^{+2} – low resistance, since plant associated Enterobacteriales were highly Mn-tolerant. This notwithstanding, *K. radicincitans* DSM16656^{T} establishment could be favourable at soil pH >7 were Mn^{+2} availability is reduced.

Figure 2-11 Effect of selected microelements at two levels [+,-] on kinetic growth of *K. radicincitans* DSM16656^T (OD_{600} nm) and biomass production

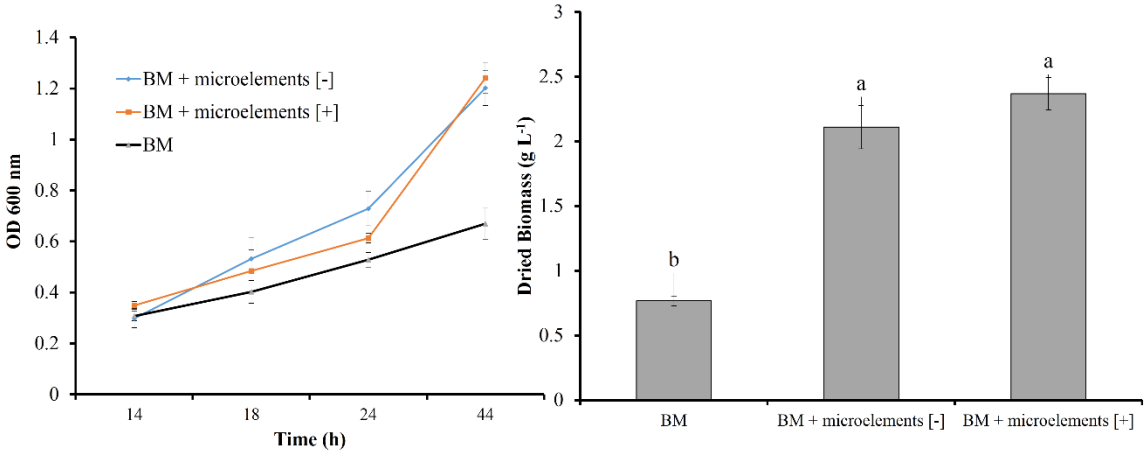
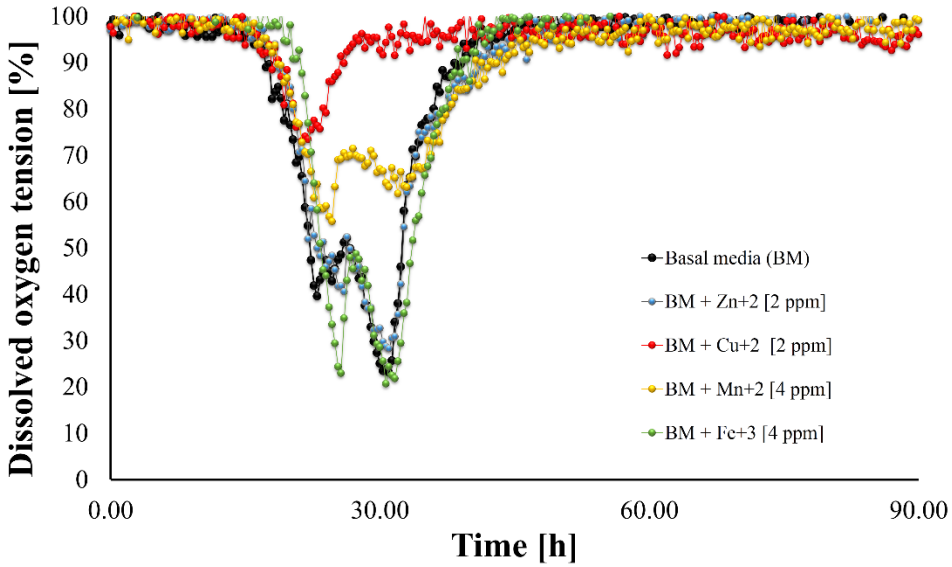
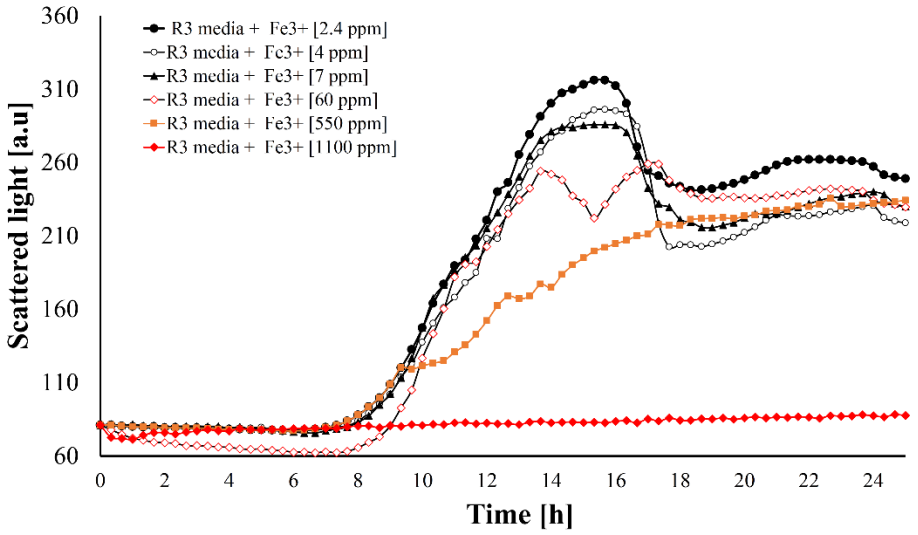


Figure 2-12 Effect of selected microelements [0.2 – 4 ppm] on *K. radicincitans* DSM16656^T On dissolved oxygen tension (DOT) in basal media. Sensibility test by microfermentation (Biolector®). Two concentration levels for each metal [-, +]. Zn [0.4, 2.0 ppm]; Fe [0.2, 4.0 ppm]; Mn [0.7, 4.0 ppm]; Cu [0.4, 2.0 ppm].



Regarding Fe^{+3} abundance in plant-based culture for isolating rhizobacteria (Nour *et al*. 2012), further Fe^{+3} resistance assay in bacterial cells was carried out. Then, in R3 media, Fe^{+3} was adjusted at 4, 12, 18, 70, 550 and 1100 ppm, to elucidate its susceptibility against this metal-ion (Figure 2.13).

Figure 2-13 Effect of selected microelements Fe^{+3} on *K. radicincitans* DSM16656^T kinetic growth



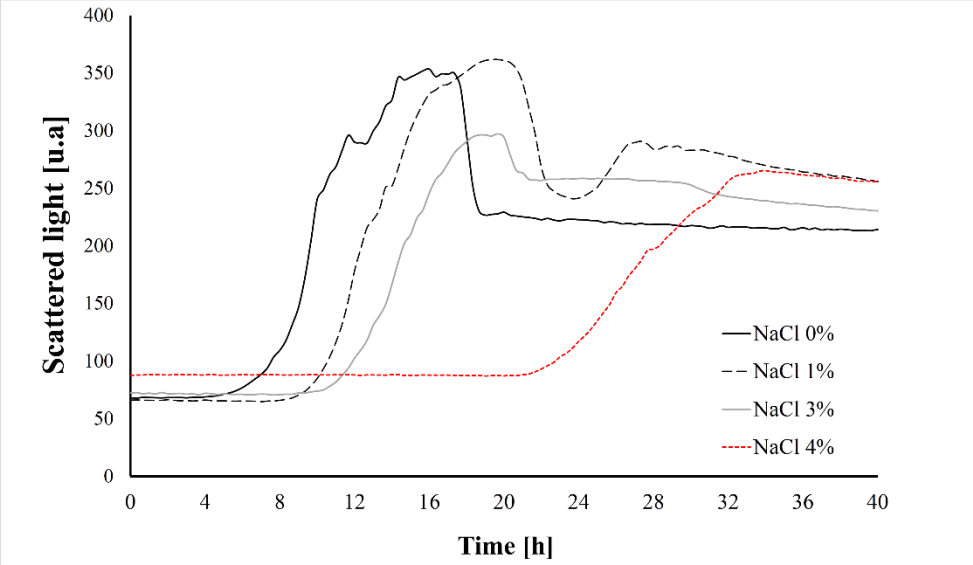
Metal ions are critical for many reactions; however, metal excess can be toxic. Metal-ion resistance in *K. radicincitans* DSM16656^T is an interesting aspect, since studies regarding the metal toxicity in bacteria suggest that in general Fe^{+3} is more toxic than Cu^{+2}, Zn^{+2} and Mn^{+2} (Cornelis *et al*. 2011; Chandrangsu *et al*. 2017). The high resistance against Fe^{+3} , even proliferating at 550 ppm, is surprisingly interesting, and comparable with other Gram-negative bacteria, such as the halophyte *Chromohalobacter salexigen* which can proliferate at high iron concentration at 50 µM $FeCl_3$ (2.8 ppm Fe^{+3}) (Argandona *et al*. 2010) (Figure 2.13). Finally, studies considering metals resistance in DSM16656^T generate important knowledge, upon bioremediation alternatives and future applications may emerge.

2.3.5 Effects of osmotic stress and compatible solutes on *K. radicincitans* DSM16656^T cultivation

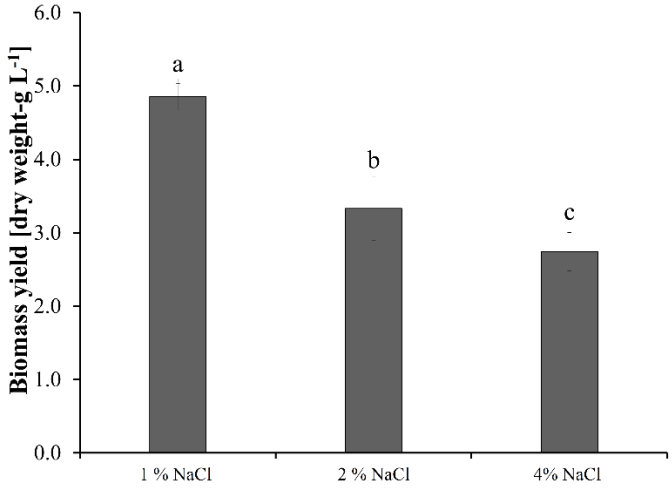
To assess the osmoadaptation capability of *K. radicincitans* DSM16656^T, the cultivation at high salinities was conducted. Moreover, the affinity for compatibles solutes uptake exerted

by osmotic stress was also tested. In this research, the capability of the bacterium endophyte for proliferating at 4% NaCl was demonstrated (Figure 2.14 A). Assays were conducted in the BioLector® micro-fermentation system with 48-well BH microplates with 1 ml R3-buffered media at 1200 rpm and 30°C. The increment of salt in media strongly impaired the growth of the bacteria. Thus, biomass yield decrease from 4.85 ± 0.18 to 2.74 ± 0.25 g L^{-1} for non-stresses cells to 4%NaCl added-media respectively (Figure 2.14 B). However, after 30 h of cultivation both cultivation reached 10^{11} CFU mL^{-1}, with concentration (log_{10}) values at 11.13 ± 0.11 and 11.02 ± 0.07 (n=3) for 1% NaCl and 4% NaCl treated cells respectively.

Figure 2-14 Salt stress effect on *K. radicincitans* DSM16656^T growth. **A.** Effect on kinetic growth by Biolector® approach in culture media R3 amended with NaCl [1, 3, 4%]. **B.** Effect on biomass production at 24 h cultivation. Lines represent the mean values of three replicates (n=3).



A

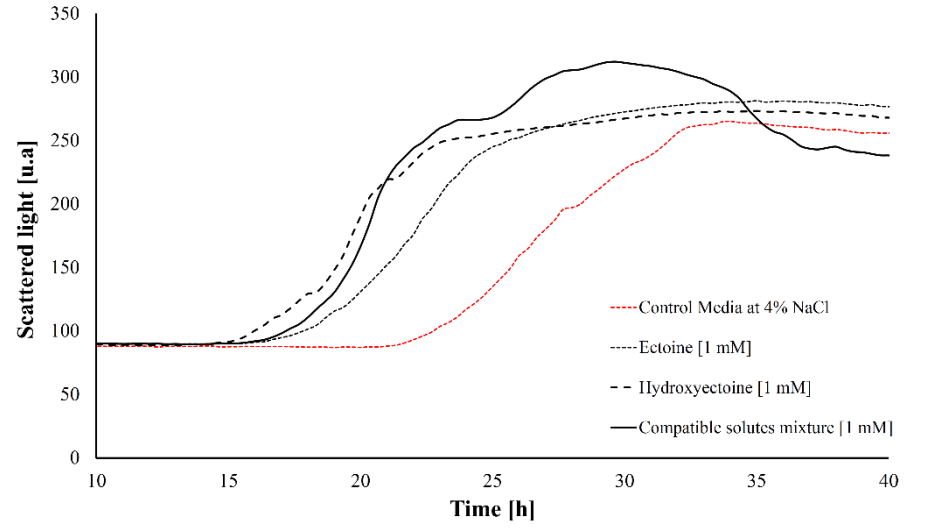


B

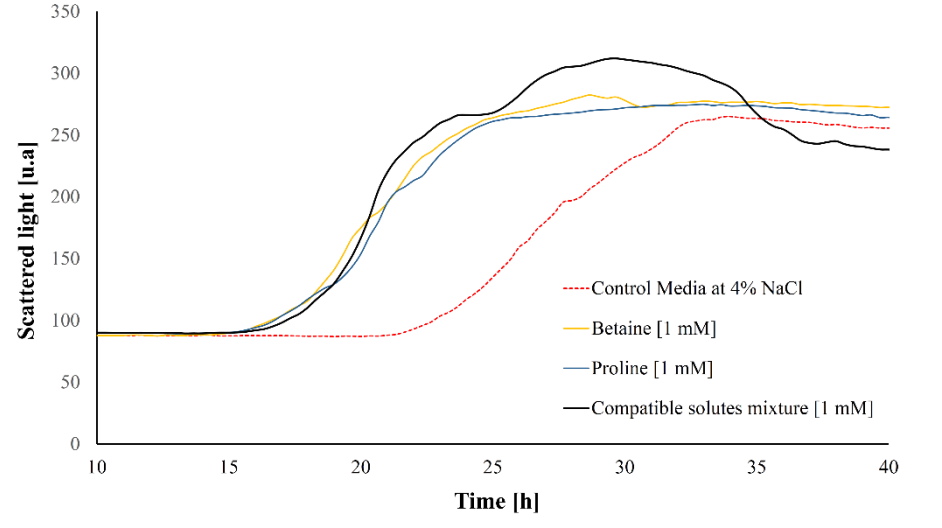
The positive role of compatible solutes during cultivation at high salinities is shown in Figure 2.15. Osmolytes such as ectoine and hydroxyectoine at 1 mM reduced the lag phase ~ 2 h in comparison to control media (Figure 2.15 A). The upper growth limit was also increased by the exogenous supply of these osmolytes in a mixture or independently. The osmoprotective effect of ectoines (1 mM) was reported in other Gram-negative bacteria to cope the proliferation at high salinities (1.2 M NaCl), such *Streptomyces coelicolor* A3 (2) (Bursy *et al*. 2008).

The amino acids proline, betaine, glutamine and glycine as osmolytes also provided advantages for conferring a rapid osmoadaptation (Figure 2.15 B-C). At a lower level, trehalose and glutamate could serve as a compatible solute for the bacterium but with a smaller effect (Figure 2.15 D). Betaine was reported as osmoprotectant for *Gluconacetobacter diazotrophicus* PAL5, which under high salinities (50-300 mM NaCl) led to increased kinetic parameters, reducing lag phase and biomass yields (Boniolo *et al*. 2009). Osmostress protection by amino acid uptake is reported in bacteria as physiological process, with the conversion to proline as a key trait. Hence in the absence of NaCl, proline is utilized as a substrate for protein synthesis, but at high salinities serves as a compatible solute in Gram-negative bacteria (Nagata *et al*. 2005).

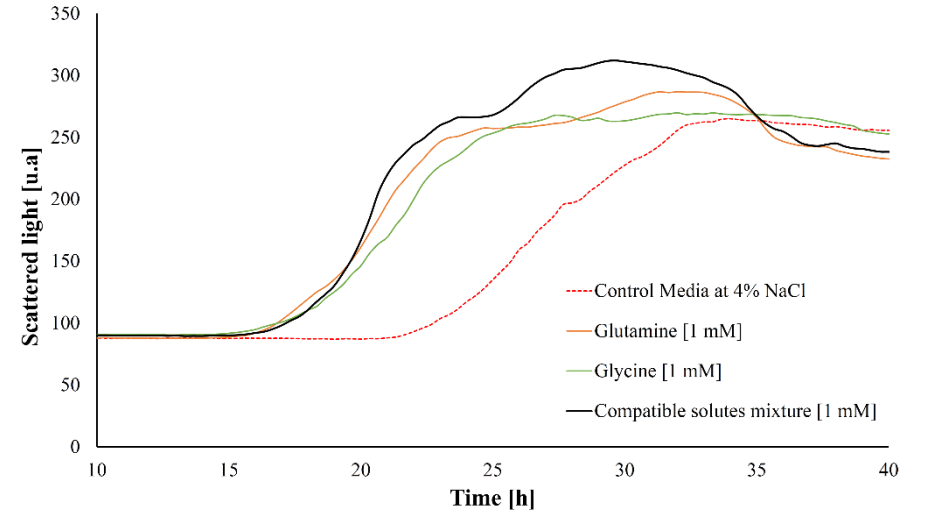
Figure 2-15 Effect of compatible solutes addition [1 mM] on *K. radicincitans* DSM16656^T growth at high salinity at 4% NaCl. **A**. Ectoine and hydroxyectoine. **B**. Proline and Betaine. **C**. Glutamine and glycine. **D**. Glutamate and trehalose. Scattered light curves of cultivations in a MTP in the BioLector® system. Lines represent the mean values of three replicates (n=3).



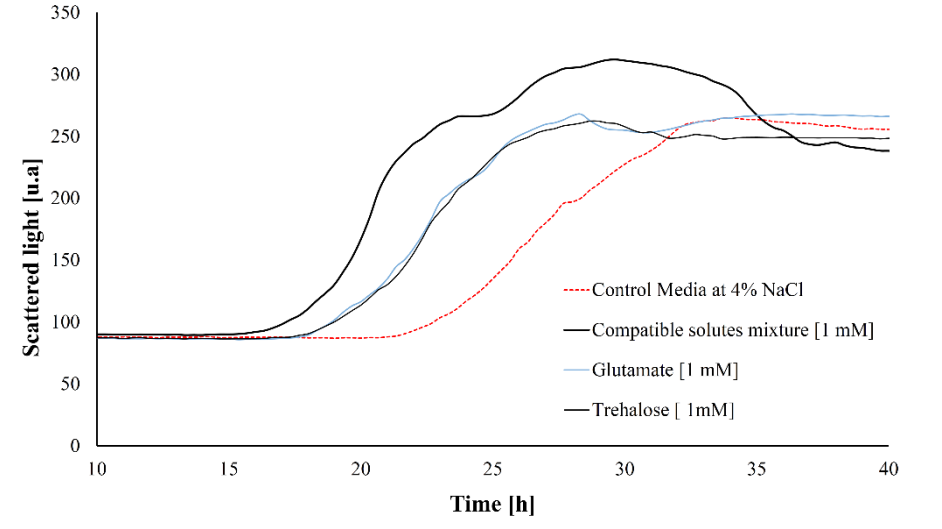
A



B



C



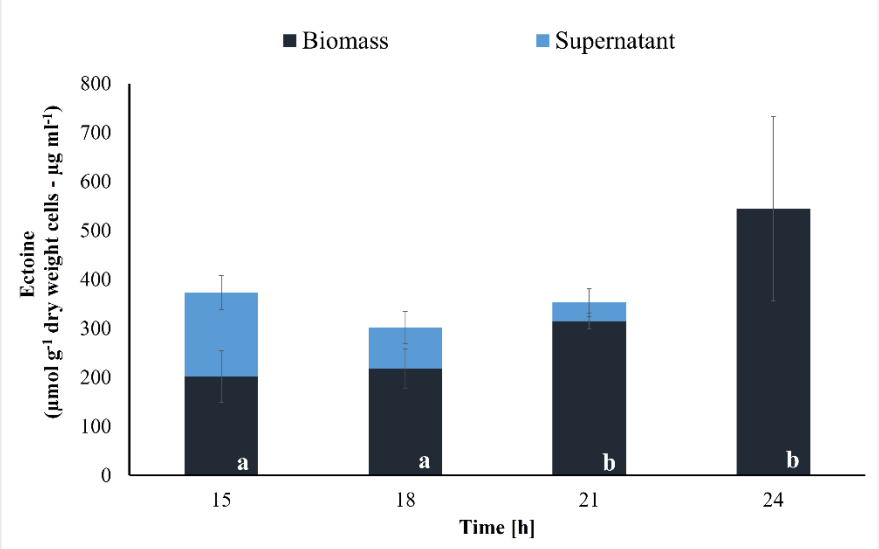
D

Figures 2.16 A–B shows the intracellular accumulation dynamics for ectoine and hydroxyectoine respectively. For both osmolytes, it was found a significant effect by cultivation time, ectoine ($F_{3,\ 15}$=16.05, p=0.0002, Figure 2.16 A) and hydroxyectoine ($F_{3,\ 15}$=36.45, p<0.001, Figure 2.16 B). After 24 h, the intracellular concentration of ectoine and hydroxyectoine reached values up to 544.4 ±187 and 528.6±165 µmol g^{-1} of dry biomass, equivalent to 77.39 µg mg^{-1} and 83.06 µg mg^{-1}. These yields are comparable to other studies

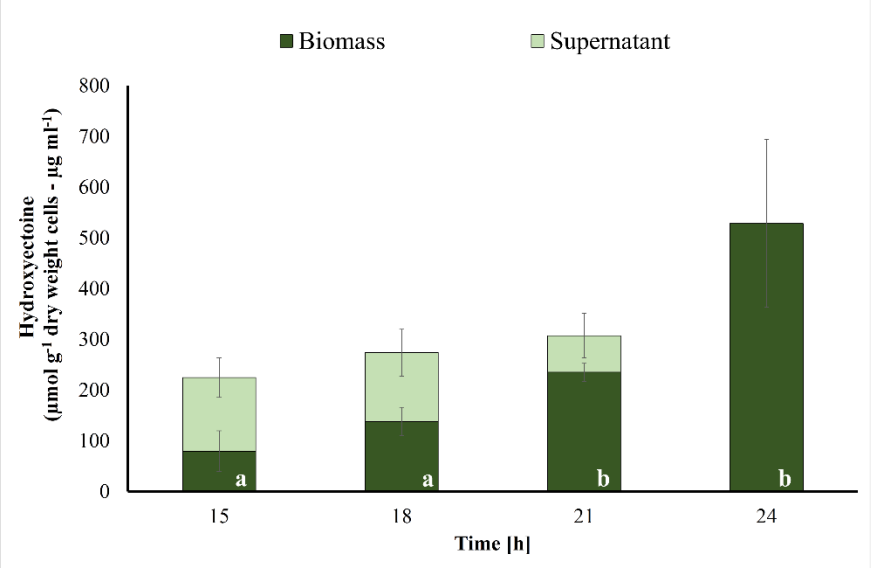
which reported intracellular concentration in *Halomonas elongata* KS3 at 120 µg mg^{-1} and 45 µg mg^{-1} of dry cells for ectoine and hydroxyectoine respectively (Ono *et al*. 1998). These results demonstrate the capacity of *K. radicincitans* DSM16656^{T} to accumulate these substances under conditions of osmotic stress. Control cells without osmotic stress did not accumulate ectoine or hydroxyectoine. It is observed that the solute uptake was driven by osmotic stress during the exponential growth phase, where the osmolyte was detected.

For the case of Proline, the confirmation of its uptake was conducted through GC-MS. Figure 2.17 showed the GC-MS spectra for intracellular extracts for control cells and proline-added cells. The peak at 20.19 min was identified as L-proline According to the NIST 98 database (NIST, MD, USA), and occurred in cells when this amino acid was added.

Figure 2-16 Intercellular uptake of selected compatible solutes by *K. radicincitans* DSM16656^{T} cells osmoadapted at 4% NaCl in culture media R3. **A.** Ectoine, **B.** Hydroxyectoine. Different letters indicate significant differences between biomass sampling times according to Tukey post hoc test (n=4, $p<0.05$).

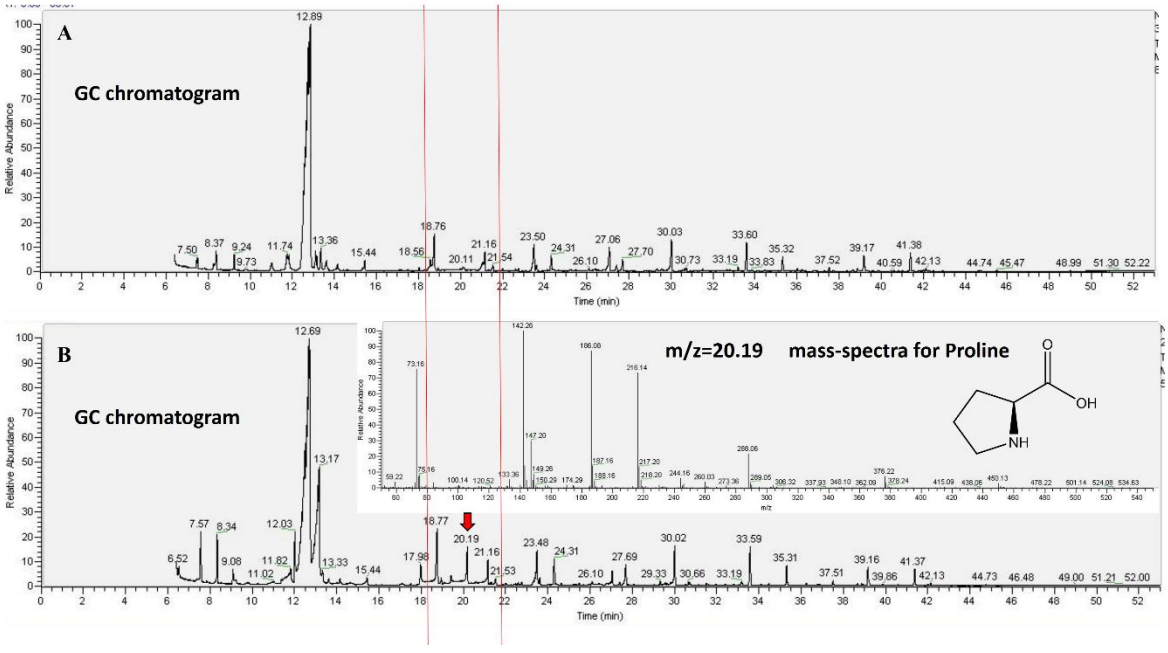


A



B

Figure 2-17 Intracellular identification of L-proline by GC-MS, after the uptake by *K. radicincitans* DSM16656^{T} cells after 24 h of cultivation. **A**. R3 media at 4% NaCl. **B** R3 media at 4% NaCl 4%: plus the addition of L-proline at 1 mM.



2.4 Discussion

Cultivation of bacterial endophytes represents a mandatory step prior to their exploitation as potential biological inoculants with a sustainable production at semi-industrial and industrial scales. The use of endophytic bacteria-based inoculants on increasing plant nutrition and protection has a great potential in developing countries (Brader *et al.* 2014; Santoyo *et al.* 2016), especially if the lower cost of liquid media could be developed for producing microbial biomass.

The relative new species *Kosakonia radicincitans* is a heterogeneous group of bacteria that occupy different natural ecosystems, providing plant growth-promoting features and playing an increasingly essential role in the study of endophytic bacteria (Witzel *et al*. 2017; Becker *et al.* 2018). Here, the research focused on the elucidation of advantageous nutrient sources for the endophyte *K. radicincitans* DSM16656^{T}. The strategy included the use of different approaches for revealing the affinity of this bacterium to multiple nutrients. Thereby, the initial step was to characterize the bacteria phenotypically using a Biolog GEN III MicroPlate^{TM} system, followed by the screening of different culture media available in the literature for rhizobacteria and endophytes. Further, the selection and optimization of media components by a Plackett-Burman design. Moreover, the inclusion of high-throughput microfermentation studies in BioLector® to investigate the effects of amino acids, macro and microelements on kinetic growth. Finally, the evaluation of osmotic stress and the addition of compatible solutes as potential pre-conditioning mechanisms for integrating the proliferation of the endophyte with the formulation step.

The technology GEN III of Biolog effectively allowed the phenotyping of *K. radicincitans* DSM16656^{T}. Our data revealed some key phenotypic traits related to substrate assimilation, such the utilization of carbon sources including polyols (D-mannitol, glycerol and sorbitol), sugars (D-maltose, gentiobiose and L-rhamnose), organic acids (mucic acid, acetic acid and gluconic acid), and nitrogen sources mainly amino acids (L-alanine, L-serine, proline and L-aspartic acid). The poor assimilation of histidine was consistent with previous studies in bacterial endophytes (Loiret *et al.* 2009). Beyond the GEN III results, the selection of glycerol as the main C-source in culture media relied on the cost of this substance and

benefits to provided osmotic stress to cells by decreasing water activity in media. Besides, *K. radicincitans* DSM16656^T possess a large plasmid gene for 1,2 propanediol degradation (PDD) and glycerol transformation (Becker *et al.* 2018). According to GEN III phenotyping, the bacterium can oxide D-galacturonic acid, the primary monomer in pectins, which provide knowledge that support the integration of this biopolymer as a potential carrier within the formulation step. Pectin also contains neutral sugars such as L-rhamnose, which is either inserted or attached to the main chains (Liu *et al.* 2003; Sriamornsak 2011). Interestingly, L-rhamnose was also discovered as a useful nutrient C-source for the endophyte. Noticeably, the presence of pdu genes (pduABDEFLMPQ) for propanediol degradation (PDD) and dha genes (dhaBDKLMT) for glycerol transformation (GT) on the large plasmid suggests that *K. radicincitans* DSM16656^T has the potential to anaerobically degrade rhamnose and fucose in plant cell walls and glycerol in plant lipids (Becker *et al*. 2018). GEN III results provided useful insights for the selection of culture media for screening and further phases of this research.

The screening of culture media supports the advantages of glycerol as C-source in comparison to starch or lactose for this endophyte. From an economic point of view, glycerol concentration at 1.5 % is enough to reach biomass yield similar to the higher concentration of the polyol, suggesting that yeast extract is limiting the biomass production. It is corroborated by the positive and significant effect found with the Plackett-Burman design.

Microfermentation in BioLector® revealed the affinity of endophytic bacterial cells to selected amino acids; thus L-alanine, L-proline and glutamic acid were identified as essential for growth, suggesting an activation of the alanine-aspartate-glutamate metabolism. Besides, provide insights into the potential amino acid derivatives that the bacterium may produce such antibiotics (i.e., Ecomycin) (Singh, Kumar *et al*. 2017). Moreover, these findings are in line with studies which report that alanine, aspartate and glutamate facilitate the proliferation of endophytic nitrogen-fixing bacteria, providing increments in ammonium production and nitrogenase activity to *Pantoea* sp. (Loiret *et al*. 2009). Likewise, plant-based culture media used for culturing rhizobacteria contains mostly these amino acids (Youssef *et al*. 2016).

Microelements represents a critical component in culture media, conferring grow co-factors and serving as a stabilizer of enzymes and intermediates of metabolites. Metals cannot be synthesized or degraded, then homeostasis primarily relies on modulating transport into and out the cell (Chandrangsu *et al*. 2017). For *K. radicincitans* DSM16656^{T} was established that relative high concentration of Fe^{+3} and Zn^{+2} did not impair the growth of the bacterium. Conversely, Mn^{+2} and Cu^{+2} must be used in liquid culture at lower concentrations than 2 ppm, since the DOT curves in BioLector® revealed an inhibitory effect of these minerals at higher concentration.

The potential iron tolerant cells of the endophyte may provide advantages through siderophore biosynthesis, iron import pathways and iron chelation in high concentrated plant microbiomes (Kandel *et al.* 2017). The tolerance to Fe^{+3} and Zn^{+2} can be explained by more efficient metal homeostasis mechanisms and storage events such ferritin-related proteins (Chandrangsu *et al*. 2017). Besides, according to the high contents of Fe^{+3} and Zn^{+2} in plant extracts using to proliferate bacterial endophytes, is expected the natural affinity of the endophyte for them (Nour *et al.* 2012; Youssef *et al*. 2016). Noteworthy, *K. radicincitans* DSM 16656^{T} can proliferate in iron (III) concentration higher than 500 ppm (Figure 2.13), suggesting efficient sequestration proteins, Fe^{+3}-siderophore ABC transporter permease activity, and efflux pumps. DOT curves suggest that Fe^{+3} can be used by the bacterium as an electron acceptor under anoxic conditions. In parallel, to the potential iron uptake, bacterium controls the formation of dangerous reactive oxygen species (ROS) *(Cornelis et al.* 2011).

2.4.1 Effects of osmotic stress and compatible solutes on *K. radicincitans* DSM16656^{T} cultivation

According to the cultivation under osmotic stress assessments, *K. radicincitans* DSM16656^{T} can be considered slightly halotolerant, since it can proliferate at 4% NaCl and showed respiration activity in GEN III profiling at 8% NaCl (Appendix 2). Although kinetic parameters are impaired along salt content in media, the use of exogenous compatible solutes alleviates the cell adaptation. Hence, ectoine, hydroxyectoine and proline

demonstrated the highest osmolyte effects, by reducing lag phase and in some cases extending the upper growth limit.

The analysis of the chromatograms in HPLC-UV confirmed the intracellular accumulation of ectoine and hydroxyectoine. Interestingly, according to its genome, *K. radicincitans* DSM16656^{T} lacks ectoine synthase and ectoine hydrolase, in consequence, cannot synthesize or catabolize neither ectoine nor hydroxyectoine (Witzel *et al.* 2012; Czech *et al.* 2018), indicating that the uptake molecules are not metabolized within the cell. On the other hand, proline is considered another potential compatible solute during the osmoadaptation of cells, its accumulation was confirmed via GC-MS. Considering the beneficial effect of compatible solutes, it is hypothesized that the inclusion of these substances as supplements in salt-added liquid media may support the strength of cells prior to formulation processing and subsequent may provide advantages during plant-endobiome establishment.

To summarize, after the different approaches carried out in order to elucidate advantageous nutrient sources for *K. radicincitan*s DSM16656^{T}, here it is proposed that culture media must contain a polyol as glycerol as main C-source at 1.5% when the aim is harvesting at 24 h, as N-source, yeast extract which may provide the amino acids and minerals required for proliferation. Hence, supplementation of this media can contain at 1 mM amino acids such as proline, glutamic acid and L- alanine. Microelements addition [Zn^{+2}, Fe^{+3}, Mn^{+2}, Cu^{+2}] are needed for enhancing kinetic growth parameters. Finally, at high salinities, a rapid adaptation and growth of this bacteria endophyte require the inclusion of compatible solutes; thus ectoine and hydroxyectoine can provide the best performances at 4% NaCl with a low risk to be assimilated as C-source compares to proline and betaine.

2.5 Conclusions

The phenotypic characterization in GEN III technology and the assessment of culture media for *K. radicincitans* DSM16656^{T} revealed the affinity of bacterial cells for polyols as advantageous carbon sources. The amino acids L-alanine, proline and glutamic acid as N-sources enhance the kinetic parameters of the endophyte significantly. Microelements such

as Fe, Zn, Mn and Cu are required in liquid culture media for f bacteria proliferation. After the systematic application of microfermentation and, the obtained cost-effective liquid media permitted 32-fold increment of cells concentration up to 2x 10^{11} CFU mL^{-1} at 24 h in comparison to the typically used standard nutrient broth.

Compatible solutes such as ectoine, hydroxyectoine, proline and betaine are a feasible strategy to accelerate bacteria growth and to increase biomass yields under high salinities. These findings comprise valuable knowledge upon the integration of cultivation formulation and plant interaction can be developed.

3. CHAPTER 3. OSMOTIC STRESS AND HYDROXYECTOINE INCREASE PHOSPHATE SOLUBILIZATION AND PLANT COLONIZATION CAPACITY OF *K. radicincitans* DSM16656^{T}

3.1 Introduction

Bacterial endophytes have attracted considerable attention because of their capability to promote plant growth through direct or indirect mechanisms. Direct mechanism includes the nutrients acquisition facilitation or hormones modulation, and the indirect mechanism by pathogen inhibition (Reinhold-Hurek and Hurek, 2011; Santoyo *et al.* 2016). Many bacterial endophytes can support host plants by counteracting negative impacts on the environment (high salinities, heavy metal concentration, etc.) and are classified as plant growth-promoting endophytic bacteria (PGPEB) (Rosenblueth and Martinez-Romero, 2006; Ryan *et al.* 2008). Colonization by PGPEB is essential for providing benefits to host plants. Endophytic colonization involves the entry, growth and proliferation of bacterial populations within the plant (Reinhold-Hurek and Hurek, 2011; Kandel *et al.* 2017).

The colonization patterns of PGPEB in plant tissues are strongly dependent on several biotic and abiotic factors (Hallmann, QuadtHallmann *et al.* 1997; Gaiero *et al.* 2013). Moreover, rhizosphere microbial communities and metabolic-pathway profiles may be entirely different from those of endophytes (Timm *et al*. 2015). Hence, variations in abiotic factors such as temperature (Mocali *et al.* 2003), soil type (Gottel *et al*. 2011), pH (Hardoim *et al.* 2012), and nutrients availability have been described to influence bacterial plant colonization behavior. Especially, soil salinity alters bacterial community composition and their functional activities (Yaish *et al*. 2016; Kandel *et al.* 2017; Szymanska *et al.* 2018). Thus, bacterial endophytes may help the plant to withstand such sudden osmolarity peak conditions in their ecological niche, by regulating hormones such as auxin, cytokinin, and ethylene.

Organic osmolytes enable organisms to adapt to environmental conditions by protecting cells or molecules against salt stress. These organic osmolytes are generally ‘‘compatible” with the metabolism of the cell without adversely affecting macromolecules or physiological processes and are referred to as compatible solutes (Brown, 1978). These solutes accumulate either by synthesis or by transport from the extracellular medium through osmotically regulated transporters and mechanosensitive channels (Wood *et al*. 2001). Moreover,

compatible solutes provide beneficial enzyme functions, protecting against high temperature, desiccation, salinity, freeze-thaw procedures and even drying (Lippert and Galinski, 1992; Sleator and Hill, 2002; Manzanera *et al*. 2004). Among these, intracellular solute such hydroxyectoine is well known in halophytic bacteria (del Moral *et al*. 1994; Ono *et al.* 1998), and the latter is considered a protein protectant (Wang *et al.* 2006). Despite these benefits, the effect of hydroxyectoine enrichment in bacterial cells on bacterial plant colonization behavior is still unknown.

The ability of endophytic bacteria to solubilize phosphorus has been amply demonstrated (Estrada *et al.* 2013; Joe *et al.* 2016). These studies have in many cases, corroborated the *in vitro* results of phosphorus solubilization with *in vivo* studies. Hence, the monitoring of the capacity to solubilize phosphorus by *K. radicincitans* DSM16656^{T} is proposed during the phases of media design, pre-conditioning and formulation as a model for evaluating the *in vitro* biological activity of the endophyte. This monitoring would be used taking into account the potential genotypic or phenotypic changes that the bacterium may have in each phase, depending on the different environments (Rainey and Travisano, 1998). Likewise, it will serve as criteria to address the research and further serves as a tool for the explanation or correlation of the results in the efficacy test in the presence of the plant system.

The course of nutrients use including the phosphorus uptake, emphasizes the necessity to stimulate its availability during early plant growth stages during the endophytic establishment (Schilling *et al.* 1998; Singh *et al*. 2013). Thus, beyond the well-documented plant growth-promoting benefits of this bacterial strain, and its inorganic phosphate solubilization capability, the response of this endophyte to both the osmotic stress and the accumulation of compatible solutes, and how these conditions may influence its phosphate solubilization ability and subsequent plant colonization activity are still unknown. Here, it is hypothesized that providing exogenous hydroxyectoine during the adaptation at high salinities in culture media may synergistically influence the phosphatase enzymes of *K. radicincitans* DSM16656^{T}, enhancing its physiological machinery for phosphate solubilization (*in vitro*) and plant colonization activity.

Members of the *Brassicaceae* family are economically important crops, which the benefits of associated bacterial endophytes have tested successfully (Card *et al.* 2015). Previously studies demonstrated the ability of *K. radicincitans* DSM 16656^T to colonize internal tissue of *Brassicaceae* species such as radish (*Raphanus sativus L. var. sativus*) (Berger *et al.* 2015), independently of the site of application. Radish has features that favor the assessing of effects caused by environmental variables such as the short growth cycle (24-30 days), small size enabling a large number of treatments, easy determination of growth parameters and nutrient uptake from soil (Kostka-Rick and Manning, 1993). In particular, radish easily responds to the application of PGP and especially P-solubilizing bacteria with leave and tuber growth improvement and P-uptake responses (Lara *et al.* 2013). Therefore, this plant was selected as a model for testing plant growth-promoting and endophytic activity of pre-conditioned cells.

3.2 Materials and Methods

Compatible solute standard hydroxyectoine (H-ectoine) was acquired from Sigma Aldrich (Cat: 70709, Sigma Aldrich Corporation, Darmstadt, Germany). All other materials used were of analytical reagent grade and used as received.

3.2.1 Bacterial and growth conditions

The bacterial strain *K. radicincitans* DSM16656^T was provided by the Leibnitz Institute of Vegetable and Ornamental Crops in Grossbeeren, Germany. Chemically defined growth medium (DM) was routinely used (g L^{-1}): glycerol (15), yeast extract (8), K_2HPO_4 (2.74), KH_2PO_4 (1.31), $MgSO_4$ $7H_2O$ (0.5), $FeSO_4H_2O$ (60 ppm), $MnSO_4$ (10 ppm) at pH 7.4. Pre-conditioning of bacteria before plant colonization assays was carried out by amending the DM with NaCl [1 and 4%] and providing hydroxyectoine [1 mM] to DM 4% NaCl.

Pre-conditioned bacterial cells for plant colonization experiments were prepared as follows: DM (100 mL) was poured into 250 mL baffled Erlenmeyer flasks that were autoclaved at 121°C under 1.5 atm, for 30 min. The initial inoculum concentration in the media was adjusted at 10^6 cells mL^{-1}. The cultures were maintained at 190 rpm in a rotary incubator at

30 ± 1°C (IKA KS 4000 IC Control, Staufen, Germany). Hydroxyectoine was sterilized separately by filtration through a 0.2 µm membrane filter (Durapore® 0.2 µm polyvinylidene fluoride (PVDF), Millipore, Darmstadt, Germany). Actively growing cells were harvested at the exponential phase after 20 h (OD_{600} of 0.7-0.9) by centrifugation at 6875 x g for 15 min (Centrifuge 5810R, Eppendorf, Wesseling, Germany), and the obtained pellet of bacteria was washed and centrifuged twice with corresponding NaCl solution [1 or 4%] to maintain the osmotic pressure. The three pre-conditioned bacteria type cells [NaCl 1%, 4% and 4% + H-ectoine] were stored in the same NaCl solution adjusted to OD_{600} ~ 1.0 until use in plant colonization assays. The intracellular hydroxyectoine concentration before endophytic establishments experiments was determined via HPLC (Teixido *et al.* 2005).

3.2.2 Osmoadaption and effect of hydroxyectoine: Microfermentation procedure

The influence of different NaCl concentrations [0, 1, 3, 4%] in DM and the addition of hydroxyectoine on *K. radicincitans* DSM16656^T kinetic growth was monitored online in microtiter plate cultivations (MPCs), with a microbioreactor, the RoboLector-BioLector® system (m2p-labs, Baesweiler, Germany). Hydroxyectoine was sterilized separately by filtration through a 0.2 µm membrane filter (Durapore® 0.2 µm PVDF, Millipore, Ireland), and added at 1 mM final concentration to the DM media (See chapter 3). The microtiter plate (MTP) assays were conducted in 48-well flower plates, and the plates were enclosed with an adhesive gas-permeable membrane with evaporation reduction layer, but allowing for sufficient and uniform aeration (F-GPR48-10, m2p-labs, Baesweiler, Germany) (Huber *et al.* 2009).

The BioLector® instrument was used for non-invasive online assessment of scattered light (signal representing the biomass formation) during cultivations for 48 h. Signals were acquired by irradiating each well with a light of a defined wavelength in a filter (excitation) and detecting and interpreting the reflected/scattered light. The monitoring of all BioLector® cultivations used the following adjustments: Scattered light (filter 620 nm, Gain 20), pO_2-optode (filter 500 nm, Gain 33). The experiments were carried out at 30°C under constant stirring (1200 rpm, shaking diameter = 3 mm, orbital) in 48-well MTP-48-BO

flower- plates, Lot No: 1711 (mp2-labs, Baesweiler, Germany) with an adjusted volume of 1000 µl DM. Each treatment was composed of three replicates. High densities of bacterial cells were necessary to correlate scattered light intensities and biomass concentrations in BioLector®. A concentrated starter culture of *K. radicincitans* DSM16656^T (~ 5 g dry matter L^{-1}) was diluted in DM and measured at the same operating conditions as in the cultivation assessments (Kensy *et al.* 2009) (Appendix 3).

3.2.3 Mineral phosphate solubilization in liquid media

The efficiency of *K. radicincitans* DSM16656^T of both pre-conditioned osmoadapted cells and osmoadapted hydroxyectoine-added cells for phosphate solubilization were measured via Pikovkaya's (PVK) liquid media (pH 7.2 ± 0.2) (Schoebitz, Ceballos *et al.* 2013). PVK media were amended with 4% NaCl and 4% NaCl plus hydroxyectoine [1 mM] to reveal the effects of osmotic stress and the osmolyte on bacterial phosphate solubilization ability. Each flask containing 100 mL of PVK was inoculated with 100 µL of a bacterial suspension at 10^8 cells mL^{-1}. The flasks were incubated for approximately nine days at 30 ± 1°C under shaking at 190 rpm on a rotary shaker (IKA KS 4000 IC Control, Staufen, Germany). Four independent replicates per treatment were tested. Quantitative spectrophotometric analysis of the soluble phosphate was performed at 24 h, 48 h, 120 h and 200 h according to a standard protocol (Murphy and Riley, 1962) (Appendix 4). In parallel, the influence of osmotic stress and the addition of hydroxyectoine on pH evolution in PVK medium was monitored online, using microtiter plate cultivations (MPCs) (RoboLector-BioLector system, m2p-labs, Baesweiler, Germany). The following adjustments were used: Scattered light (620 nm filter, gain 3), pH-optode (Filter pH[HP8] Ex (nm)=470 nm; Em(nm)=525, gain 7), and 1000 µL of PVK media incubated in 48-well MTP-48-BH flower- plates, Lot No: 1808 at 30°C and 1200 rpm.

3.2.4 Phosphatase enzyme activity

Phosphatase activity was determined using p- nitrophenyl phosphate disodium (PNPP, 0.025 M) as a colorimetric substrate. For the assay, 2 mL of 0.5 M modified universal buffer (MUB) buffer adjusted to pH 6.5 (acid phosphatases) and 11 (alkaline phosphatases), and

0.5 mL of the substrate were added to 0.5 mL of PVK supernatant medium. Cell-free supernatant samples were obtained by centrifuging 2 mL of culture at 21382 x g for 10 min (Mikro HT 200R, Hettich GmbH & Co. KG, Tuttlingen, Germany). Reactions were carried out at 37±1°C for 60 min and stopped by the addition of 0.5 mL of 0.5 M $CaCl_2$ and 2 mL of 0.5 M NaOH. Samples were filtered by using filter paper (grade 401) retention time 12-15 µm pore size. The p-nitrophenol (PNP) formed was measured spectrophotometrically at 400 nm (Genesys 10S UV-Vis, Thermo Fisher Scientific, Waltham, MA, USA) (Tabatabai and Bremner, 1969; Murphy and Riley, 1962) (Appendix 4). Four independent replicates per treatment were tested. A control treatment including a hydroxyectoine solution [1 mM], were analyzed under the same experimental conditions. The unit of enzyme activity (U) was expressed as micrograms of PNPP released per milliliter per hour (µg mL^{-1} h^{-1}) and normalized to dry biomass (mg) produced after 24 h.

3.2.5 Promotion of radish plant growth by osmoadapted *K. radicincitans* DSM16656^T cells

The effects of both osmoadaptation and the inclusion of hydroxyectoine into *K. radicincitans* DSM16656^T on plant growth promotion were tested under glasshouse conditions. Radish (*Raphanus sativus* L. var. *sativus*) seeds of cultivar Rondar (an F1 hybrid; S & G GmbH, Kleve, Germany) were used in all experiments. Radish inoculation with osmoadapted bacterial cells and hydroxyectoine-added cells was conducted by immersing radish seeds into a bacterial suspension (10^8 cells mL^{-1}) for 5 min, for ensuring spermosphere establishment (Figure 3.7). Afterward, 10 inoculated seeds were placed in pots (10 pots per treatment), filled with 1.5 L of a 1:1 (v/v) quartz-sand soil mixture (Fruhstorfer Erde type T25: P_2O_5: 200-300 mg L^{-1}, Hawita Gruppe GmbH Vechta, Germany). The pots were then placed randomly on trivets to avoid the transfer of bacteria between individual pots (Berger *et al.* 2015) (Figure 3.1). Seeds treated with NaCl solution [1 and 4%] were used as controls. Seedlings were irrigated manually with 50 mL of tap water per day (conductivity 0.005-0.05 S/m). Plants were maintained under natural light conditions in the glasshouse at an average temperature of 18 ± 4°C and an air humidity > 45%. At one and four weeks post-planting, the plants were harvested.

One-week-old seedlings samples from three different locations per pot were taken, rinsed thoroughly with sterile water for removing soil and loosely adhered bacteria and flash frozen for nucleic acid extraction. Afterwards, the plants were equally thinned to five plants per pot. Total fresh plant material (tuber and leaves), as well as the tuber diameter of each plant were measured at four weeks old plants. The leaves were separated from the roots, and both of them oven-dried at 60°C (WTC Binder 342, Germany) until constant weight, after which the dry weight of tubers and leaves were measured. The complete experiment was repeated twice.

Figure 3-1: Arrangement of pots during the plant growth promotion experiments in radish under glasshouse conditions

3.2.6 Nucleic acid extraction and quantification of *K. radicincitans* DSM16656^T *in planta* using qPCR

DNA was extracted from approximately 50 mg of lyophilized plant root material (one-week-old seedlings) using DNeasy Plant Mini Kit (Qiagen, Hilden GmbH, Germany) according to the manufacturer's instructions. The lysis of plant tissue and bacterial cells was ensured by the addition of 5 mm sterile metal beads and by mechanical cell disruptor (Retsch MM200, Haan, Germany) at 30 rpm for 5 min. DNA quality and purity were assessed photometrically (NanoDrop, Thermo Fischer Scientific, Darmstadt, Germany). Quantitative

real-time PCR (qPCR) was conducted using Advanced TM Universal SYBR® Green I Dye Supermix (Bio-Rad Laboratories, Hercules, CA, USA).

Considering the genome of *K. radicincitans* DSM16656^T (Witzel *et al.* 2012) and its comparison with other Enterobacteriaceae genomes. A DNA repair protein was found to be specific to *Kosakonia radicincitans* and closely related taxa. Following Witzel *et al* (2017), specific primers were designed using Geneious from Biomatters and Oligo Calc from Northwestern University. Primer details are given in (Appendix 5).

K. radicincitans DSM16656^T species-specific primers and the plant TEF reference gene were used for *in planta* bacterial quantification (Witzel *et al*. 2017). The fold colonization of *K. radicincitans* treated plants with respect to the reference gene and the control plants were calculated and represented with the $2^{-\Delta\Delta cq}$ method (Livak and Schmittgen, 2001). This method is a convenient way to analyze the relative changes in gene expression from real-time quantitative PCR experiments.

Statistical analysis

The data were analyzed using SPSS Statistics v.2 software (SPSS, Chicago, IL). Data are presented as mean values ± standard deviations (SD) or standard error (SE). The means were tested for significant differences by one-way analysis of variance (ANOVA) followed by the Tukey post hoc test. Repeated measures ANOVA was carried out for the phosphate solubilization data. The level of significance was set at $p<0.05$.

3.3 Results

3.3.1 Osmoadaption and effect of hydroxyectoine: Microfermentation procedure

To investigate the osmotic pressure effect on kinetic growth response of *K. radicincitans* DSM16656^T, a high-throughput microfermentation strategy using a microbioreactor system was followed. The scattered signal demonstrated that bacteria cells showed growth variability under different a_w [0.97, 0.96, 0.955, 0.95] conditions, generated by the ionic solute NaCl at 0, 1, 3 and 4% in DM, respectively. Interestingly, bacterial cells were able to grow in media with a low a_w, simulating environmental stress conditions (Figure 3.2 A). At 16 h, *K. radicincitans* DSM16656^T in DM 1% NaCl (0.96 a_w) reached a similar growth of cells density relative to the control without NaCl addition in DM at 0.97 a_w.

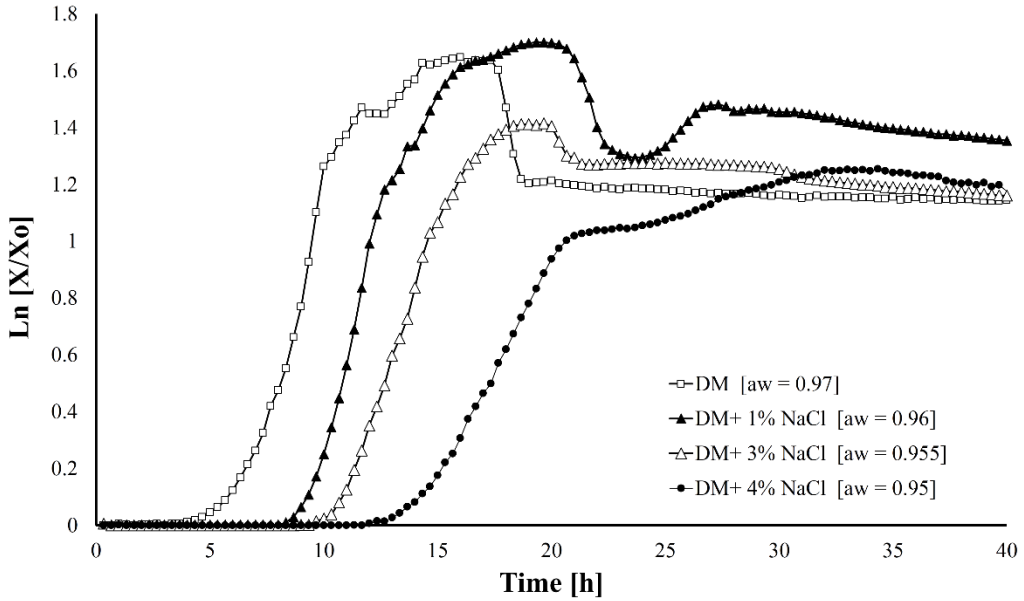
Relative to the biomass (X) evolution with the highest value of Ln (X/Xo) data, the maximum growth of 88.91% ± 1.49 % and 75.99% ± 1.82 % of the control was obtained at 19.3 h and at 33 h for 0.955 a_w and 0.95 a_w respectively (Figure 3.2 A). However, at 0.95 a_w proliferation of bacteria was strongly impaired. Thus, further kinetic parameters were also affected by increasing the salinity in media such maximum specific growth rate (μ_{max}). The μ_{max} which was taken at the point of highest slope or maximum exponential growth stage in the range at 7-10 h, 10-12.3 h, 12-14.3 h and 17-20 h for NaCl at 0, 1, 3 and 4% in DM, respectively (Figure 3.2 A). The μ_{max} for no amended media and 1% NaCl were 0.334 ± 0.004 $h^{-1}$and 0.361 ± 0.008 h^{-1}, decreasing significantly to 0.2849± 0.015 h^{-1} in DM at 3 % NaCl and 0.1529 ± 0.0026 h^{-1} in DM 4% NaCl ($F_{3, 11}$=319.5, p<0.001). *K. radicincitans* DSM16656^T was not able to grow at a_w lower than 0.94. Glycerol as main C-source was depleted after 18 h and 30 h in DM 1% and 4% NaCl respectively (data not shown).

Dissolved oxygen tension (DOT) signal displayed no oxygen limitation throughout the whole cultivation in DM (a_w = 0.97) (DOT≥ 60 %). DOT curves dropped until complete consumption of the glycerol after 10 h, 19.3 h and 21.3 h for DM without salt and supplemented with 1 % and 3 % NaCl respectively (Figure 3.2 B). Interestingly, DOT curve at 4% NaCl showed in the range of 20-47.3 h an extended plateau at levels lower than 8 %

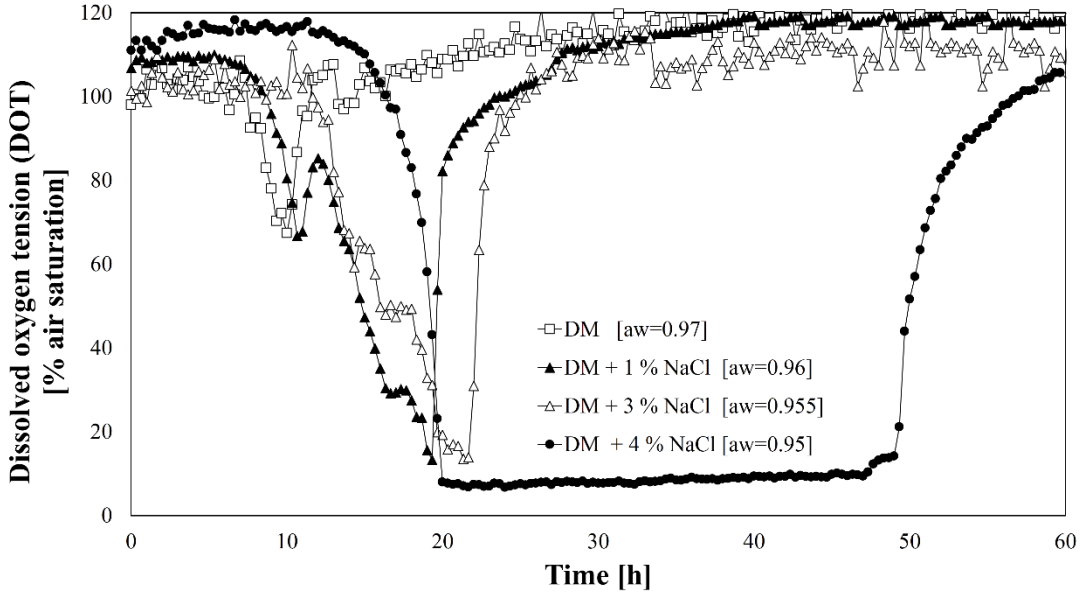
of air saturation, indicating a high oxygen consumption rate during the exponential and early stationary phases.

K. radicincitans DSM16656^T osmoadaptation improved by adding hydroxyectoine at 1 mM. Hence, exogenously provided hydroxyectoine extended the upper growth limit of *K. radicincitans* DSM16656^T under high-salinity growth conditions. Thus, after 24h of incubation in the presence of hydroxyectoine, the biomass increased significantly by 15.18 ± 3.82 %, compared to that obtained at DM 4% NaCl. Furthermore, the lag-phase in DM 4% NaCl [a_w 0.95] lasted 14 h and it decreased by 3.1 h with the inclusion of hydroxyectoine (Figure 3.2 C). The specific growth rate was also significantly higher in hydroxyectoine-added cells at 0.1808 ± 0.004 h^{-1} (range at 18-20 h) in comparison to DM at 4% NaCl at 0.1562 ± 0.004 h^{-1} ($F_{1,5}$=18.98, p=0.0121).

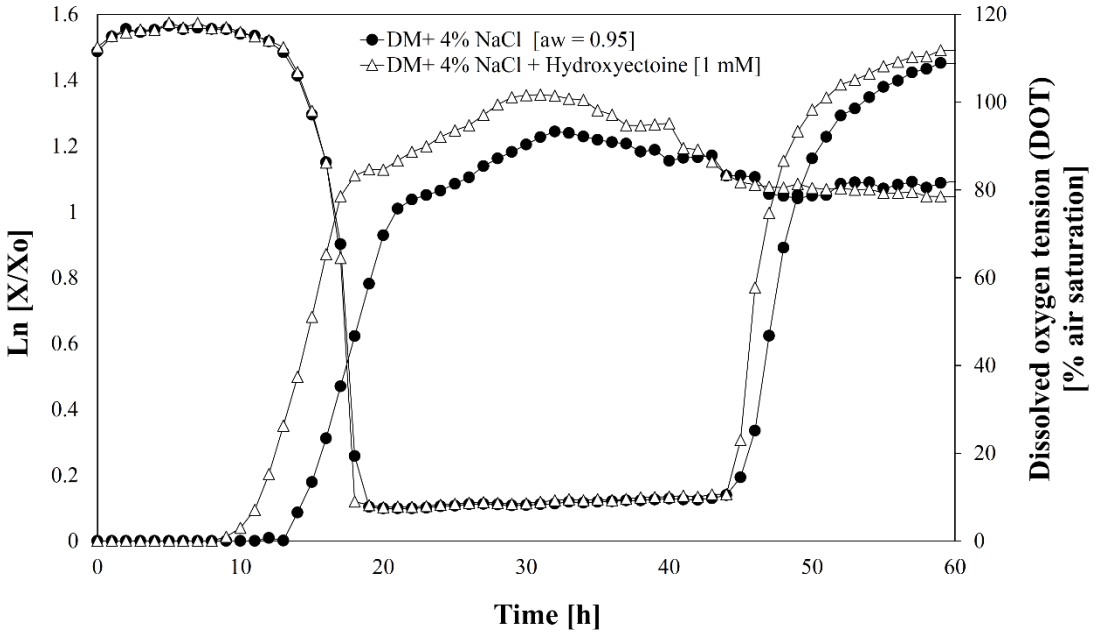
Figure 3-2: A. *K. radicincitans* DSM16656^T osmoadaptation at different NaCl concentrations in defined media (DM). **B.** Effect of NaCl concentration in DM on dissolved oxygen tension (DOT). **C.** Effect of addition at 1 mM of hydroxyectoine on *K. radicincitans* DSM16656^T kinetic growth during osmoadaptation in DM at a_w 0.95 (4% NaCl). Ln [X/Xo] values were calculated by calibrating scattered light intensities and biomass (X) concentration curves of cultivations in a MTP in the BioLector® system. Mean values, n=3.



A


Dissolved oxygen tension (DOT)
[% air saturation]
Time [h]
DM [aw=0.97]
DM + 1 % NaCl [aw=0.96]
DM + 3 % NaCl [aw=0.955]
DM + 4 % NaCl [aw=0.95]

B


Ln [X/Xo]
Dissolved oxygen tension (DOT)
[% air saturation]
Time [h]
DM+ 4% NaCl [aw = 0.95]
DM+ 4% NaCl + Hydroxyectoine [1 mM]

C

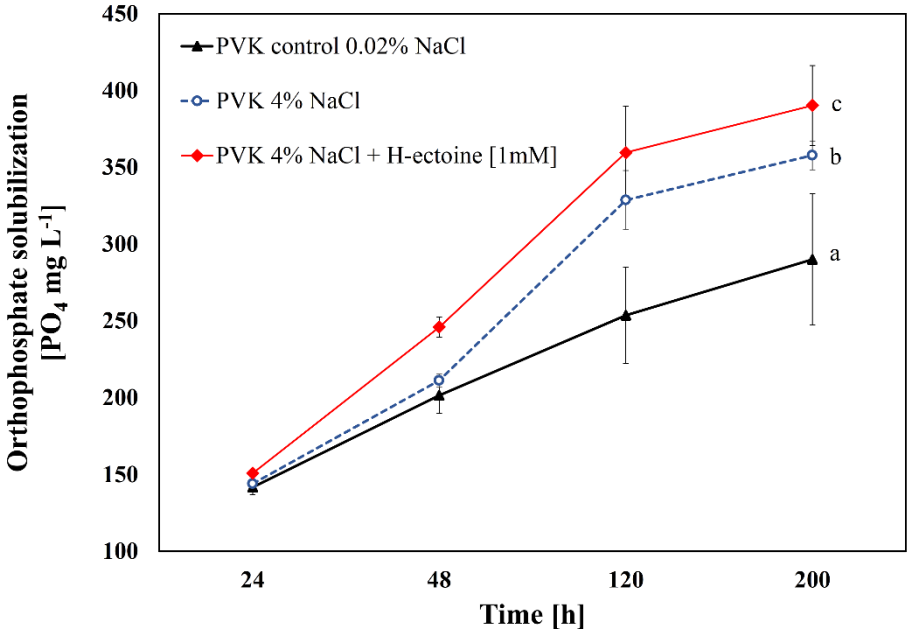
3.3.2 Effects of osmotic stress on phosphate solubilization capability (*in vitro*)

To look into the influence of osmotic unbalance on phosphate solubilization capability of *K. radicincitans* DSM16656^T a PVK-amended liquid media with NaCl was used. Figure 3.3 A shows the positive effects on bacteria ability to solubilize calcium phosphate in the PVK media under osmotic stress caused by 4% NaCl, compared with that in the PVK control media (NaCl 0.02%), solubilization in the salt-amended media increased by up to 23.3%. The bacterial uptake of hydroxyectoine was detected by HPLC analysis after 15 h in response to high salinity, reaching more than 500 µmol g^{-1} of dry biomass at 24 h. No hydroxyectoine was detected in cells grown in PVK in the absence of salt. Interestingly, repeated measures ANOVA indicated significant differences ($F_{2, 9} = 28.229$; $p<0.001$) in response to the accumulation of hydroxyectoine on phosphate solubilization capability during cultivation in PVK medium at 4% NaCl, and additional phosphate release increased by up to 9.39% after five days (Figure 3.3 A). Thus, *K. radicincitans* DSM16656^T can solubilize phosphate in a range of 150-400 mg L^{-1} after eight days of cultivation. Comparable yields are congruent with other pure culture experiments with Gram-negative bacterial endophytes such *Pseudomonas* isolates with phosphate solubilization capacities ~400 mg L^{-1} (Oteino *et al.* 2015).

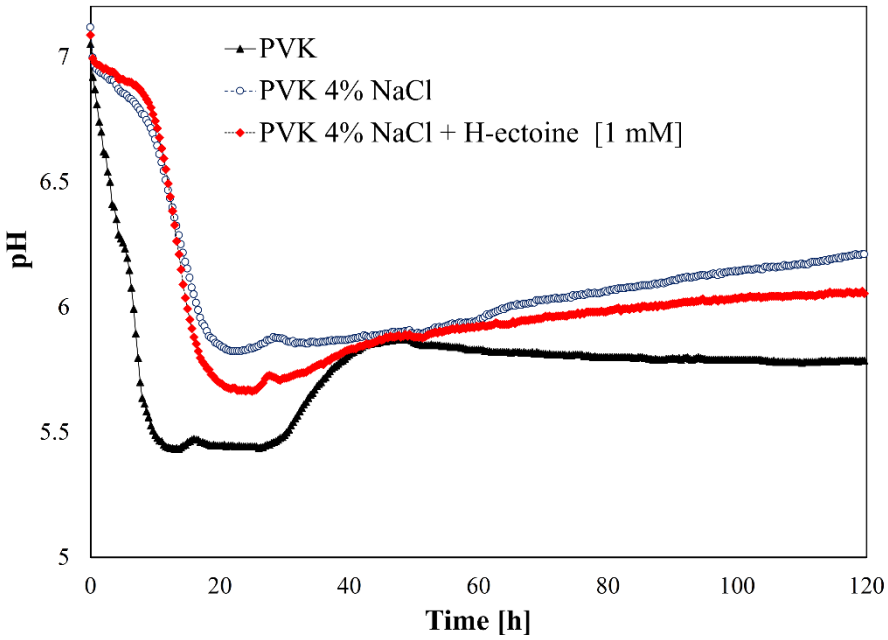
Online pH monitoring during cultivation in PVK media, demonstrated a decrease in pH with increasing salinity in the media, with minimal pH values of 5.43, 5.66 and 5.82 recorded after 12.3 h, 21.6 h and 22.3 h for PVK control, PVK 4% NaCl with hydroxyectoine and PVK 4% NaCl media respectively (Figure 3.4 B). The decline in pH (< 5.5) during the cultivation of endophytic pseudomonas in $Ca_3(PO_4)_2$ rich media was also reported as a response on organic acid production (Oteino *et al.* 2015).

Figure 3-3: A. Effects of the pre-conditioning of *K. radicincitans* DSM16656^T in culture media by osmoadaptation and by the addition of hydroxyectoine [1mM] on the solubilization of orthophosphate, when grown in salt and hydroxyectoine amended PVK medium. **B.** BioLector® pH online monitoring during the cultivation of *K. radicincitans* DSM16656^T in PVK. The Statistics were determined using repeated measures ANOVA and

the Tukey-HSD post hoc test at $p<0.05$. The sphericity of the matrix was assessed with the Mauchly sphericity test.



A



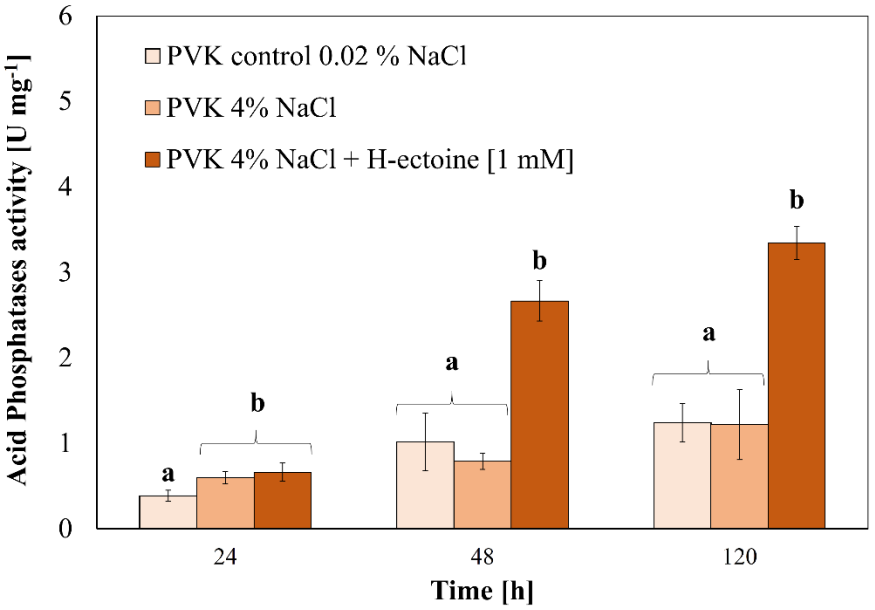
B

3.3.3 Phosphate enzyme activity

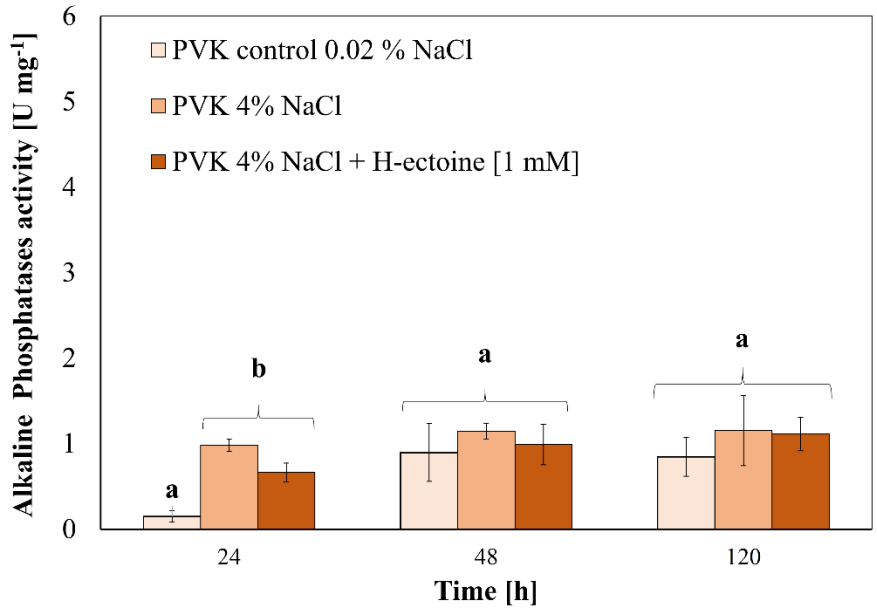
Compared to the PVK control and the PVK 4% NaCl media, the addition of hydroxyectoine induced a positive effect on phosphatase enzyme activity. Acid phosphatase activity was highly induced by the presence of hydroxyectoine in the PVK media (4% NaCl) after 24 h ($F_{2, 11}$=12.00; p=0.0029), 48 h ($F_{2, 11}$=70.67; p<0.0001) and 120 h ($F_{2, 11}$=35.22; p=0.0001) (Figure 3.4 A). Conversely, alkaline phosphatase activity was affected only after 24 h ($F_{2, 11}$=35.52; p=0.0001), since at 48 h ($F_{2, 11}$=1.17; p=0.3542) and at 120 h ($F_{2, 11}$=1.32; p=0.3140), all treatments presented similar activities (Figure 3.4 B). Chemical control hydroxyectoine had not a detectable reaction for inducing P-solubilization.

Phosphatases activation results upon osmotic stress are similar to those reported with *Aeromonas hydrophila*, whose phosphatases increased along with high salinity and the inclusion of betaine (Lim *et al.* 1996). Another study with rhizobacteria revealed increments up to 30% higher acid phosphatases production upon increasing NaCl (100 mM) in liquid media (Shazia and Shabida, 2000).

Figure 3-4: Phosphatase enzyme activity of *K. radicincitans* by osmoadaptation and by the addition of Hydroxyectoine [1mM] in PVK medium. **A.** Acid phosphatases; **B.** Alkaline phosphatases. Different letters above bars indicate significant differences according to the Tukey post hoc test at p<0.05 (means ± SD, n= 4).


Acid Phosphatases activity [U mg-1]
PVK control 0.02 % NaCl
PVK 4% NaCl
PVK 4% NaCl + H-ectoine [1 mM]
0
1
2
3
4
5
6
a
b
a
b
a
b
24
48
120
Time [h]

A


Alkaline Phosphatases activity [U mg-1]
PVK control 0.02 % NaCl
PVK 4% NaCl
PVK 4% NaCl + H-ectoine [1 mM]
0
1
2
3
4
5
6
a
b
a
a
24
48
120
Time [h]

B

3.3.4 Plant growth-promotion in radish

Generally, plant growth promotion by non-osmoadapted and osmoadapted *K. radicincitans* DSM16656^T cells was observed in all inoculated radish plants. Interestingly, when bacterial cells before plant seed inoculation where in contact with hydroxyectoine [1 mM] during the cultivation, the fresh matter of tuber and leaves increased significantly by 41.1% ($F_{3, 39}$=9.80, p=0.0001) and 5.4% ($F_{3, 39}$=3.86, p=0.0172) in comparison to the non-inoculated control (Figure 3.5 A).

Notably, compared with the osmoadapted cells in DM 4% NaCl, the hydroxyectoine amended cells increased the dry matter of both tubers and leaves by 16.20% ($F_{3, 39}$=3.01, p=0.0426) and 3.96% ($F_{3, 39}$=3.60, p=0.0672) respectively (Figure 3.5 B). In line with the plant weight increase, the tuber diameter also significantly increased in all cases of *K. radicincitans* cells compared to the non-inoculated control ($F_{3, 34}$=6.70, p=0.0013). The tuber diameter varied from 15.74 ± 1.82 mm in the native control up to 19.18 ± 1.41 mm for the hydroxyectoine pre-conditioned treatment (Figure 3.5 C). Figure 3.6 shows the evident plant growth promoting effects of pre-conditioned *K. radicincitans* DSM16656^T cells in comparison to the native control.

K. radicincitans DSM16656^T plant growth-promoting benefits were also demonstrated in different plat host. Field cultivars of winter wheat and pea showed increments in grain yield up to 20% , and peas increased dry matter yield about 100 % (Remus *et al*. 2000). In tomato, the mass of ripened fruits increased by 24 % as well as the number of fruits per plant by 18 % (Berger *et al*. 2017). On grain maize, the *K. radicincitans* inoculated plants increased up to 29.3% under field conditions and aerial plant mass with 27.8% increments (Berger *et al*. 2018).

Berger *et al* (2015) also used radish as a plant system for revealing plant growth promotion effects of *K. radicincitans* DSM16656^T. In that study, seed-inoculated plants were compared to two-leaf sprayed plants. Interestingly, they reported that plants inoculated from seed germination gained double weight rather than spray-leaves plants. These findings supported

the bacterium inoculation technic used in this research, which longer exposure times upon seed inmersion in bacteria suspesion seem to determine the magnitude of growth increase.

Figure 3-5: Radish growth promotion in a glasshouse inoculated with pre-conditioned *K. radicincitans* DSM16656^T cells by osmoadaptation at 4% NaCl and by the addition of hydroxyectoine at 1 mM. **A.** Fresh mass of tubers and leaves. Fresh tuber mass ($F_{3, 39}$=9.80, p =0.0001); leaves fresh mass ($F_{3, 39}$=3.86, p =0.0172) **B.** Dry mass of tubers and leaves**.** Dried tuber mass ($F_{3, 39}$=3.01, P=0.0426) and dry leaves mass ($F_{3, 39}$=3.60, p =0.0672) (means ± SE, n=10). **C.** Tuber diameter after 4 weeks of planting ($F_{3, 34}$=6.70, p =0.0013) (means ± SD, n>5 of 5 plant measurements each). Different letters represent significant differences according to Tukey post hoc test at p<0.05.

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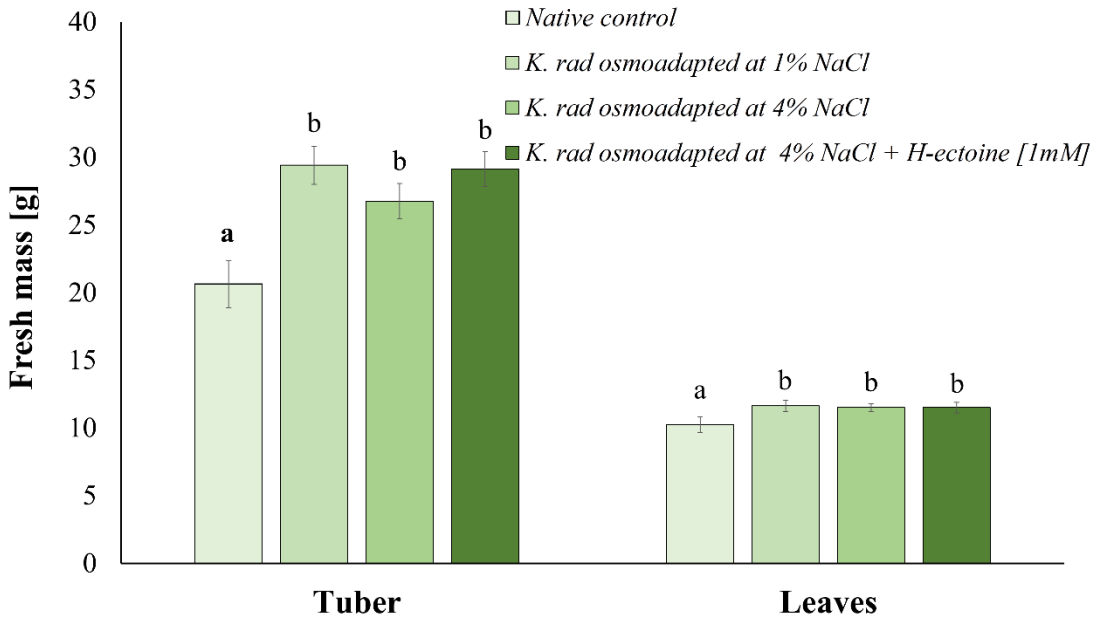
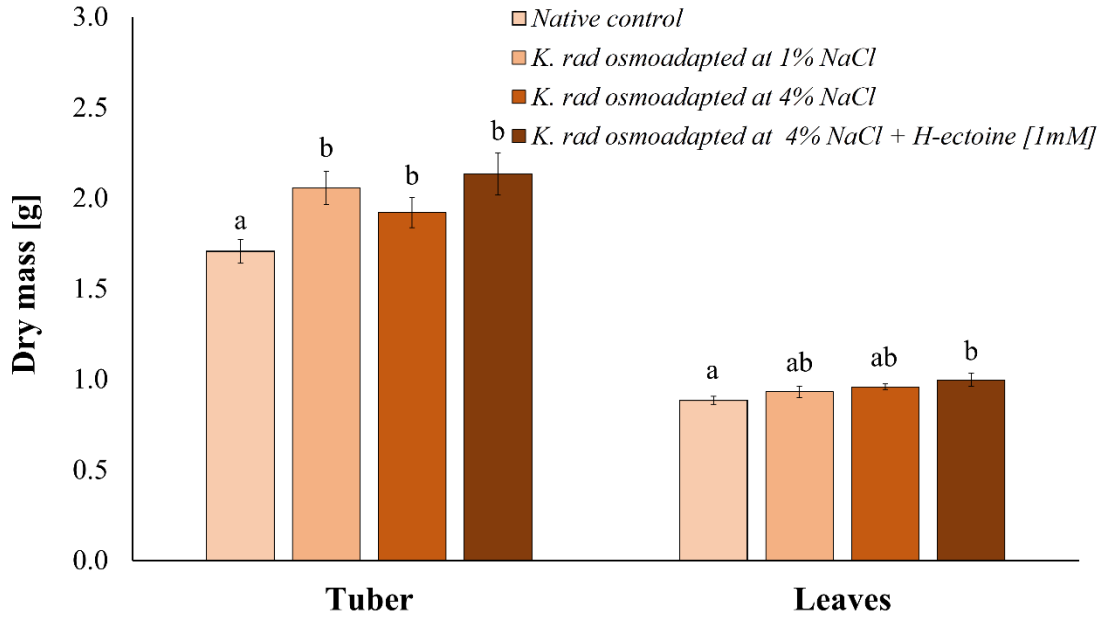
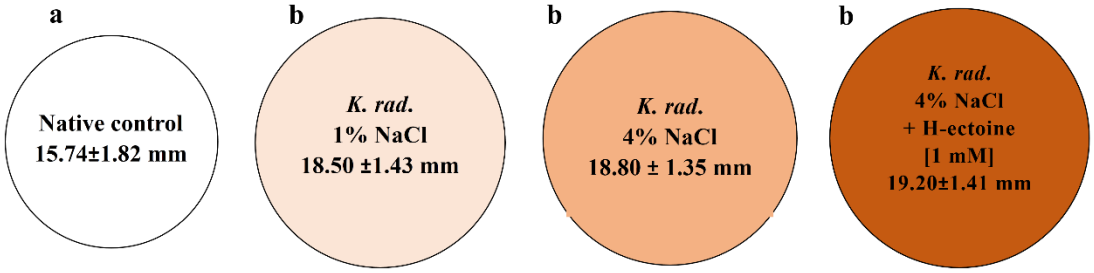


A

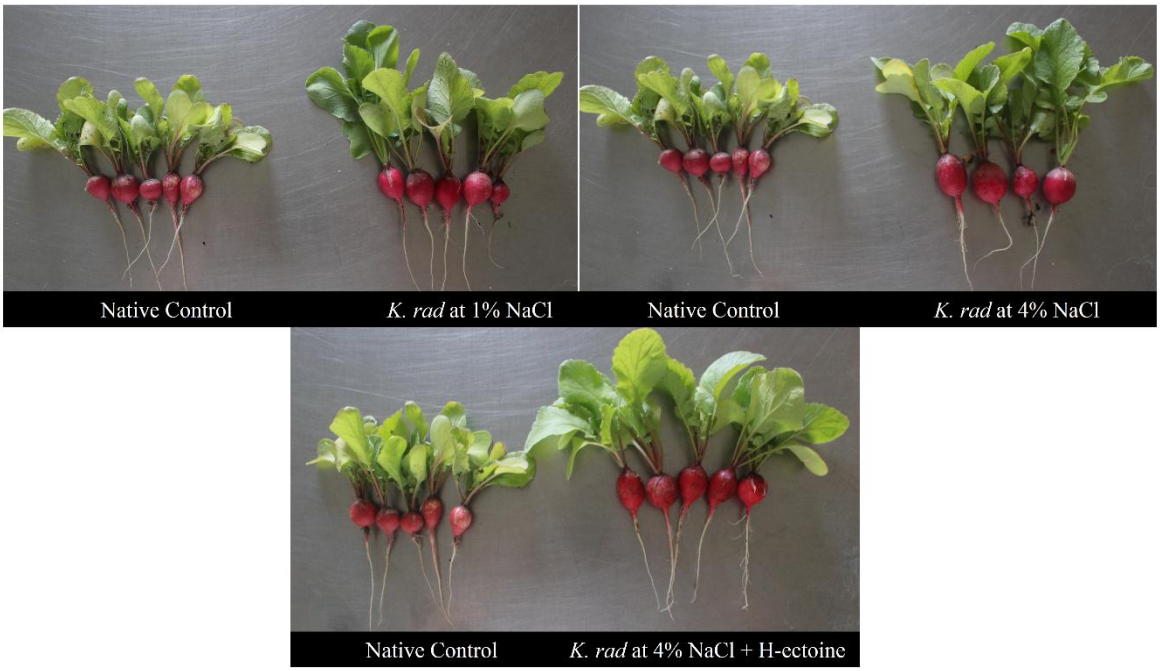

3.0
2.5
2.0
1.5
1.0
0.5
0.0
Dry mass [g]
Native control
K. rad osmoadapted at 1% NaCl
K. rad osmoadapted at 4% NaCl
K. rad osmoadapted at 4% NaCl + H-ectoine [1mM]
a
b
b
b
a
ab
ab
b
Tuber
Leaves

B


a
Native control
15.74±1.82 mm
b
K. rad.
1% NaCl
18.50 ±1.43 mm
b
K. rad.
4% NaCl
18.80 ± 1.35 mm
b
K. rad.
4% NaCl
+ H-ectoine
[1 mM]
19.20±1.41 mm

C

Figure 3-6: Plant growth promoting effects of pre-conditioned *K. radicincitans* DSM16656^T cells treatments in comparison to the native control after 4 weeks of planting.



3.3.5 *K. radicincitans* DSM16656^T plant colonization

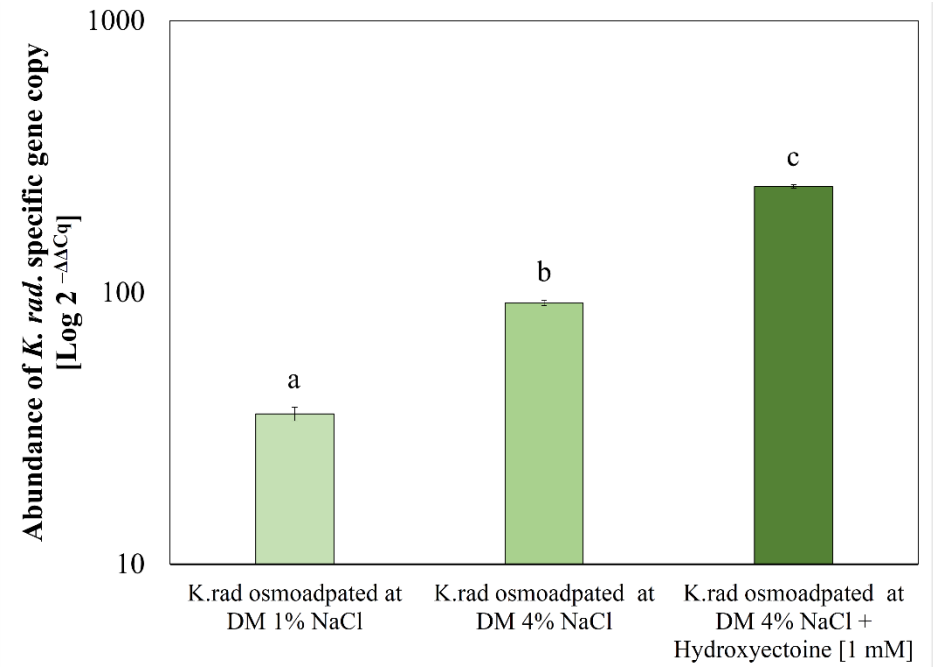
Regarding the relative gene expression response, compared with non-pre-conditioned bacteria cells at 1% NaCl, bacteria cells pre-conditioned with 4% NaCl colonized significantly tissue of eight-day-old seedlings ($F_{2, 14}$=10.803; p=0.033), in comparison to non-pre-conditioned cells at 1% NaCl (Figure 3.8). Consistent with the biomass production of radish plants, the endophytic colonization was relatively strong in the presence of intracellular hydroxyectoine in *K. radicincitans* DSM16656^T cells that were osmoadapted at 4% NaCl*;* the colonization was approximately 3-fold greater under the evaluated treatment conditions ($F_{2, 14}$=10.803; p=0.002) (Figure 3.8).

As reported in other colonization studies, DSM 16656^T is capable of establishing in different plants tissues (roots, leaves and stems) such as in maize (Ruppel *et al.* 2006), wheat (Ruppel *et al.* 1992), *Arabidopsis thaliana* (Witzel *et al.* 2017) and cruciferous vegetables phyllosphere such as *Brassica juncea*, *Brassica campestris*, *Brassica oleracea*, *Brassica rapa* and *Nasturtium officinal* (Schreiner *et al.* 2009).

Accumulation of *K. radicincitans* DNA in radish was also investigated by Berger *et al* (2015). They reported successfully colonization by actin copy number (LecActin PCR) as a reference, with up to 0.7-fold induction of 16 rDNA-gene copy number of bacterium. Interestingly, after 5 weeks, they reported that the endophyte mainly accumulated in tuber tissue rather than aerial parts on the plant. These previous findings indicate that site of application, for instance seed-contact approach, may influence bacterium colonization ability. In this study, the spermosphere colonization in seeds was observed after inoculation, indicating a vertical transmission mechanism (Figure 3.7)

Figure 3-7: SEM images of radish seed surfaces showing the colonization by *K. radicincitans* DSM16656^{T} cells before glasshouse experiments

Figure 3-8: Abundance of *K. radicincitans* DSM16656^T specific gene copy numbers in inoculated radish plants with osmoadapted and hydroxyectoine pre-conditioned cells. Relative gene abundance measured using qPCR and calculated according to the methods of Livak (Livak and Schmittgen, 2001). Different letters above the bars indicate significant differences between treatments according to Dunnett's post hoc test at $p<0.05$, (means ± SE).



3.4 Discussion

Beyond the undeniable plant growth-promoting capability of *K. radicincitans* DSM16656^T, results of this study suggest that it is feasible to significantly improve plant colonization ability of bacterial endophytes, by physiologically growing them under external osmolarity conditions that promote the uptake or synthesis of advantageous compatible solutes such as hydroxyectoine.

Microfermentation studies in the BioLector® revealed that *K. radicincitans* DSM16656^T cells could consider osmoadapted after growing at a_w lower than 0.955 when the osmotic pressure considerably affects the kinetic behavior and the oxygen transfer rate for growing. The viability of bacteria at stationary phase was slightly affected along with higher NaCl concentrations; however, all treatments reached 10^{11} CFU mL^{-1} after 30 h of cultivation, indicating that viability and cellular structures of the endophyte cells were not disturbed under high salinities. Similar results regarding the bacteria endophyte *Sphingomonas* sp LK11 and high salinities were previously described (Halo *et al.* 2015).

DOT curves for DM and DM 1% NaCl demonstrated the natural course of oxygen utilization over time for a non-oxygen limited *K. radicincitans* DSM16656^T cultivation. Thus, the DOT curves showed no plateau, which reveals that the growth of cells was not subjected to oxygen limitation at any time (Wewetzer *et al*. 2015). Nevertheless, at higher salinities such as 4% NaCl, a plateau existence with DOT < 20 % suggested an oxygen limitation during around 26 h. These also indicate that bacterial cells proliferation requires higher oxygen transfer rates along with the salt increment into the media. Since bacteria cells at high salinities can proliferate under low dissolved oxygen tension (DOT), the osmoadapted phenotypes could have intrinsic changes in their intracellular metabolites and metabolic pathways.

The reduction of lag phase and the slight shift of DOT curve by the addition of hydroxyectoine in DM at 4% NaCl, indicate the amelioration effect of this compatible solute at accelerating the response of bacterial endophyte cells to high salinities. The reduction in lag phase caused by this osmolyte is consistent with other reports in bacteria (Bursy *et al.* 2008; Tao *et al*. 2016). Growth profiles permit to confirm that hydroxyectoine have a robust osmoprotective impact since the addition of this osmolyte showed a significant effect on kinetic growth in comparison to DM at 4% NaCl. It is clear in Figure 3.2 C, that the presence in media and further active transport of this compound counteracts the energy demand caused by the adaptation to the osmotic pressures unbalance. It seems that metabolic energy requires priory the cellular homeostasis over growth at a_w 0.95. Results prove that exogenously provided hydroxyectoine extend the upper growth limit of *K. radicincitans* DSM16656^T under high-salinity growth conditions. Moreover, hydroxyectoine uptake > 150 µmol g^{-1} dry weight into bacteria cytoplasm may be sufficient to shift metabolic response,

likely altering pool composition. The amount of accumulated hydroxyectoine agrees in concept with several reports, who demonstrated uptake of this osmolyte from 100 up to 400 µmol g^{-1} dry weight in early and late stationary phase in bacteria upon salt stress cues (Bursy *et al.* 2008; Tao *et al.* 2016). Besides, hydroxyectoine in Gram-negative bacteria such as *Pseudomonas putida* or *Halomonas elongata* offsets the detrimental events of high salinity on cell growth (Grammann *et al*. 2002; Manzanera *et al*. 2004).

The bacterial ability to solubilize phosphate has been extensively reported as a vital mechanism that promotes plant growth (Gyaneshwar *et al*. 2002; Hayat *et al*. 2010). In this study, was demonstrated that *K. radicincitans* DSM16656^T cells under osmotic stress could increase its phosphate solubilization capability up to 357.7 ± 9.38 mg L^{-1} in comparison to non-stressed cells with 290.3 ± 42.6 mg L^{-1} after eight days of cultivation. Therefore, bacteria may respond to high salinity no only by accumulating exogenous osmolytes such as hydroxyectoine but also by up-regulating central metabolic pathways involved in the organic acid synthesis, which is well known for its role as crucial factors in phosphate solubilization (He *et al.* 2017). Since the pH values were lower in the treatment with hydroxyectoine compared to the treatment in which the bacterial cells were grown solely in PVK 4% NaCl media, a higher concentration of organic acids or the consumption of alkaline components within the media is feasible in conjunction with the osmoadaptation process (Schilling *et al.* 1998). Organic acid detection could confirm these assumptions.

Widely distributed in microorganisms, plants and animals, phosphatases are hydrolases that catalyze the hydrolysis of orthophosphate monoesters, thereby releasing phosphate (Bull *et al.* 2002). The general classification of acid and alkaline phosphatases relies only on the optimum pH for enzymatic activity. A superior production of enzymes may be correlated with the intracellular content of amino acids, since phosphatases are built on the basis of amino acid sequences and the phosphoryl group acceptors. It is expected that the physiological changes caused by either osmoadaptation or the addition of hydroxyectoine trigger metabolic pathways for induction of amino acids, either aromatic or branched-chain ones, which can favor enzyme biosynthesis and secretion. At high salinity, as a homeostasis

response, trehalose biosynthesis in *K. radicincitans* DSM16656^T cells may also lead to high concentrations of trehalose-6-phosphate phosphatase (T6PP), which catalyzes the hydrolysis of trehalose 6-phosphate (T6P) to not only trehalose but also inorganic phosphate (Kong *et al*. 2001; Liu *et al*. 2017). The elucidation of these metabolic profiling aspects will be addressed in Chapter 6.

A greater production of enzymes, including acid phosphatases, may also be attributed to the construction of osmo-remedial mutations-epimutations in *K. radicincitans* DSM16656^T cells, since some proteins are nonfunctional when the cells are grown in media of low osmotic strength but regain activity at elevated osmolarities, suggesting that cells may be undergoing phenotypic modulation (Csonka, 1989; Kunin *et al.* 1993). On the contrary, alkaline phosphatases in Gram-negative bacteria are located in the periplasmic space or over the cell surface, and can be released upon osmotic stress. In addition, specific activation of alkaline phosphatases may depend on growing phase and membrane phospholipid composition that may not change at high salinities (Badyakina *et al.* 2003).

Here the increase in acid phosphatase activity of the endophyte *K. radicincitans* DSM16656^T exposed to osmotic shock in the presence of hydroxyectoine was evident. Nonetheless, further research is needed to elucidate the types of phosphatases (nonspecific vs. specific for certain substrates) that have been up-regulated in the production process.

Since osmoadaptation in this bacterium leads to increased acid phosphatase enzyme production, it can be expected that this sub-lethal pre-conditioning procedure also supports the metabolite-rich arsenal to face the extremely competitive rhizosphere conditions before entering the plant. Hence, osmoadaptation could trigger other proteins that *K. radicincitans* DSM16656^T can encode to facilitate plant establishment, including pectinases, cellulases, glycoside hydrolases and glucanases (Kampfer *et al*. 2005a; Becker *et al.* 2018). Moreover, it has been demonstrated with bacteria such as *Pantoea agglomerans*, that osmotic stress can alter quorum sensing or the quenching of produced metabolites such as 1,3-propanediol, which are suggested to act as signals in the plant microbiome, resulting in tolerance to abiotic stress (van Kessel *et al.* 2015; Bacon and White, 2016). Therefore, *K. radicincitans*

DSM16656^T has a fraction of genes involved in propanediol degradation (PDD-pduABDEFLMPQ) and dha genes (dhaBDKLMT) involved in glycerol transformation (GT) (Becker *et al.* 2018).

K. radicincitans DSM 16656^T cells were able to promote growth in radish plants. These findings are in line with previous studies (Berger *et al.* 2015), in which the weight of radish tubers and leaves increased from 20 to roughly 50%, in response to either seed-inoculated or two-leaf sprayed plants with fresh cultivated cells. Nevertheless, since in these pot experiments the majority of plant growth yields were not significantly different between *K. radicincitans* DSM16656^T cells grown in DM 1 % and DM 4% NaCl, the physiological advantages conferred to the osmoadapted cells may affect mainly during early colonization stages, as shown here for one-week old plants. Thus, the relative high endophytic lifestyle preference for salt-stressed bacteria cells and hydroxyectoine-added cells may be due to the alteration of signaling types of metabolites that bacteria can secrete into the microenvironments surrounding plant roots, modifying plant-defense and plant-competition mechanisms along with plant metabolite synthesis (Brader *et al.* 2014). Besides, external stimuli such as the presence of root exudates may up-regulate the expression of the chemotaxis and motility-related genes of these bacteria (Becker *et al.* 2018).

Consistent with the biomass production of radish plants, plant colonization was stronger in the presence of intracellular hydroxyectoine within *K. radicincitans* DSM16656^T cells, in which the approximately 3-fold increase in colonization was significant under the evaluated conditions. Generally, these results may indicate that symbiotic performance with plants increased by synergistic effects of pre-conditioned cells by osmoadaptation and the physiological changes caused by hydroxyectoine. This could be due to the chemical chaperone properties of hydroxyectoine. Hence, the influence of hydroxyectoine on the local water structure exerts pronounced effects on protein-DNA binding interactions, an essential step that might alter the transcriptional profile of salt-stresses bacteria cells *(*Hahn *et al.* 2015; Czech *et al.* 2018). Besides, hydration of cytoplasm upon hydroxyectoine

accumulation affords a major protective influence on protein stability and macromolecules functionality (Knapp *et al.* 1999).

Despite the endophytic colonization ability of the strain, it also growth and colonizes the root surfaces (Ruppel *et al.* 1992; Ruppel *et al.* 2006). These cells may contribute to phosphorous solubilization and improved plant P-uptake, which has been previously demonstrated (Schilling *et al.* 1998). Nevertheless, additional studies are required to determine the performance of halotolerant bacterial endophytic cells on plant phosphorus uptake under saline conditions.

The reinforcement of *in vitro* phosphate solubilization capability, including the production of acid phosphatase enzymes and the accumulation of hydroxyectoine, also suggests an intracellular metabolic re-ordering within bacterial cells in response to high salinity (see Chapter 6). It is proposed that these substantial and significant alterations in metabolites levels represent the activation of a phenotypic shift as an osmoadaptation mechanism for conferring advantages during rhizosphere competition establishment, orchestrating nutrient exchange and mediating associations within the plant. To the best of our knowledge, the current study is the first to address the pre-conditioning of bacterial endophytic cells as an alternative to increasing plant colonization abilities.

3.5 Conclusions

The results of this study showed that physiological modifications of *K. radicincitans* DSM16656^T by osmotic stress treatments and by the accumulation of compatible solutes during cultivation constitute a feasible strategy to improve the ability of the bacterium to solubilize phosphate and to promote the plant tissue colonization. Moreover, pre-conditioning could be a promising alternative prior to formulating bacterial endophytes.

4. CHAPTER 4. DRYING SURVIVAL AND PRE-FORMULATION STUDIES FOR *K. radicincitans* DSM16656^{T}

4.1 Introduction

The design of endophytes-based bio-formulations has drawn the attention of the agro biotechnological industry which has become interested in commercial inoculant production. Such bio-formulations are considered more effective because once the microbe is inside the plant, it avoids the high competitive soil surroundings, which is commonly faced by the rhizosphere microbes (Lodewyckx *et al*. 2002; Santoyo *et al.* 2016). Most of the studies on bacteria endophytes including *K. radicincitans* DSM16656^T have used fresh cultivated cells prior to reveal beneficial effects on plants, lacking a formulation proceeding which could guarantee a longer shelf life and further efficacy reproducibility (Berger *et al.* 2015; Garima and Nath, 2015; Berger *et al.* 2017). Moreover, for commercial proposes, the next-generation bio-formulations must address the understanding of the genetic and molecular bases of plant-endophytes interactions, the mode of transmission and further the designs of formulation-drying strategies to protect the endophyte and to establish a symbiotic association between endophyte and host plant.

The regular application target for endophytes such as soil or phyllosphere represents harsh environments, where conditions of microbial interactions, temperature, UV radiation, free moisture, pH, organic matter and limited nutrient supply can fluctuate significantly at nanometer and micrometer levels (Lindow and Leveau, 2002; Paul, 2015). Gram-negative bacteria are generally less tolerant of such environmental conditions than Gram-positive bacteria, yeasts or filamentous fungi (Wieland *et al*. 2001; Ren *et al.* 2018). Although many endophytic strains have been reported, most of these isolates belong to the sensitive Gram-negative bacteria (Table 4.1). Thus, for endophytic Gram-negative bacteria, preventing the rapid loss of viability during the formulation-drying process is essential to achieve cost-effective products and further exploitation at industrial agriculture scale (Singh and Arora, 2016; Le Cocq *et al.* 2017).

Table 4-1: Bacteria genera reported with endophytic capabilities in different plan host (Lodewyckx *et al.* 2002).

Gram-negative Bacteria endophytes	**Gram-positive Bacteria endophytes**
Acidovorax, Acinetobacter, Aeromonas, Afipia, Agrobacterium, Agromonas, Alcaligenes, Alcanivorax, Allorhizobium, Alteromonas, Aminobacter, Aquaspirillum, Azoarcus, Azomonas, Azorhizobium, Azotobacter, Azospirillum, Beijerinckia, Blastobacter, Blastomonas, Brachymonas, Bradyrhizobium, Brenneria, Brevundimonas, Burkholderia, Chelatobacter, Chryseomonas, Comamonas, Delftia, Derxia, Devosia, Enterobacter, Flavimonas, Flavobacterium, Flexibacter, Halomonas, Herbaspirillum, ***Kosakonia****, Matsuebacter, Mesorhizobium, Moraxella, Nevskia, Ochrobactrum, Pectobacterium, Phenylobacterium, Photobacterium, Porphyrobacter, Pseudoalteromonas, Psychrobacter, Pseudomonas, Pantoea, Ralstonia, Rhizobacter, Rhizobium, Rhizomonas, Rhodanobacter, Shewanella, Sinorhizobium, Sphingobacterium, Sphingomonas, Spirillum, Stenotrophomonas, Thauera, Variovorax, Vibrio, Xanthomonas, Xylella, Zoogloea, Zymobacter and Zymomonas*	*Actinomyces, Arthrobacter, Aureobacterium, Bacillus, Chromobacterium, Corynebacterium, Frankia, Nocardia, Renibacterium, Rhodococcus,* and *Streptomyces*

Noteworthy, some microbiome studies indicate that under natural conditions (adult plant leaves, stems, roots of seedlings), Gram-negative endophytes are predominant over Gram-positive in a proportion of 70:30 respectively (Vega *et al.* 2005). Altogether, Gram-negative bacteria endophytes such as *Kosakonia radicincitans* require research efforts on increasing the ability to withstand abiotic stresses during formulation-drying processing such high temperatures, elevated osmolalities and dehydration while conserving biological activity and further endophyte capacity.

Preservation of microorganisms by desiccation is the preferred formulation method to achieve long-term storage (Berninger *et al.* 2018). Considering that, one of the main concerns for PGPBE from the laboratory to industrial development comprises the tolerance for drying (Santoyo *et al.* 2016; Mei *et al.* 2017). The challenge is higher when the bacterium presents physiological disadvantages for desiccation as in the case of Gram-negative bacteria (Miyamoto-Shinohara *et al.* 2008). Besides, there is limited knowledge regarding strategies aiming at enhancing drying survival for non-sporulating PGPBE. Hence, the enhancement of tolerance for drying, by using integrated novel cultivation-formulation approaches could be the key for further popularization and application of PGPBE.

A considerable number of studies have demonstrated that exposing bacterial cells as *Lactobacillus* sp. or *Pseudomonas* sp. to sub-lethal conditions improves resistance to deleterious effects caused by abiotic stresses such as drying (McIntyre *et al*. 2007; Cabrefiga *et al.* 2014; Shao *et al.* 2014; Barbosa *et al.* 2015). Thus, studies of epiphytic bacteria, which live non-parasitically on the surface of a plant, suggested that tolerability of environmental factors could improve by eco-physiological manipulation of growth conditions, pre-conditioning or the accumulation of compatible solutes. The latter ones are low-molecular weight compounds that accumulate primarily under hyperosmotic stress. Considered also as osmolytes, commonly share the properties of being polar, highly water-soluble, do not interact with proteins and do not carry a net charge at physiological pH (da Costa *et al.* 1998; Kempf and Bremer, 1998; Sevin *et al.* 2016a). In addition to equilibrate intracellular osmotic balance, compatible solutes operate as useful stabilizers of enzyme functions, so protecting whole cells against high temperature, desiccation, salinity, freeze-thaw procedures and even

drying in bacterial cells (Lippert and Galinski, 1992; Sleator and Hill, 2002; Manzanera *et al.* 2004). Hence, osmoadaptation by compatible solute accumulation as a pre-conditioning mechanism may be a feasible strategy to strength PGPBE cells before formulation-drying processes.

An advantageous compatible solute is hydroxyectoine, which appears intracellular in halophytic bacterial pools (del Moral *et al.* 1994; Ono *et al.* 1998), acting as a protein-protecting agent. Its hydroxylated nature has superior properties compared to its precursor ectoine in many applications (Wang *et al.* 2006). So far, few studies have dealt with anhydrobiotic engineering in PGPBE to confer tolerance for drying (Berninger *et al*. 2018). Thereby, there is currently no study on the influence of exogenously compatible solutes during the osmoadaptation in bacterial endophytes and ensuing desiccation tolerance. In particular, the influence of this compatible amassing strategy on drying survival upon formulation in polymeric materials by encapsulation is unknown.

Even though many indications pointed out that polymeric inoculants are perhaps the future of inoculants, few formulations of PGPB or rhizobia have passed the threshold of industrial approval, as happened in other industries. Notwithstanding, bio-encapsulation is a sizeable emerging field in pharmaceutical, nanotechnology, medicine, aquaculture, and cosmetics (Bashan *et al.* 2014), which can be used as a platform for applying the PGPR endophytes. The production of an air-dried formulation containing viable Gram-negative PGPBE cells could be more cost-effective to produce, but remains a challenging and essential step in developing commercial bioproducts.

Few studies of formulating-drying Gram-negative bacteria endophytes are available in the literature. Thus, so far, the successfully dried formulations of Gram-negative bacteria are mostly described the talc-based formula for *Paraburkholderia phytofirmans* PsJN (Bejarano *et al.* 2017) and *Burkholderia* sp in coir dust-based inoculant (Bazilah *et al.* 2011). In particular, the encapsulation of Gram-negative PGPBE with polymeric materials has mostly used alginate cross-linking with divalent cations (Ca^{2+}), including bacteria such as *Enterobacter* spp. MN17 (Aziz *et al.* 2018), *Pseudomonas fluorescens* (Lally *et al*. 2017) and

Pseudomonas consortia (Grobelak *et al.* 2018). However, more work is needed on polymeric materials and cross-linkers usage, for enhancing cell survival, shelf life and scalability within convective capsules-drying processes (Humbert, Przyklenk, Vemmer and Patel, 2017; Humbert, Przyklenk, Vemmer, Schumann *et al.* 2017).

Pectins are a diverse family of biopolymers extracted from the plant cell wall, with an anionic polysaccharide backbone of α-1,4-linked D-galacturonic acids in common. Uronic acids present in the backbone have carboxyl groups, which can be substituted by methyl esters and/or carboxamide groups. Thus, pectin can be characterized by its degree of esterification (DE) and/or degree of amidation (DA), which are both expressed as the percentage of carboxyl groups esterified and/or amidated, respectively (Huynh *et al*. 2017). In particular, amidated pectin as biopolymer is extensively studied for scaffolding drug delivery systems, considering their versatility by targeting specific sites and releasing rates (Liu *et al.* 2003; Kosaraju 2005; Sriamornsak, 2011). Noteworthy, the incorporation of calcium salts as cross-linker to pectin matrices could enhance the reactivity of the polysaccharides to the enzymatic arsenal of bacteria, since many pectinases require calcium ions or are stimulated by their presence (Sunnotel and Nigam, 2002; Yadav *et al*. 2008). Therefore, apart from a recent study by encapsulating a fungal endophyte (Krell, Jakobs-Schoenwandt, Vidal *et al.* 2018), the potential utilization of pectin-based systems as a carrier of PGPBE remains unexplored. So, despite these benefits, a detailed investigation regarding the influence of amidated pectin cross-linking with calcium salts as the carrier of bacteria endophytes has so far been missing.

Here, it is hypothesized that amidated pectin cross-linked with calcium salts is an advantageous material for encapsulating *K. radicincitans* DSM16656^T, serving as a carrier for cells, fillers and additives, and providing a nutrient source for bacteria. Additionally, supporting the positive influence of pre-osmoadapted cells on drying survival and on plant colonization. In this chapter, the research aimed at determining the influence of osmoadaptation and hydroxyectoine on drying survival, the effect of cross-linker on providing desiccation tolerance to entrapment cells, and the ability of encapsulated pre-osmoadapted cells to survive an air-drying process.

4.2 Materials and Methods

Chemicals used in this study were obtained from Carl Roth GmbH (Karlsruhe, Germany) or AppliChem GmbH (Darmstadt, Germany) if not specified otherwise. Amidated pectin references used in this study were provided by Herbstreith & Fox KG (Neuenbuerg/Wuertt, Germany). Details are given in Table 4.2.

4.2.1 Drying survival assessments for free cells

Bacteria suspensions for drying survival assessments were prepared as follows: DM (100 mL) was poured into 250 mL baffled Erlenmeyer flasks that were autoclaved at 121°C, 1.5 atm, for 30 min. The initial inoculum concentration in media was adjusted at 10^6 cells mL^{-1}. The cultures were maintained at 190 rpm in a rotary incubator at 30°C (IKA KS 4000 ic control, Staufen, Germany). Actively growing cells were harvested at exponential phase after 20 h (OD_{600} 0.6) by centrifugation at 5252xg for 15 min (Mikro HT 200R, Hettich GmbH & Co. KG, Tuttlingen, Germany), and the obtained pellet of preadapted bacteria was washed and centrifuged twice with a corresponding NaCl solution [0, 1, 2, 3 4 %] to maintain the osmotic pressure. The bacteria were stored in the same NaCl solution adjusted at OD_{600} ~ 1.0 (~ $1.0x10^{10}$ CFU mL^{-1}) for the ensuing drying test. 100 µL of each bacterial suspension was spread evenly as a thin layer onto culture microplates-6 wells (VMR 10062-892, Stockholm, Sweden). Samples were allowed to dry under oxic conditions during 2 h in a laminar flow hood at 25 ± 1°C, an airflow at 0.4 m s^{-1} with relative humidity at 45 ± 2 % (Nocker *et al*. 2012). After drying, dried bacteria cells were recovered from microplates by adding 5 ml of NaCl solution [0, 1, 2, 3, 4 %] to wash off the dried biofilm in a rotatory shaker at 120 rpm, 20°C for 1 h. To assess the number of viable cells, serial dilutions were plated by the microdrop (10 µL) technique on standard nutrient agar media (Merck, Darmstadt, Germany), incubated at 30°C for 24 h and counted to determine the number of viable cells (CFU) (Naghili *et al.* 2013). Bacterial cells with added hydroxyectoine were treated with the same procedure.

4.2.2 Preparation of beads for cross-linker selection

To assess the influence of cross-linker agents on *K. radicincitans* DSM16656^T drying survival, two calcium salts, calcium chloride ($CaCl_2$) and calcium gluconate (CG) were tested in an alginate-based prototype. Cross-linker solutions of $CaCl_2$ and CG were prepared at 0.1 M final concentration. Sodium alginate (Manugel GMB, FMC Corporation, PA, USA) was dissolved in ultrapure water to a final concentration of 4.0 % and autoclaved for 6 min at 121 °C. Afterward, a sodium alginate solution (4%) was thoroughly mixed for 15 min adjusted to a final concentration of alginate 56.2%, native crystalline starch at 22.5% (CIF GmbH, Siegburg, Germany) and *K. radicincitans* DSM16656^T suspension at 21.3%, composition is given in %w/w wet basis (Humbert, Vemmer *et al.* 2017).

After 15 min of stirring the suspension was transferred into a 20 mL syringe and dripped through a cannula (diameter 2.1 x 0.8 mm, Sterican, B. Braun Melsungen AG, Melsungen, Germany) into the stirred cross-linker solution. The ionic gelation reaction took place immediately, and the resulting hydrogel beads were kept for 10 min in the stirred cross-linker. Further, beads were separated from the cross-linker using a sieve (mesh size: 1.0 mm) and finally washed gently with ultrapure water. Schematic representation of the ionic gelation reaction is given in Figure 4.1.

A standardized *in situ* laboratory drying test was conducted for the hydrogel beads. Briefly, the excess of water was removed from the surface with cellulose paper, and the beads were spread on aluminum trays. The beads and 2 kg freshly regenerated silica gel were put in an oven for 24 h at 35 °C and in a second step the beads were dried in a desiccator filled with silica gel for 48 h at room temperature to reach a water activity close to or even smaller than 0.3 (Humbert, Przyklenk, Vemmer and Patel, 2017; Humbert, Vemmer *et al.* 2017). To evaluate the drying process, the water activity of the beads was measured by means of a water activity meter (LabMaster-aW, Novasina AG, Lachen) (Humbert, Przyklenk, Vemmer and Patel, 2017).

4.2.3 Screening of pectin materials as a nutrient source

For assaying pectin as a potential nutrient source, a high-throughput screening through microfermentation was carried out. Pectin materials with a wide range of degrees of esterification, degrees of amidation and galacturonic acid contents were selected (Table 4.2). These pectin materials were used for screening their potential for serving as a nutrient source for *K. radicincitans* DSM16656^T cells. Briefly, 1 mL of 2% pectin material supplemented with 1% yeast extract were placed in a microtiter plate (MTP) for conducting cultivations (RoboLector-BioLector system, m2p-labs, Baesweiler, Germany). The dissolved oxygen tension and Green Fluorescent Protein (GFP) signal intensity were selected as parameters for detecting bacterial growth activity. Previously, electrocompetent bacterial cells were transformed with plasmid pMP4655 (Lagendijk *et al.* 2010) to produce colonies of eGFP mutants of *K. radicincitans* DSM16656^T (Appendix 6).

All BioLector® cultivations were monitored online with a pO_2-optode (filter DO [Pst3] Ex (nm) =520; Em=600) and GFP filter (filter GFP Gemini [Pst3], Gain=5, Ex (nm) =470; Em=525). The experiments were performed at 30°C under constant agitation (1200 rpm, shaking diameter = 3 mm, orbital) in 48-well MTP-48-BO flower- plates, Lot No: 1711 (mp2-labs, Baesweiler, Germany) with a working volume of 1000 µL DM. Each treatment was composed of three replicates.

Table 4-2: Psychochemical properties of selected pectin references used for screening in microfermentation at BioLector®

Pectin Reference	DE	DA	Galacturonic acid content
Pectin Classic CU 902	5.7 %	-	75 %
Pectin Classic AU-L 061/10	40 %	-	85 %
Pectin Classic AU-L 062/10	30 %	-	84 %
Pectin Amid AU-L 063/10	33 %	15%	83 %
Pectin Amid CU-L 065/10	44 %	11 %	89 %
Pectin Amid CU-L 066/10	24 %	24 %	91 %

Degree of esterification (DE); Degree of amidation (DA)

4.2.4 Formulation prototypes based on calcium alginate and calcium pectin hydrogel beads

After the selection of both the cross-linking agent to improve desiccation tolerance and the pectin reference for its benefits as a nutrient source, two groups of formulation prototypes based on sodium alginate and pectin were developed. For alginate prototype, the encapsulation suspension was prepared by mixing 2.0 % sodium alginate, 14% of filler, 1% sorbitol and 1% monosodium glutamate. Maltodextrin (Applichem Pancreac, A4804) and microcrystalline cellulose (MCC) (Avicel RC-591 NF, IMCD, Cologne, 102 Germany, batch No. 110240) were tested as filler in the beads. They were selected according to their potential benefits on drying survival and supporting endophytic colonization (Berninger *et al.* 2018; Krell, Jakobs-Schoenwandt, Vidal *et al*. 2018).

K. radicincitans DSM16656^{T} cells were added into a final concentration of 15%, and the suspension was gently stirred for 5 min. For bead formation, the suspension was dripped into a sterile calcium gluconate solution (0.1 M) by using a syringe with a cannula (diameter 2.1 x 0.8 mm, Sterican, B. Braun Melsungen AG, Melsungen, Germany). For pectin prototype, alginate solution was replaced by the amidated low methoxy pectin (ALMP) called Amid CU-L 066/10 (DE 25% and DA 23%) at 4% (w/v) (Herbstreith & Fox KG, Neuenbürg, Germany), and beads were manufactured by ionotropic gelation method as described previously. For all prototypes, alginate as well as pectin, the gelled beads instantaneously formed were allowed to cure in the calcium gluconate solution for 10 min; then beads were separated by filtration before air drying procedures. Details of the prototypes are given in Table 4.3.

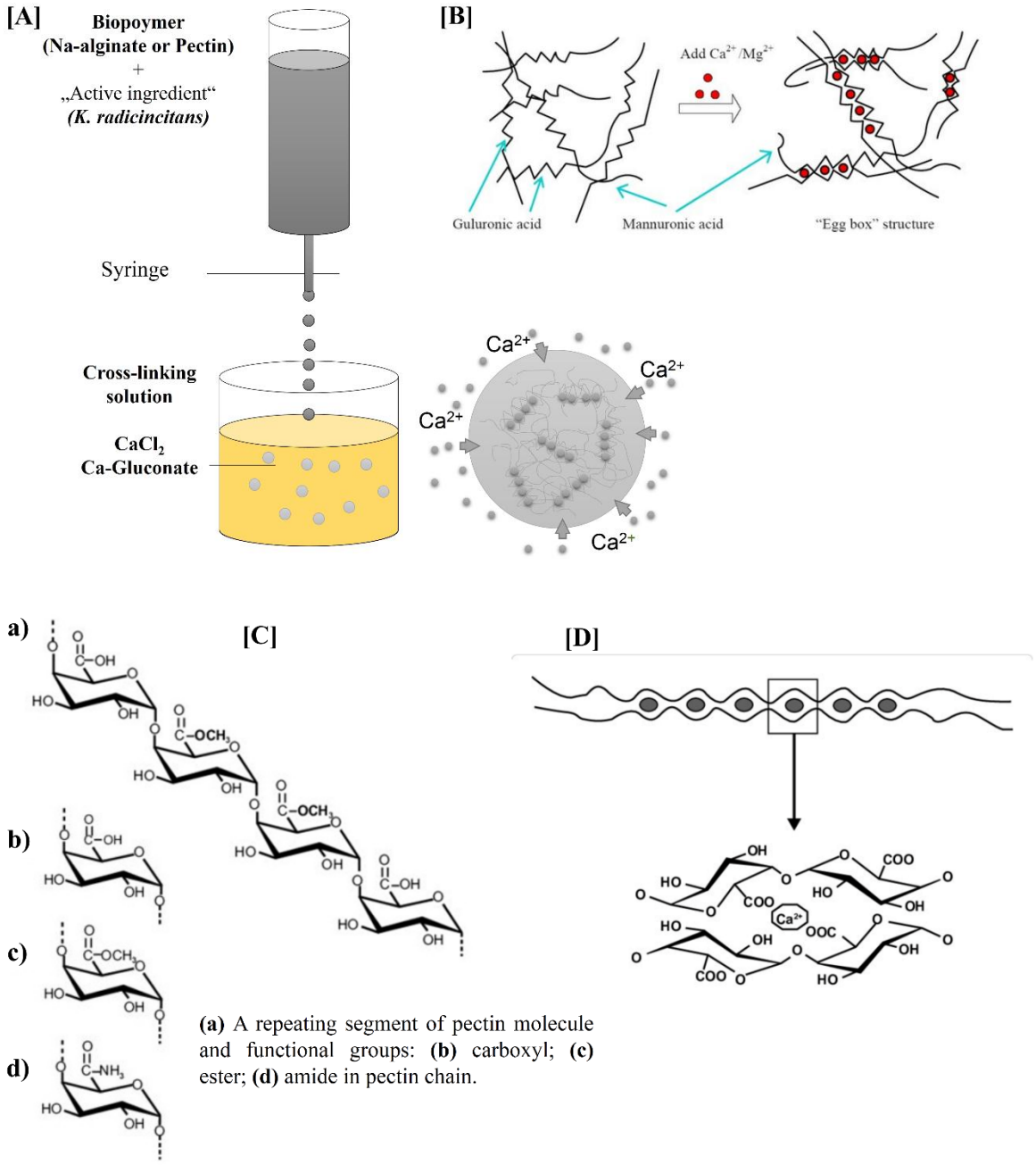
Table 4-3: Formulation prototypes for hydrogel beads preparation to encapsulate *K. radicincitans* DSM16656^T cells

Prototype	*Biopolymer	Cross-linker	Filler	C-Source	N-source
P1	Alginate	CaGlu	Maltodextrin	Sorbitol	Na-Glutamate
P2	Alginate	CaGlu	MCC	Sorbitol	Na-Glutamate
P3	Ami-Pectin	CaGlu	Maltodextrin	Sorbitol	Na-Glutamate
P4	Ami- Pectin	CaGlu	MCC	Sorbitol	Na-Glutamate

*Amidated pectin corresponding to the reference CU-L-066/10

Sorbitol and sodium glutamate were selected as advantageous nutrient sources for *K. radicincitans* DSM16656^T cells according to the phenotyping fingerprint in GEN III microplate (see Chapter 2).

Figure 4-1: [A]. Schematic representation of the ionic gelation reaction during the encapsulation of *K. radicincitans* cells in biopolymeric prototypes based on alginate and pectin. **[B]**. Gelation of alginate solution with the addition of calcium ions and the formation of "Egg box " structure (Wan *et al.* 2008). [**C]**. Pectin molecule structure based on polygalacturonic acid. **[D].** Schematic representation of calcium binding to polygalactoronate sequences: 'egg box' dimer and 'egg-box' cavity (Thakur *et al.* 1997).



4.2.5 Determination of viable cell number in formulation prototypes

Before and after drying assessments, bacterial viable cell number within the beads was determined by dissolving ten beads in 10 mL of a solution containing 0.03 M citric acid and 0.05 M sodium carbonate (pH 7±2) for 1 h in a rotatory shaker at 150 rpm (Mater *et al.* 1995). After complete dissolution, diluted samples were plated on Merck media agar and incubated at 30°C for 24 hours. The results are given in % mean drying survival from five replicates with N as CFU of dry beads and N_0 as CFU of moist beads:

$$Survival\,(\%) = \frac{N}{N_0} x\,100$$

Pre-conditioning of bacteria in cultivation before formulation step was carried out by amending DM with NaCl [0, 1, 2, 4 %] as required to obtain water activities (a_w) at 0.98, 0.97, 0.965 and 0.96 respectively (LabMaster-a_w, Novasina AG, Lachen, Switzerland) (See 4.2.1).

4.2.6 Air drying process for testing formulation prototypes

To look into the effectiveness of strengthening approaches for conferring a high drying survival rate on *K. radicincitans* DSM16656^T, a more scalable drying process for the beads was assessed. The formulation prototypes at a scale of 500 wet g were dried during 4 h at T_{in} 30 ± 2°C, using relative humidity (In=30 ± 2%, out=50 ± 2%) and airflow at 9 g.m^{-3} in a rotatory drying system at 1200 drum speed (Fredhelm Stechel GmbH D-31031 Alfeld, Germany), an up to a_w <0.4. After drying, viable cell number and survival rate in beads was established as described before.

4.2.7 Physicochemical characterization of formulation prototypes

Determination of hygroscopicity

The hygroscopicity of dry beads prototypes was evaluated based on the equilibrium of moisture content achieved when they were exposed to 80% relative humidity at 30°C in a closed container. A saturated ammonium sulfate solution was used to control the relative humidity (Chan *et al*. 2011). The moisture content was measured gravimetrically for each sample at the desired time intervals. The moisture content was expressed as a percentage in dry weight (% d.w.).

Determination of bead shape

The bead shape was quantified using the sphericity factor (SF), which was given by the following equation:

$$Sphericity \quad \text{factor} \ (SF) = \frac{d_{\max} - d_{\min}}{d_{\max} + d_{\min}}$$

where d_{max} is the largest diameter and d_{min} is the smallest diameter perpendicular to d_{max} (Chan *et al.* 2011). The d_{max} and d_{min} were measured using an image analyzer software (Digimizer image, MedCalc Software, Ostend, Belgium) and the sphericity factor was computed.

Bead size and scanning electron microscopy (SEM)

Beads surface morphology was analyzed by scanning electron microscopy. Briefly, beads were fixed by 4% (w/v) glutaraldehyde and 0.5% (w/v) formaldehyde for 2 h. The fixed beads were sequentially dehydrated in a series of acetone (20, 40, 60, 80, 2*100%) 20 min each, and dried on stubs. Dried samples were coated with gold under an argon atmosphere using a gold sputter module (Balzers, SCD 050, 40 mA, 80 seconds) providing a coating of 20-25 nm. Then, samples were then observed with a scanning electron microscope Zeiss 1450VPSE equipped with Everhart-Thornley SE detector at 5 kV.

4.2.8 Selection of pectin bead prototype, pre-conditioning of cells and drying survival

Considering the positive effects in free cells upon osmoadaptation and hydroxyectoine inclusion on drying survival (see section 4.3.1), it was essential to establish the effectiveness of this strategy integrated with the encapsulation in amidated pectin beads. After the selection of prototype P3, the *K. radicincitans* DSM16656^T cells were pre-treated during growth and encapsulated according to the methods described before (see sections 4.2.2 and 4.2.4). Drying survival test was carried out under *in vitro* conditions according to (Humbert, Przyklenk, Vemmer and Patel, 2017; Humbert, Vemmer et al. 2017).

Statistical analysis

The data were analyzed using SPSS Statistics v.2 software (SPSS, Chicago, IL). Data are presented as mean values ± standard deviations (SD) or standard error (SE). The means were tested for significant differences by one-way analysis of variance (ANOVA) followed by the Tukey post hoc test. Repeated measures ANOVA was carried out for the hygroscopic data. Concentration values were logarithm transformed before analysis. The level of significance was set at $p<0.05$.

4.3 Results

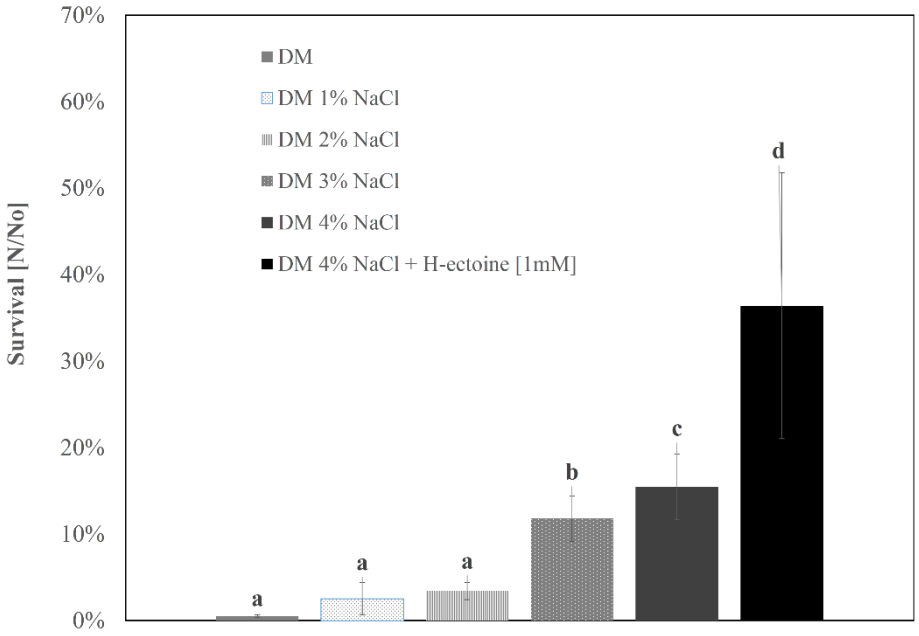
4.3.1 Drying survival for bacteria free cells

The survival of *K. radicincitans* fresh suspension after desiccation was influenced significantly by the increase of salt concentration in the culture media DM. Thus, bacterial cells pre-conditioned in DM 4% NaCl showed the highest drying tolerance in comparison to non-preconditioned cells. Interestingly, exposing bacteria to persistent high osmolarity surroundings during cultivation at 4% NaCl, resulted in a nearly two orders of magnitude higher survival rate (Figure 4.2) with 15.5 ± 3.7 % of living cells recovered, compared to 0.51 ± 0.12 % in control media grown in DM.

The inclusion of hydroxyectoine at 1 mM in DM 4% NaCl provided a further desiccation tolerance in comparison to cells pre-conditioned with DM 4% NaCl. Hence, the combination

of ionically amended media and hydroxyectoine significantly increase drying survival to 36.42 ± 1.53% (Chi^2=16.01; df=18; *p*=0.00681, Figure 4.2).

Figure 4-2: Survival of *K. radicincitans* DSM16656^T cells after 2 h of drying (25 ± 2°C, RH 45 ± 3.5 %). Columns with different letters are significantly different (p <0.05) according to Kruskal-Wallis post hoc test (means ± SD, n= 4)



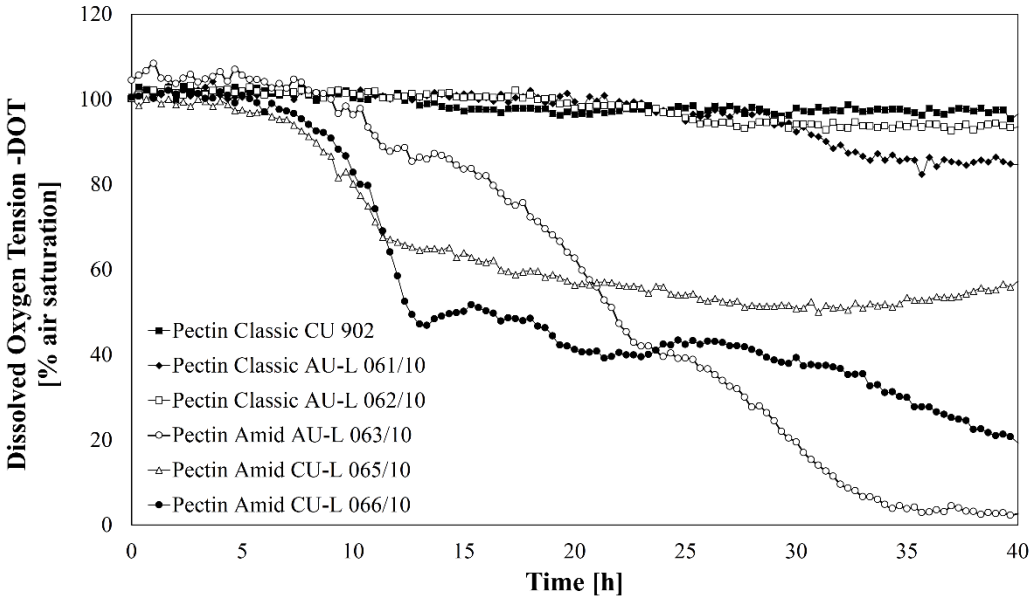
Results indicated that growth conditions at high salinities in DSM16656^T determine tolerance acquisition. Comparative studies revealed bacteria drying survival upon osmotic stress. Hence, Cabrefiga *et al* (2014) demonstrated that culture osmoadaptation in *Pseudomonas fluorescens* EPS62e provide a higher drying survival (70%) in a lactose-based wettable powder. (Malek *et al.* 2012). Besides, hyperosmotic stress along the amendment of growth media with glycine betaine increased by 10-100-fold cell survival (Bonaterra *et al.* 2007).

4.3.2 Screening of pectin references as a nutrient source

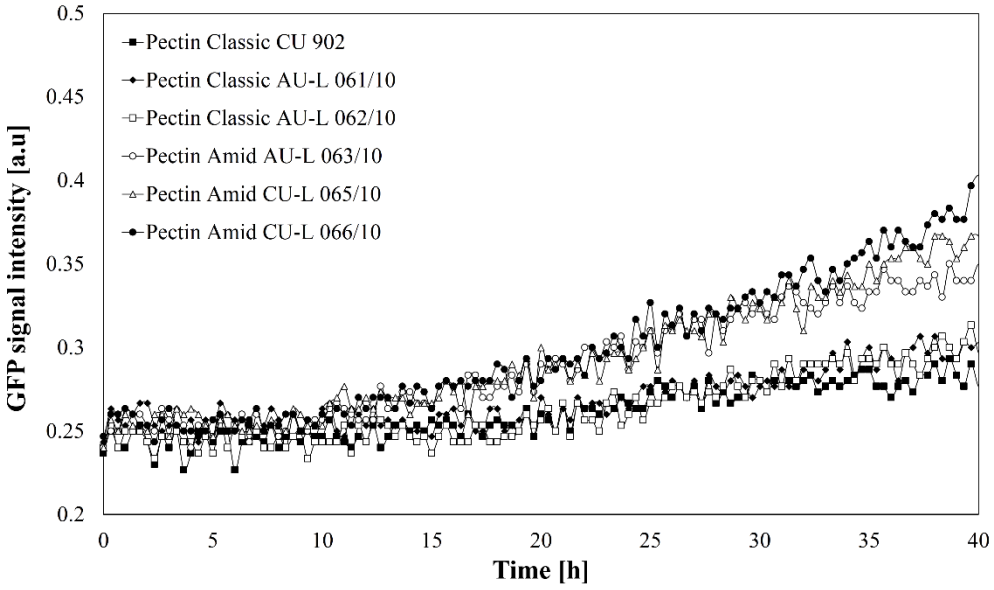
To assess the affinity of selected pectin references to serve as a nutrient source for further encapsulation procedures, a high-throughput microfermentation study was applied. Control with only yeast extract did not show growth activity. Therefore, dissolved oxygen tension and GFP signal intensity curves demonstrated that pectin references with a degree of

amidation favored DOT activity and further bacterial growth ($F_{3,\ 17}$=59.21; $p<0.05$). Noticeably, the galacturonic acid content in pectin has a significant effect on kinetic growth and oxygen consumption during *K. radicincitans* DSM16656^T cultivation ($F5_{,\ 17}$=30.45; $p<0.05$, Figure 4.3 A-B). Thus, GFP intensity curves confirmed that the pectin amid references AU-L 063/10, CU-L065/10 and CU-L-066/10, which included the combination of a DA and galacturonic acid content, are assimilated by *K. radicincitans* DSM16656^T (Figure 4.3 B). Interestingly, CU-L-066/10 showed the shortest lag phase among the biopolymers tested with ca 6 h in comparison to 9.3 h required for AU-L 063/10. With a high oxygen consumption and further longer anoxic conditions periods (< 20 % DOT), the rapid assimilation of AU-L 063/10 may be detrimental in cell viability, as was shown in protein formation in GFP curves compared to CU-L-066/10. Regarding the balance performance on rapid pectin material assimilation by DOT curves conserving cell viability in GFP kinetics, CU-L-066/10 was selected for further analysis.

Figure 4-3: Screening of selected pectin references as a nutrient source. **A)** Dissolved oxygen tension and **B)** Fluorescence intensity of GFP signal during the cultivation of *K. radicincitans* DSM16656^T in different pectin references. BioLector® approach (means ± SD, n= 3).



A



B

4.3.3 Cross-linker determination assessments

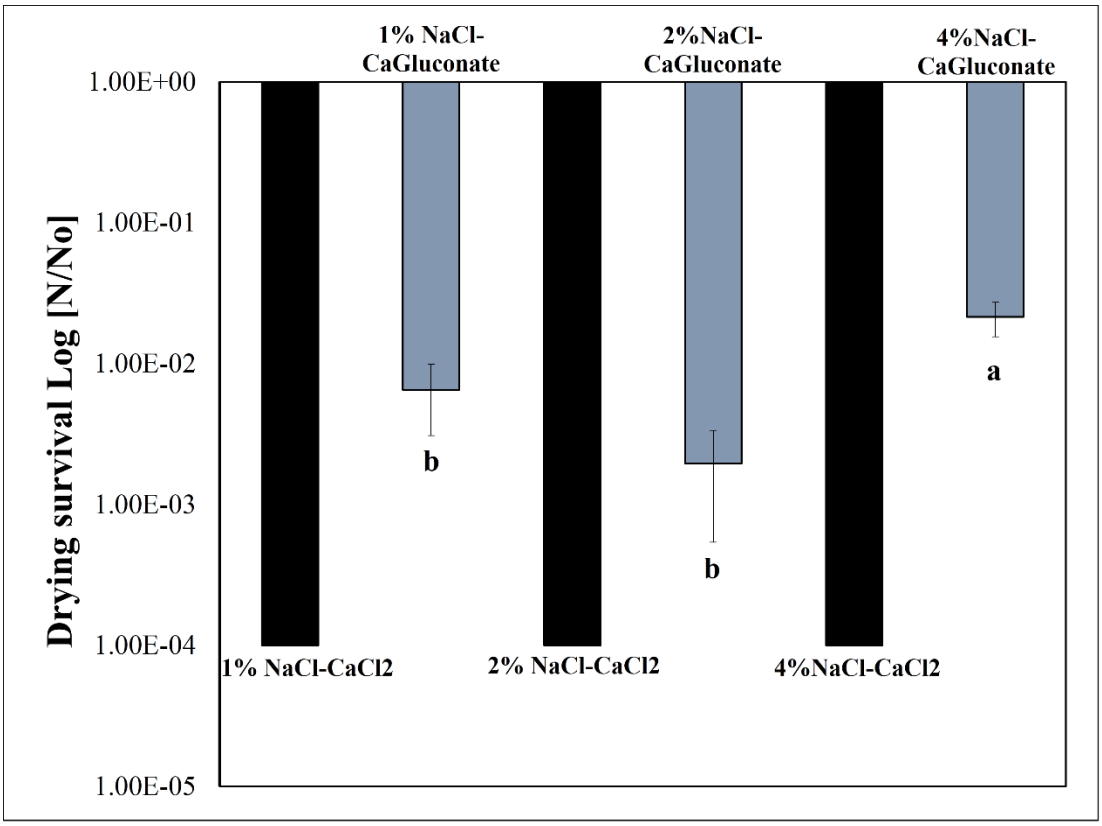
After the cross-linking reaction and formation of beads, the moisture content and water activity (a_w) were determined for each type of beads. There were no significant differences between types of beads regarding the initial moisture content and water activities before and after drying (n =5, p<0.05). It is essential to point out that a bacterial free cells suspension (10^7 CFU mL^{-1}) was used as control during the drying procedure. Upon drying and the evaluated conditions, any viable cells of the free bacterial treatment were detected at 10^6 dilutions. Details of these results are given in Table 4.4.

Based on the results of Figure 4.4, calcium gluconate was selected as the best cross-linker during the gelation reaction, providing a significant effect on the survival of *K. radicincitans*DSM16656^T cells after drying ($F_{3, 11}$ = 267.01, p <0.001, α = 0.05). Thereby, viable cells after drying were not detected in all cases where $CaCl_2$ was used as cross-linker during the formation of beads, suggesting a decrease > 4-log units on cells viable number. Additionally, it was determined that pre-conditioning by osmotic stress at 4% NaCl during cultivation could induce higher tolerance to dehydration conditions, with a viable cell number of up to $4.2x10^9$ CFU g^{-1} of dry bead or a reduction of roughly 2-log units. The drying protection of calcium gluconate was also validated in pectin base beads (data not shown).

Table 4-4: Formulation prototypes for beads preparation to encapsulate *K. radicincitans* DSM16656^{T} cells

Type of beads **Salt Pre-condition-Cross-linker**	**Initial Bead** **Moisture content (%)**	**a_w** **before drying**	**a_w** **after drying**
1%NaCl-CaCl2	74.98 ± 0.93	0.9806 ± 0.002	0.2662 ± 0.035
2%NaCl-CaCl2	75.17 ± 2.12	0.979 ± 0.001	0.2824 ± 0.052
4%NaCl-CaCl2	75.57 ± 2.58	0.9826 ± 0.002	0.286 ± 0.068
1%NaCl-Ca-Gluconate	74.93 ± 4.14	0.9822 ± 0.002	0.3018 ± 0.001
2%NaCl- Ca-Gluconate	74.97 ± 1.55	0.9794 ± 0.001	0.2968 ± 0.056
4%NaCl- Ca-Gluconate	76.84 ± 1.22	0.9812 ± 0.003	0.2792 ± 0.040

Figure 4-4: Effect of the cross-linker calcium chloride ($CaCl_2$) and Calcium Gluconate at 0.1 M, in combination with the salt pre-conditioning on *K. radicincitans* DSM16656^T drying survival. Columns with different letters are significantly different according to Tukey post hoc test (n>4, mean ± SD, $p < 0.05$).

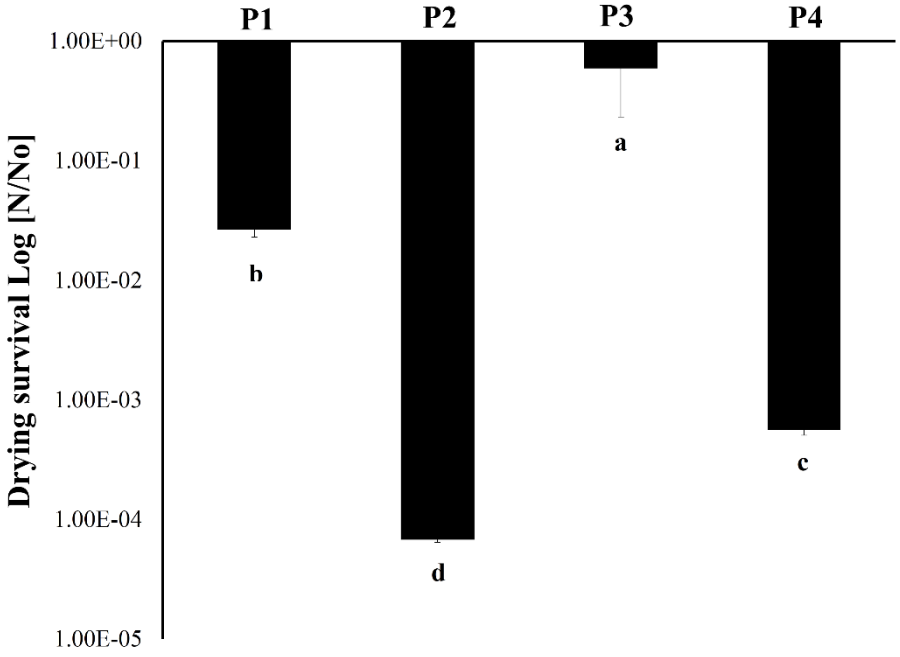


Other studies reported that calcium gluconate provides desiccation tolerance to entrapped cells of *R. terrigena* in starch-alginate based bead up to 31% after drying (Schoebitz *et al*. 2012). Similarly, Humbert *et al* (2017) showed that calcium gluconate as gelling agent increased drying survival of *M. brunneum* and *S. cerevisiae* cells by 75% and 50% respectively.

4.3.4 Drying survival of encapsulated bacteria in formulation prototypes

Adaptability is crucial for survival in nature, and various organisms take extensive measures to minimize the stress of a hostile environment. After the air drying process in the drum dryer at 30 ± 2°C, cell survival in prototypes based on amidated pectin and maltodextrin showed significant higher values in comparison to alginate, with values of 3.69 ±1.52 % and 9.94 ±3.17 % for the prototypes P1 a P3 respectively ($F_{3, 11}$ = 267.01, p<0.001, α = 0.05). In contrast, alginate prototypes reached drying survival of 0.0053 ±0.0013% and 0.0136 ±0.0028 % for P2 and P4 respectively. Interestingly, with the prototype P3, the reduction of viable cell number after drying was a roughly 1.2-log unit, conversely in P2 was 4-log units (Figure 4.5). Final bead moisture content were 2.90 ± 0.22%, 2.83 ± 0.16%, 2.53 ± 0.16% and 2.69 ± 0.25% for prototype P1, P2, P3 and P4 respectively.

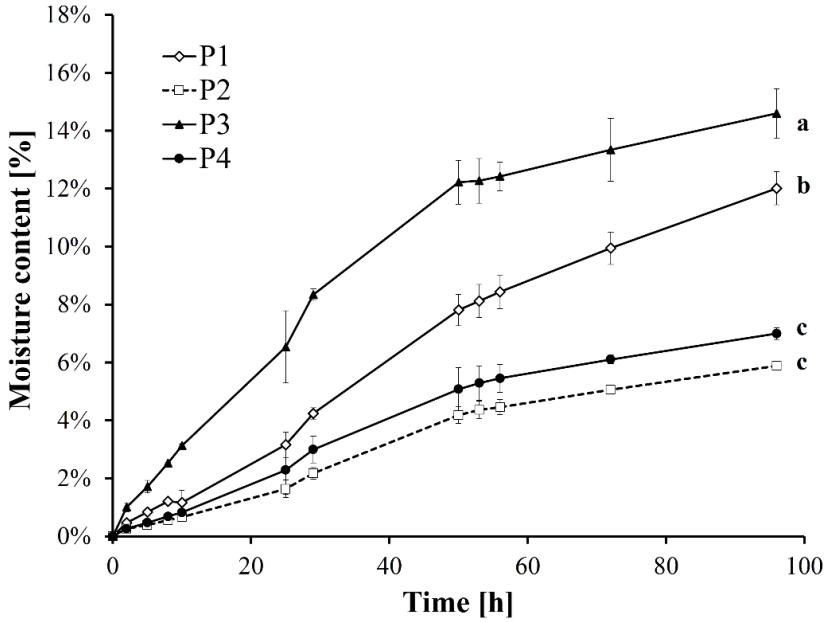
Figure 4-5: *K. radicincitans* DSM16656^T survival after air drying (30 ± 2°C, airflow at 9 g.m^{-3}) in alginate base and pectin base formulations prototypes. Columns with different letters are significantly different according to Tukey post hoc test (n>3, mean ± SD, p < 0.05).



Hygroscopic properties

In order to characterize the moisture transfer by sorption of the dry beads prepared with either amidated pectin or alginate, their hygroscopicity was investigated by determination of its sorption kinetics (Figure 4.6). There was a significant effect of the prototype ($F_{3, 143}$=1236; p<0.001), the prototype-time interaction ($F_{33,143}$=45.34; p<0.05) as well as time ($F_{11,143}$=1101.7; p<0.001) on the moisture content of beads. Formulates containing maltodextrin (P1, P3) inside the beads showed different moisture sorption in comparison to MCC-based formulates (P2, P4). The rate of water uptake from the environment was significantly different (p<0.05). Then, the moisture sorption had a significantly different course for all tested beads (p<0.05). Remarkably, the beads of the P3 prototype revealed a higher hygroscopicity. After 96 h the moisture content of beads were 12.01 ± 0.57, 5.88 ± 0.01, 14.60 ± 0.85 and 7.01 ± 0.20 for the prototypes P1, P2, P3 and P4 respectively (Figure 4.6).

Figure 4-6: Hygroscopicity of hydrogel beads containing *K. radicincitans* DSM16656^T *cells* cross-linked with calcium gluconate at 0.1M and at 80% relative air humidity and 22 ± 2°C (n=4; mean ± SD).



Determination of bead shape and morphology by SEM microscopy

To establish the bead morphology characteristics for each type-bead prototype, the measurement of bead diameter before and after drying and SEM micrographs were carried out. In Figure 4.7, it is observed the macroscopic shape that corresponds mainly to spherical beads, in both alginate and pectin based formulas, with P4 providing the most spherical beads (SF=0.09, Table 4.5). Some tear-shape and egg-shape events occurred in P2 and P3, indicating a high surface tension within the drop (Chan *et al.* 2009).

SEM micrographs confirmed a structural homogeneity of beads prepared either with alginate or pectin as a biopolymer. The surfaces of prototype samples were rough and covered with characteristic wrinkles of different size and frequency (Figure 4.8). The cross-sections micrographs showed the sponge-like inner structure of P3 and P4 with several channels and voids, possibly formed as a result of the migration of water molecules, and further facilitating the permeability. Microscopy assay allowed to clarify that beads P1 and P3 have porous and cracks inside; these hollowed structures are presented more frequently than in P2 and P4. The prototypes P2 and P4 containing MCC presented a heterogeneous surface, unlike P1 and P3 which have maltodextrin in common possess smooth and clean surface.

Figure 4-7: Macroscopy morphology of hydrogel beads prototypes containing *K. radicincitans* DSM16656^T cells cross-linked with calcium gluconate at 0.1M.

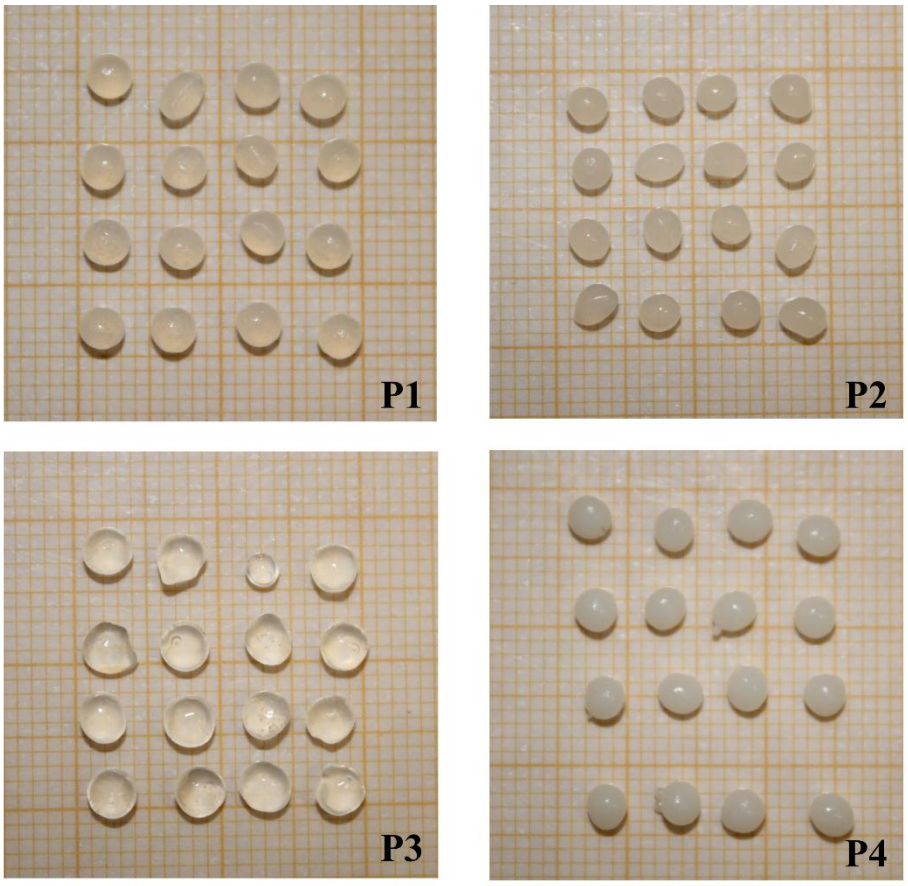
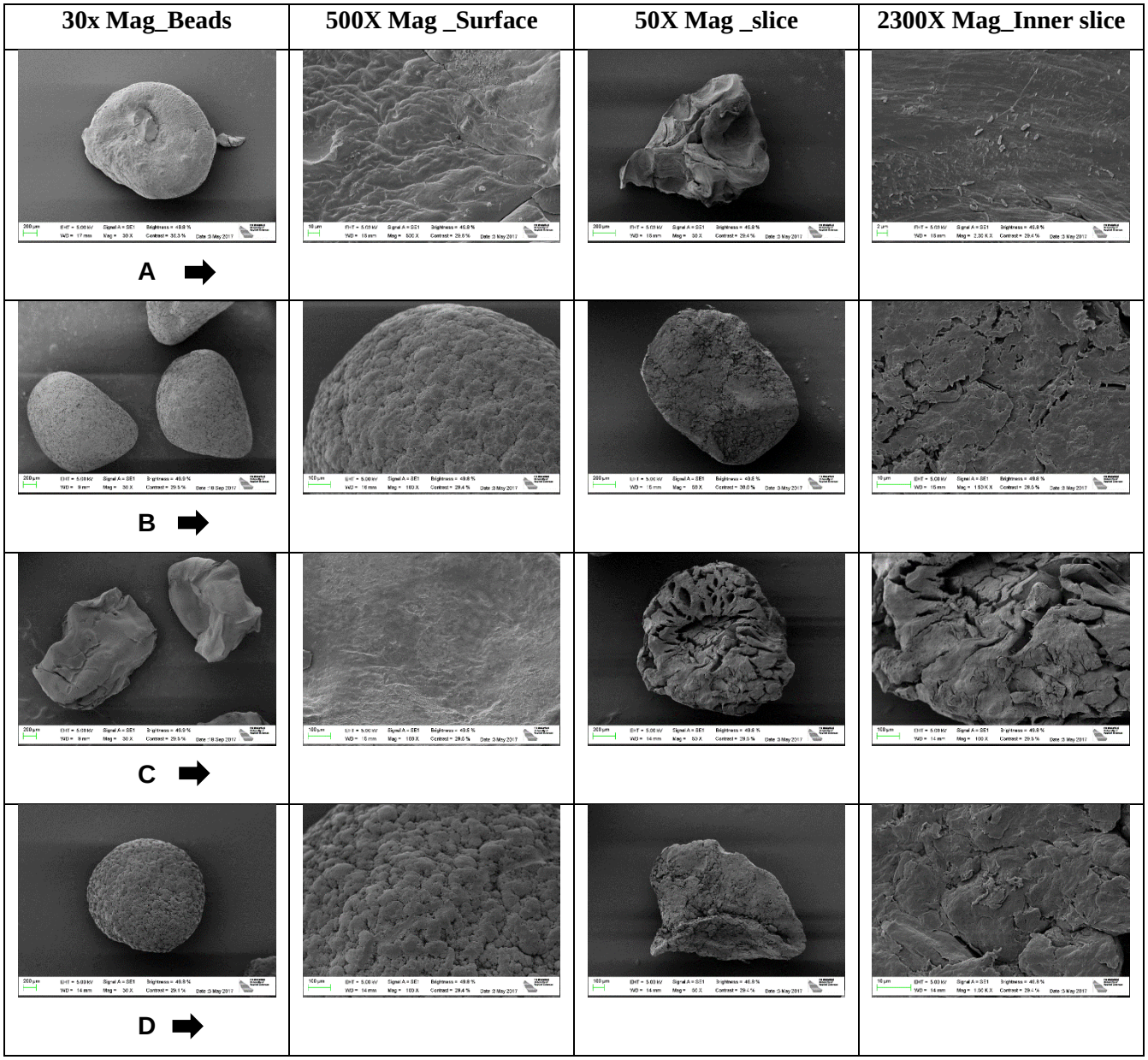


Table 4-5: Formulation prototypes for beads preparation to encapsulate *K. radicincitans* DSM16656^T

	Wet beads		**Dry beads**	
Prototype	mm	SF	mm	SF
P1	3.14±0.16	0.13	1.87±0.30	0.33
P2	2.98±0.26	0.17	1.72±0.28	0.26
P3	3.38±0.31	0.25	2.26±0.36	0.36
P4	3.05±0.13	0.09	1.81±0.11	0.10

SF=sphericity factor

Figure 4-8: Scanning Electron Micrographs (SEM) of *K. radicincitans* DSM16656^{T} beads formulations: (A) Prototype 1, (B) Prototype 2, (C) Prototype 3, (D) Prototype 4.

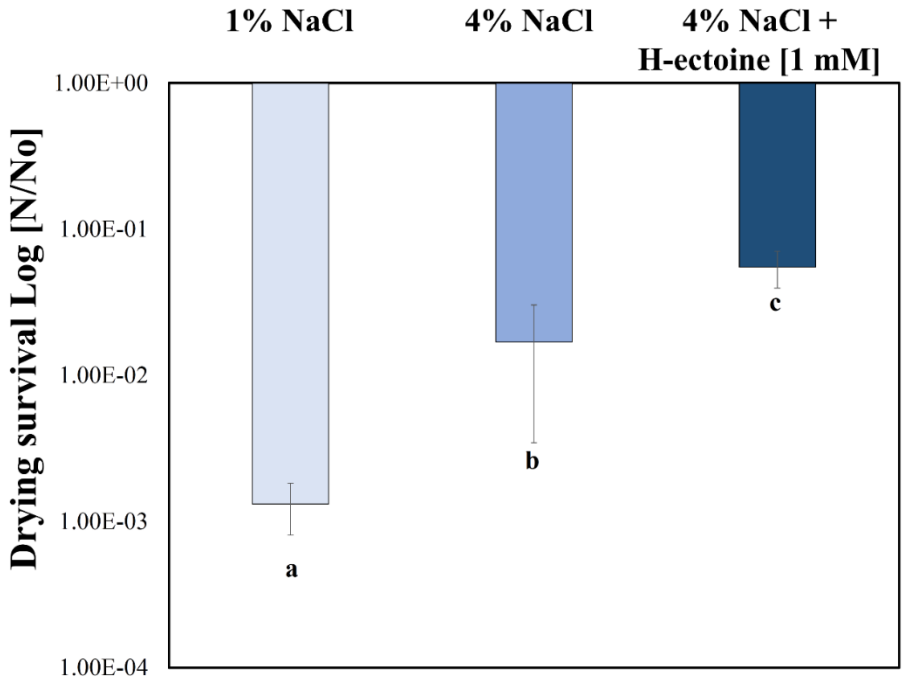


4.3.5 Selection of pectin bead prototype, pre-conditioning of cells and drying survival

In Figure 4.9, it is evident that the osmoadaptation and hydroxyectoine supply previous to encapsulation (P3 prototype) increase the drying survival of entrapped *K. radicincitans* DSM16656^{T} cells. Results suggest that osmotic –stressed cells at 4% NaCl, survive roughly two orders of magnitude more than 1%NaCl treated cells. Noteworthy, hydroxyectoine-

added cells reduced their viable number after drying only 1.27 ± 0.12 log units in comparison to 2.9 ± 0.18 and 1.86 ± 0.33 log units for 1% and 4% NaCl treated cells respectively. The differences in population survival were significantly different ($F_{2, 8}$=39.06; p=0.0004).

Figure 4-9: Drying survival of pre-conditioned *K. radicincitans* DSM16656^T cells entrapment in amidated pectin beads. Different letters indicate significant differences according to Tukey post-hoc test (p<0.05).



4.4 Discussion

An effective establishment of Gram-negative bacterial endophytes-based formulates is linked with the drying survival and the use of advantageous additives. Encapsulation in biopolymeric materials is an emerging alternative for ensuring cell protection and subsequent delivery systems for plant growth-promoting bacteria (Bashan *et al.* 2014). Thus, research on hydrogel bead technology development, claims positives effects on increasing bacterial cells tolerance against unfavorable surroundings caused by biotic and abiotic factors, such as antagonist or dryness (Vemmer and Patel, 2013; Krell, Jakobs-Schoenwandt, Persicke *et al.* 2018; Krell, Jakobs-Schoenwandt, Vidal *et al.* 2018). Ionic gelation as encapsulation technique is a potentially scalable alternative for agro-industrial applications (Humbert, Przyklenk, Vemmer, Schumann *et al.* 2017). The capsules or beads development requires pre-formulation approaches considering the screening of polymeric materials, cross-linkers and additives candidates. These bead components must be compatible with the endophytes, and support phenotypic alterations that strength cells.

It is reported that stress adaptation provokes the modulation of cell physiology to adapt to the perceived environmental stress and thereby indirectly enhances desiccation tolerance (Berninger *et al.* 2018). In general, Gram-negative bacteria respond to elevated-ionic-strength media by synthesizing or accumulating a range of osmolytes, including trehalose, various ectoines and amino acids derivatives such as glycine-betaine and L-proline (Kempf and Bremer, 1998; Sleator and Hill, 2002). To date, this principle has been applied to industrially relevant bacteria rather than to plant-associated endophytes. Thus, osmoadaptation as pre-conditioning mechanism provides an exciting and underused tool to strength bacterial endophytes cells through compatible solutes accumulation, previously formulation-drying processing and application.

As demonstrated, *K. radicincitans* DSM16656^{T} cells grown in low a_w-media altered ionically with NaCl, pose superior drying tolerance in comparison to cells grown in an unmodified basal medium. An osmolality threshold could cause significant differences in drying survival along salinity levels in media, likely between 2 and 3% NaCl, where bacteria cells could prefer the accumulation of ions (salt-in strategy), over the physiologically compliant organic osmolytes (salt-out strategy) (Kempf and Bremer, 1998; Czech *et al.* 2018). It is also

hypothesized that metabolic pool alterations in cells could drive the desiccation tolerance mechanism in osmotic treated cells.

The superior drying survival of *K. radicincitans* DSM16656^T cells upon adding hydroxyectoine during the osmoadaptation procedure probably occurred due to the physiochemical features of hydroxyectoine as drying protectant. So, hydroxyectoine decreases water activity coefficients (Held *et al.* 2010) and provides superior glass-forming properties and redox stability (Tanne *et al.* 2014). Besides, hydroxyectoine is more efficient than ectoine by increasing the hydration and mobility in lipid membranes, giving an advantage for cell membranes to withstand severe surrounding environments like osmotic unbalance, accelerating cellular repair mechanisms (Harishchandra *et al*. 2010). These properties may confer to Gram-negative bacterial cells more extended life stability, higher desiccation tolerance and protection against drying (Manzanera *et al.* 2002; Manzanera, *et al*. 2004; Pastor, *et al.* 2010).

Calcium gluconate (CG) as cross-linker showed remarkably benefits for conferring desiccation tolerance of encapsulated endophytic cells. Previous research showed the higher survival of *M. brunneum* conidia and *S. cerevisiae* upon the usage of CG as a cross-linking agent in comparison to calcium chloride (Humbert, Przyklenk, Vemmer and Patel, 2017). The mechanisms of CG as drying protector are not elucidated. However, studies suggest that CG conveys benefits such as increasing hygroscopicity of beads, shelf life and nutrient source for fungi cells (Humbert, Przyklenk, Vemmer and Patel, 2017).

Gluconate is classified as osmoprotectant (Schoebitz *et al*. 2012) may protect the membrane of dried cells by its cyclic ester form glucono-δ-lactone, resembling cyclic monosaccharides, which upon drying form amorphous glasses in the dry state (Crowe *et al.* 2001). This state confers a slowdown diffusion process due to the high viscosity in the glassy state and protects the membrane by different mechanisms (Wolfe and Bryant, 1999; Humbert, Przyklenk, Vemmer and Patel, 2017). It is noteworthy that this is the first report demonstrating that CG can act simultaneously as cross-linker of amidated pectins and enhancer of drying survival of encapsulated Gram-negative bacterial endophyte cells.

During desiccation, an organism turn to a state also known as anhydrobiosis, where vital functions temporary stop completely or partially becoming dormant. Upon rehydration, the organism is resuscitated and resumes its vital functions (Garcia, 2011). The rehydration rate or hygroscopic influence on cells survival is frequently under discussion, and the argumentation is contradictory suggesting strain-depend mechanisms. Here, hydrogel beads containing MCC has a lower tendency to absorb water and are therefore less hygroscopic than beads prepared with maltodextrin. It should be considered that maltodextrin is a highly hygroscopic substance, which exhibits a steady increase in the water absorbed at high values of a_w in the surroundings (Saavedra-Leos *et al.* 2015).

Conversely, MCC possess a relative lower hygroscopic performance; however, at levels above 5%, the material can exhibit significant changes consistent with a transition from glassy to rubbery state which is detrimental for bacterial cells (Amidon and Houghton, 1995). Noteworthy, maltodextrin prototypes (P1, P3) showed the highest values of drying survival, maintaining the decrease of viable cell number under 2-log units. Here, maltodextrin demonstrates its alleviation effect on Gram-negative bacteria cell survival as in previous studies (Berninger *et al.* 2017).

SEM images indicate outstanding differences in MCC and maltodextrin hydrogel beads prototypes. Hence, the absence of microparticles formation clarifies that maltodextrin keeps the amorphous-crystalline state, and the open inner cracks, especially in P3 could promote water absorption diffusion for rapid activation of bacteria cells. Though high moisture absorption rates are not desirable for the dry beads containing cells, the water uptake for the maltodextrin-based prototypes are still low and may therefore not detrimental to their shelf life for the events of maltodextrin rubbery state (Saavedra-Leos *et al*. 2015; Humbert, Przyklenk, Vemmer and Patel, 2017).

Although there is scarce research that deals with the alleviation of drying effects on Gram-negative endophytes, exist some reports regarding bacteria belonging to the same genera than endophytes strains. For instance, studies suggest that the potential fruit pathogen Acidovoraz

citrulli, enhance its survival by the embryo/endosperm localization in watermelon seeds versus the seed coat (Dutta *et al.* 2016), with 1-2 log units of viability loses. The Aminobacter sp. MSH1 and the Sphingomonas sp. PM2 desiccation tolerance was enhanced before freeze-drying with trehalose, reaching survivals between 30 and 60% (Schultz-Jensen *et al.* 2016). For the opportunistic pathogen Acinetobacter baumannii, a stationary phase phenotype with the BfmR gene expression showed higher drying stress response, this gene is stimulated by increasing osmolarity (Farrow *et al.* 2018). Another study with Alcaligenes species demonstrated that the drying resistance for the amino forming bacteria was able to produce cadaverine and putrescine (Lakshmanan *et al.* 2002). Some proteins such as late embryogenesis abundant (LEA) are reported to be critical for conferring drying survival and resistance to high osmolalities to cyst forming Azotobacter vinelandii (Rodriguez-Salazar *et al*. 2017).

Considering the immobilization of Gram-negative bacteria as drying protection strategy, few researchers have shown promising findings. Thus, the encapsulation of *Beijerinka* sp. in DE malt dextrin as wall material protected the cells during spray drying at a_w 0.252 with only 1 log unit of viability decrease (Boza *et al*. 2004). Interestingly, the immobilization of *Pantoea agglomerans* ISIB55 and *Burkholderia caribensis* ISIB40 in polyvinyl alcohol-nanofibers reduced the number of viable cells by ~ 1.5 log units (De Gregorio *et al*. 2017), nonetheless, the electrospinning technique requires developments for scaling. The survival population of *Azospirillum brasilense* MTCC125 after air-drying at 40°C cells were 27% higher in alginate beads compared to the standard grown cells, but with a reduction of 2-3 log units on cells viability at a_w <0.2 (Joe *et al*. 2012). Another drying studies on Gram-negative bacteria include the spray drying of *Enterobacter sakazakii* strains with reduction of viable number cells from 1.5 to 3 log units, using skim milk and T_{in} at 160°C and T_{out} at 90°C (Arku *et al*. 2008). Otherwise, some non-sporulating bacteria as *Flavobacterium columnare* did not survive after drying, even with protectants such as trehalose or skim milk (Peiren *et al*. 2015).

Among the drying process, air drying has demonstrated potential benefits for desiccating Gram-negative bacteria endophytes such as *Paraburkholderia phytofirmans* PsJN (Berninger *et al.* 2017). Since *K. radicincitans* DSM16656^T is quite sensitive for drying, with

only a decrease of 1 log unit of viable number cells, the approach of pectin-based hydrogel showed here is promising for endophytes dryable formulations.

The low survival rate of *K. radicincitans* DSM16656^T may be attributed to the nature of being a Gram-negative bacteria, which contain a the thinner peptidoglycan layer and the presence of lipopolysaccharides on the cell wall in the former species (Miyamoto-Shinohara *et al*. 2008). Thus, in Gram-negative bacteria, peptidoglycan layer has 5 to 10 nm thick, whereas in Gram-positive bacteria their layer thickness is about 20 to 80 nm (Gupta, 2002). In consequence, this thinner protecting layer in *K. radicincitans* DSM16656^T cells could generate a greater tendency to rupture during drought stress. Moreover, motile Gram-negative bacteria genera with peritrichous flagella such *K. radicincitans* DSM16656^T (Figure 5.5) are generally more sensitive during drying processes, because of the mechanical fragility and the protein-forming flagella apparatus sensitivity (Miyamoto-Shinohara *et al*. 2008).

Gram-negative bacteria with lipopolysaccharide (LPS) on the cell wall, with hydrophilic polysaccharide outermost chains may prevent the removal of water molecules during desiccation, enhancing drying survival (Miyamoto-Shinohara *et al*. 2008). According to its proteome, *K. radicincitans* possess putative lipopolysaccharide heptosyltransferase III and heptosyltransferase RfaC enzymes, which are involved in the biosynthesis of core regions of lipopolysaccharide (Witzel *et al*. 2012). Questions remain regarding the influence of osmotic stress cultivation on LPS biosynthesis that may drive higher drying tolerance.

Finally, the effectiveness of amidated pectin as a nutrient source and as entrapment biopolymer for *K. radicincitans* DSM16656^T cells was established. Indeed, results demonstrated that amidated pectins with a high content of galacturonic acid could provide an advantageous nutrient source for endophytic bacteria. The microplate GEN III findings in Chapter 2 corroborate the capacity of *K. radicincitans* DSM16656^T to use galacturonic acid as C-source. Besides, pectins in combination with maltodextrin upon drying are capable of forming a functional matrix suitable for sensitive substances, with high-water soluble properties, where pectin serves as a coating agent and maltodextrin mainly acts as a matrix-forming material (Sansone *et al*. 2011). The role of sorbitol and monosodium glutamate in

bead characteristics is still unknown; however, details of pectin bead performance are addressed in Chapter 5.

4.5 Conclusions

The pre-formulation and further drying survival assessments indicated that amidated pectin with a high content of galacturonic acid such as AU-L 063/10, CU-L065/10 and CU-L-066/10 are an advantageous nutrient source for *K. radicincitans* DSM16656^{T}. The combination of amidated pectins cross-linking with calcium gluconate favors the desiccation tolerance of the endophyte upon a convective drying process. After encapsulation in pectin-base hydrogel networks, the osmoadapted and hydroxyectoine-added cells resisted stronger drought conditions, integrating cultivation and formulation procedures, an essential step towards dryable formulations of Gram-negative endophytes. Finally, the amidated pectin-based prototype P3 with maltodextrin as filler was selected for further analysis, including the evaluation as delivery systems in a plant model.

5. CHAPTER 5. A FORMULATION STRATEGY FOR BACTERIAL ENDOPHYTES: THE INTEGRATION OF PRE-CONDITIONING OF CELLS WITH THE ENCAPSULATION IN AMIDATED PECTIN BEADS

5.1 Introduction

Application of plant growth-promoting endophytic bacteria (PGPEB) based formulations is considered an essential alternative to overcome soil health issues caused by the application of chemical fertilizers (Kandel *et al.* 2017). Contamination of soils and groundwater upon chemical fertilizers usage are common on current global agroindustry. Gram-negative bacteria are generally less tolerant of such environmental conditions than Gram-positive bacteria, yeasts or filamentous fungi because among others the chemical composition of outer cellular layers (Wieland *et al*. 2001; Gao *et al*. 2007; Ren *et al*. 2018). Thus, shortly after suspensions of bacteria are inoculated into the soil without a proper carrier, the bacteria population declines rapidly for most species of PGPB (Bashan *et al*. 2014). For Gram-negative bacteria, the ability to withstand abiotic stresses during technical formulation processing including high temperatures, elevated osmolality, and dehydration while conserving biological activity and further ability to colonize plant endosphere are central problems that require research efforts.

The potential of PGPBE has primarily been acknowledged and well documented across the globe in the last few decades. Hence, stimulation of plant growth and yield improvements by endophytic bacteria have been reported on laboratory, greenhouse and field level in several plant species (Garima and Nath, 2015; Lally *et al*. 2017; Verma *et al*. 2018). Some of them including growth under drought stress, nitrogen deficiency and excessive salinity (Rho *et al*. 2018). Notwithstanding, despite the reported benefits, most of the investigations regarding PGPBE including *K. radicincitans* DSM16656^{T} lack the formulation procedures, which could facilitate the step forward from the laboratory to the field (Berger *et al*. 2015; Garima and Nath, 2015; Berger *et al*. 2017).

Previous studies with promising bacteria in agriculture suggested that salt-stressed cells and compatible solutes inclusion could potentiate their biological activity. For *Staphylococcus saprophyticus* (ST1), biofilm formation and exopolysacharides (EPS) production increased along with NaCl concentration, enhancing its plant growth-promoting abilities (Qurashi and Sabri, 2011). Besides, the combination of osmoadaptation and glycine betaine amendment to

growth media increased *Pantoea agglomerans* E325 colonization in apple flowers (Pusey and Wend, 2012). In particular, this strategy may be extended to endophytic bacterial, which studies revealed that osmoadaptation and the accumulation of selected compatible solutes could drive the strengthening of bacterial phenotypes (Cabrefiga *et al.* 2011).

Encapsulation may offer an uninterrupted supply of nutrients without competing with other microorganisms, protection against environmental stress and longer shelf life in storage (Bashan *et al.* 2002; Trivedi *et al.* 2005). So, the immobilization of microorganisms in polymeric materials provides them with several significant advantages over free-living suspensions.

As it was mentioned in Chapter 4, pectins emerge as a potential alternative for producing polymeric inoculants. One of the most notable pectin features is its ability to form gels and hydrogel composites, which can provide a wide range of biological applications. Hydrogel beads are hydrophilic, three-dimensional cross-linked polymer networks, with high biocompatibility, porous structure, enabling a high water content that mimics the extracellular matrix environment *in* vivo (Zhao *et al.* 2018). Pectin is a structural plant polysaccharide, which macromolecules aggregates tend to dissociate and expand at neutral pH (Liu *et al.* 2003).

Due to differences in degrees of esterification and amidation, viscosity, gelling, mechanical properties, and stability of pectin are affected (Sato *et al.* 2008; Munarin *et al.* 2012). Pectins have been widely used as emulsifiers, gelling agents, glazing agents, stabilizers, and/or thickeners in food, pharmaceutical, personal care, and polymer products (Chan *et al.* 2017). Altogether, the potential utilization of pectin-based systems as a carrier of PGPBEs remains unexplored. Besides, a detailed investigation regarding the interaction of embedded bacterial endophytes in amidated pectin beads during plant colonization is necessary. Thereby, in this chapter, the novel combination of a bacterium endophyte with the encapsulation in amidated pectin hydrogel beads for supporting endophytic performance is addressing.

The exploitation of biostimulants based on bacterial endophytes in the context of achieving a sustainable regime of crop fertilization will require an in-depth understanding of how PGPBEs interact with the plant host. Hence, questions are remaining about the chemotaxis of entrapped endophytic cells, in addition to how these immobilized cells re-activate through the water uptake and emerge from the polymeric material towards plant tissue. Here, it is hypothesized that amidated pectin represents a diffusion system for bacterial growth, enabling root exudates-sensing, and facilitating cells to grow as spatially structured aggregates similar to those reported in roots during plant colonization by using planktonic cells.

Trough gnotobiotic and glasshouse studies, in combination with advanced microscopy imaging approaches, this research's section aimed at determining the amidated pectin dried beads capability as a delivery system of the bacterium endophyte *K. radicincitans* DSM16656^T and its modes of action. Moreover, it is proposed to establish the ability of encapsulated pre-osmoadapted and hydroxyectoine-added cells to colonize the endosphere in radish plants.

5.2 Materials and Methods

Chemicals used in this study were obtained from Carl Roth GmbH (Karlsruhe, Germany) or AppliChem GmbH (Darmstadt, Germany) if not specified otherwise. Hydroxyectoine (Hye) was acquired from Sigma Aldrich (Cat: 70709, Sigma Aldrich Corporation, Germany). Amidated pectin was provided by Herbstreith & Fox KG (Neuenbuerg/Wuertt, Germany). All other materials used were of analytical reagent grade and were used as received.

5.2.1 Encapsulation of *K. radicincitans* DSM16656^T cells

As described in chapter 4, calcium amidated pectin hydrogel beads were selected as immobilization support. Briefly, an encapsulation suspension was obtained by mixing ALM pectin solution [4% w/v Amid CU-L 066/10 (DE 24% and DA 24%)] in ultrapure water (Elix Advantage Water Purification System, Merck Millipore, Darmstadt, Germany) at 50 % w/w. Pectin solution was supplemented with 14% w/w maltodextrin (Applichem Pancreac,

A4804), 1% w/w sorbitol and 1% w/w monosodium glutamate. *K. radicincitans* cells suspension for the osmoadaptation and hydroxyectoine treatments were added in the matrix to a final concentration of 15% w/w (~ 8 x10^9 viable cells mL^{-1}) and the suspension was gently stirred for 5 min.

For bead formation, it was followed by the methods described in chapter 4 (sections 4.2.2 ad 4.2.4). Four biological replicates composed each treatment.

5.2.2 Encapsulation efficiency

The measurement of encapsulation efficiency for *K. radicincitans* DSM16656^T cells in amidated pectin hydrogel beads was carried out as followed: 10 beads were disintegrated in a solution containing 0.03 M citric acid and 0.05 M sodium carbonate (pH 7±2) for 1 h in a rotatory shaker at 150 rpm (Mater *et al.* 1995). After complete dissolution, the entrapped viable bacteria were counted by diluting samples and plated on standard nutrient agar media (Merck, Darmstadt, Germany), and incubated at 30°C for 24 hours. Bacterial cells encapsulation efficiency (BEE) was calculated by the following equation (Eq. 5.1).

$$\text{BEE } (\%) = \frac{Log_{10}\,[\text{N}]}{Log_{10}\,[\text{No}]} x100 \;\; Eq.\,5.1$$

Where N is the number of measured viable bacteria cells and N_0 displays the free viable bacteria cells before encapsulation (Haghshenas *et al*. 2015).

To establish the efficacy of pectin networks to entrap beads additives, sorbitol (182.2 g mol^{-1}) as a chemical parameter was selected. Here, the amount of sorbitol was quantified in the remaining calcium gluconate cross-linking solution. Briefly, after 10 min of hardening time, beads were separated and 1 mL of calcium gluconate solution was recovered, centrifuged at 21130 x g for 5 min (Mikro HT 200R, Hettich GmbH & Co. KG, Tuttlingen, Germany), and filtered through a 0.45 µm membrane filter. The concentration of sorbitol was determined by HPLC (EC 150/4.6 NUCLEODUR® 100-5 NH_2-RP column, RI detector). The chromatographic separation was performed at a flow rate of 1 mL min^{-1} at 30°C controlled with a column heater and using a solvent gradient established between eluents A and B (80%

acetonitrile in HPLC water). The peak areas were integrated and compared with calibration curves constructed with sorbitol [0.2-20 mg mL^{-1}]. The entrapment efficiency (EE) for sorbitol was calculated using the equation (Eq. 5.2):

$$EE\ (\%) = \frac{[Total\ sorbitol\ added - sorbitol\ in\ crosslinker\ solution}{\text{Total sorbitol added}}\ x\ 100\ Eq.5.2$$

5.2.3 Plant growth promotion in radish by osmoadapted and encapsulated *K. radicincitans* DSM16656^T cells

The efficacy of amidated pectin hydrogel beads as formulation alternative for the bacterium endophyte *K. radicincitans* DSM16656^T was assessed under glasshouse conditions.

Moreover, the extended cross-effect of pre-conditioning by osmoadaptation and the inclusion of hydroxyectoine was also addressed. The glasshouse experiments were conducted independently as those described in Chapter 3. Briefly, radish (*R. sativus* L. var. sativus) seeds of cultivar Rondar (F1 Hybrid; S & G GmbH, Kleve, Germany) were used as plant systems. Ten radish seeds were placed per pot, with ten pots per treatment, filled with 1.5 L of a 1:1 (v/v) quartz-sand soil mixture (Fruhstorfer Erde type T25: P_2O_5: 200-300 mg L^{-1}, Hawita Gruppe GmbH Vechta, Germany). Afterward, pots were randomly placed on trivets to avoid the transfer of bacteria (Berger *et al*. 2015). The final concentration of cells in dried beads considered the beneficial effects on drying survival encountered previously by salt stress and hydroxyectoine addition (see Chapter 4). Thus, *K. radicincitans* DSM16656^T inoculation with osmoadapted as well as bacteria cells pre-conditioned with hydroxyectoine was conducted by locating two dried amidated pectin beads with the same log unit of cells concentration ca 2.0 $x10^6$ CFU/bead under every single seed. Amidated pectin hydrogel beads without the endophyte were also tested as a control for the formulation components. Native seed without any treatment was used as the absolute control.

In these assays, seedlings were irrigated and fertilized manually with Hoagland solution (50 mL per day) (Hoagland and Arnon, 1950). Plants were maintained under natural light conditions. Temperature and humidity were recorded over the growth period, with an average temperature of 18 ± 2°C and with an air humidity > 45 %. (See Chapter 3).

Plant sampling was conducted at one-week post-planting from three different locations per plot. The seedlings were rinsed thoroughly with sterile water for removing soil with beads adhered to the roots. Further, samples were flash frozen prior to isolation of nucleic acid procedures. At this plant age, the root length was measured with a digital image analyzer (Digimizer image, MedCalc Software, Ostend, Belgium) and the plants were equally thinned to five plants per pot. Final sampling was carried out at five weeks post-planting, and then five radish plants from the center of each pot were harvested. Total fresh mass of tubers and leaves material and the tuber diameter of each plant were measured. The leaves were separated from roots, oven dried at 60°C during 4 days (WTC Binder 342, Germany) until constant weight and dry weight of tubers and leaves were determined (Chapter 3). The data collected are the mean values of two whole experiments.

5.2.4 Plant colonization by osmoadapted and encapsulated *K. radicincitans* DSM16656^{T}cells

Nucleic Acid extraction and quantification of K. radicincitans in planta using qPCR

The quantification of endophytic capacity followed the procedures previously described (see Chapter 3, section 3.2.6). Thereby, the fold colonization of *K. radicincitans* DSM16656^T entrapped cells in treated plants with respect to the reference gene and to the control plants was calculated and represented with the $2^{-\Delta\Delta cq}$ method (Livak and Schmittgen, 2001).

5.2.5 Encapsulated bacteria and radish seedlings interaction: GFP-tagged bacteria approach

To visualize the endophytic mode of actions and chemotactic performance of the encapsulated bacteria cells within radish seedlings an *in vitro* gnotobiotic study was conducted. Briefly, electrocompetent bacterial cells were transformed with plasmid pMP4655 (Lagendijk *et al.* 2010) (Appendix 6). Single colonies of eGFP mutants of *K. radicincitans* DSM16656^T grown on Luria-Bertani agar plus gentamycin (150 µg mL^{-1}) were inoculated in 100 mL standard nutrient broth and the encapsulation of GFP-tagged bacteria followed the procedure above.

Endophytic modes of action visualization for entrapped K. radicincitans DSM16656^T

To elucidate the interaction of encapsulated bacteria and radish plant tissues a cell imaging approach was conducted. Firstly, the activation of beads and seedlings tissues interaction events were observed under transmitted light microscopy (EVOS® XL core, Life Technologies). After, GFP-tagged bacteria from amidated beads and subsequent tissue colonization were observed with an inverted imaging system with four-color fluorescence and transmitted-light applications approach (EVOS® FL, Life Technologies). Further, the root colonization by immobilized bacterial within specific site-locations was recorded with a Zeiss LSM 510 META laser scanning confocal microscope (Carl Zeiss Jena GmbH). Bacterial eGFP fluorescence signals were captured using argon laser excitation at 488 nm (BP505-550 180 filter, Plan Apo 63/1.4 oil lens), and root images were captured using bright field settings (Witzel *et al.* 2017; Becker *et al.* 2018).

Mapping of the chemoattraction for the bead-radish seedling system

The chemotactic performance of entrapped bacteria was detected by multispectral and kinetic fluorescence imaging (PSI Open FluorCam FC 800-O, PSI, Brno, Czech Republic). Briefly, three radish seeds and three amidated pectin beads containing immobilized GFP-labelled *K. radicincitans* DSM16656^T were located in a Petri dish with 20 ml of agar media (1% w/v). After four days of incubation at 30±1°C, GFP tagged bacteria activity inside beads and their chemotactic interaction with radish seedlings was assayed. The following parameters were used for capturing fluorescence images: Reflectance mode: Blue light source (447 nm) at 5% intensity, bandpass filter (440/40 nm), shutter at 2 milliseconds and sensitivity at 0%; Fluorescence mode: GFP bandpass filter (517/20 nm): Blue excitation light (447 nm) at 100% intensity, shutter at 300 milliseconds and sensitivity at 38%.

Flagellar apparatus of *K. radicincitans* DSM16656^T was visualized as follows: Bacteria cells were taken from solid media and suspended in saline solution (0.05 M). One drop of bacterial suspension was applied in Pioloform-carbon-coated, 400-mesh copper grids (Plano GmbH) for 10 min. Further, cells were fixed with aqueous glutaraldehyde (2.5%, 1 min), stained with uranyl acetate (2.5%, 1 min), and examined by transmission electron microscopy (JEM-1400 Plus, JEOL, voltage at 120 kV) (Becker *et al*, 2018).

Statistical analysis

SPSS Statistics v.22 software (SPSS, Chicago, IL) was used for analyzing data. Results are presented as mean values ± standard deviations (SD). Data were checked for normality and homogeneity of variance using Shapiro-Wilk and Bartlett test, respectively. Means were tested for significant differences by one-way analysis of variance (ANOVA) followed by a Tukey post hoc test. Data from glasshouse experiments were subjected to Duncan post-hoc test. The significance level was set at $p<0.05$.

5.3 Results

5.3.1 Encapsulation efficiency

The utility of a biopolymer hydrogel for providing a delivery system relies on the entrapment effectiveness of the active ingredient and additives (Bashan *et al.* 2002). Hence, to look into the efficiency of amidated pectin beads with CU-L-066/10 for encapsulating *K. radicincitans* DSM16656^T cells and formulation additives, the concentration of these parameters before and after the entrapment was determined. The initial cell count of *K. radicincitans* before beads preparation was 9.46 ± 0.28 log CFU mL^{-1}. The encapsulation efficiency for bacteria cell using amidated pectin beads was 98.37 ± 1.39 % (n=4). Regarding sorbitol encapsulation efficiency, HPLC showed that 48 ± 1.39 % (n=4) of this polyol remains into the amidated pectin beads after the cross-linking reaction. Beads diameter before and after drying were 3.38 ± 0.31 mm and 2.26± 0.26 mm respectively (n>8).

5.3.2 Plant colonization by encapsulated *K. radicincitans* DSM16656^T cells

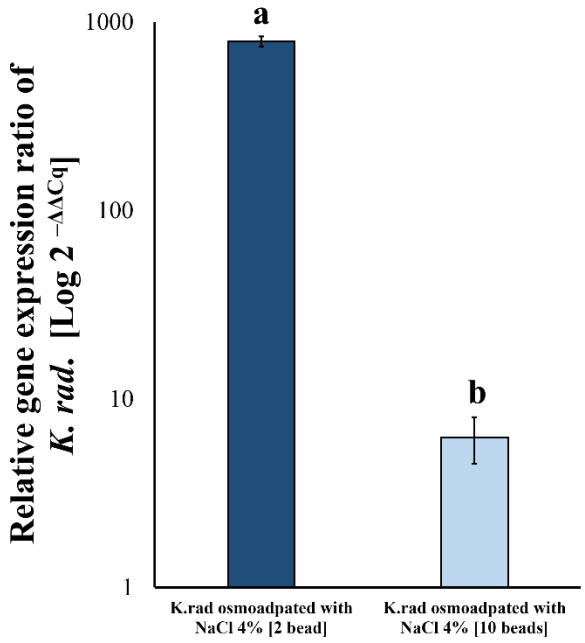
Effect of the number of beads on plant colonization

To establish the number of beads per seed that guarantee plan colonization, a comparative study with amidated pectin beads with 4% NaCl treated cells was carried out. It is important to mention that during the glasshouse trial, there was a negative effect by the number of beads per seed on plan colonization. Thus, 10 beads per seed generally had significant lower colonization capabilities, roughly 100-fold less, compared to treatments with 2 beads per

seed ($F_{1, 6}$=671.7, P<0.0001, Figure 5.1). Considering these results, 2 beads per seed method for inoculation was selected for further glasshouse trials.

It is important to point out that there are not published and comparable data regarding the number of beads and PGPBE plant colonization performance.

Figure 5-1: Accumulation of *K. radicincitans* DSM16656^T DNA in inoculated radish plants with amidated pectin beads. Effect of number of beads per seed on relative gene expression in plant tissue. Different letters represent significant differences according to post hoc Tukey post hoc test at p<0.05, (means ± SD, n>3).

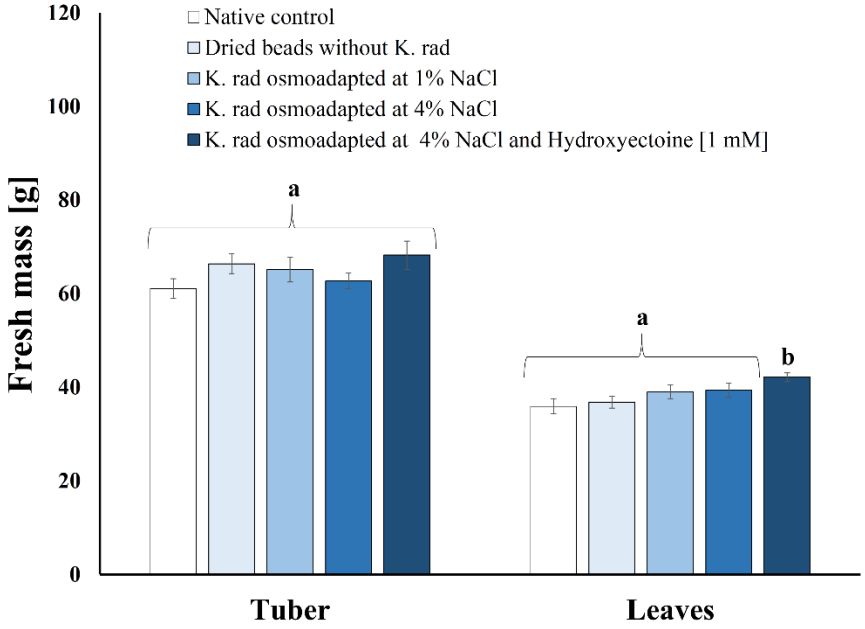


5.3.3 Plant growth promotion in radish by osmoadapted and encapsulated *K. radicincitans* DSM16656^T cells

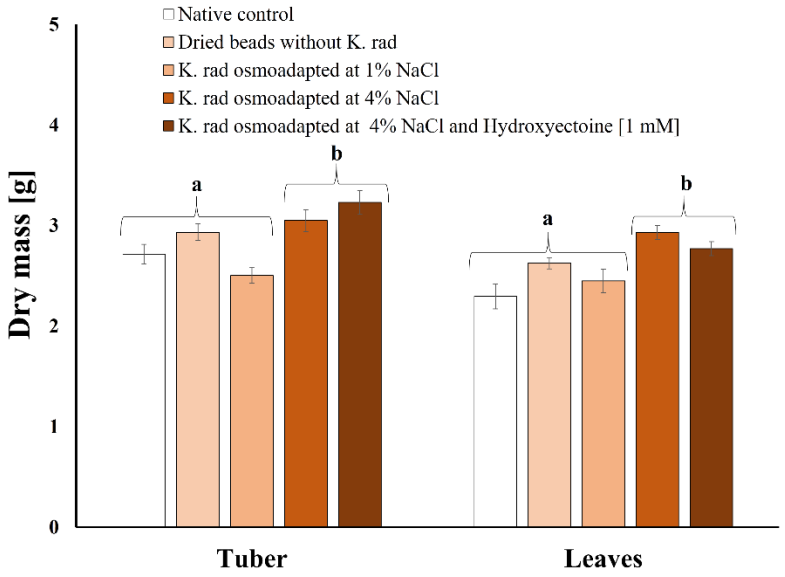
Generally, in both glasshouse experiments, plant growth promoted by amidated pectin hydrogel beads containing *K. radicincitans* DSM16656^T cells was observed for all inoculated radish plants, compared to uninoculated native plants. The dried beads for each treatment contained the same log unit of concentration by approximately 2.0x10^6 viable cells per bead. It is important to notice that when hydroxyectoine at 1 mM was added during the pre-

conditioning step, the fresh matter of leaves increased significantly by 17.45 % in comparison to the absolute control treatment ($F_{4, 39}$=3.15; p=0.0259, Figure 5.2 A, Figure 5.2 E). Noteworthy, either the dry matter of tuber or leaves increased significantly by 18.93 % ($F_{4, 39}$=9.66; p<0.0001) and 20.68 % ($F_{4, 39}$=7.74; p=0.0001) respectively, in comparison to the absolute control treatment (Figure 5.2 B). Concerning the dry beads control, the increments were 2.97 % and 5.48 % for weight gain in the tuber and leaves respectively. In line with the plant weight gain, the root length after eight days of planting was significantly longer in case of hydroxyectoine treatment at 3.99 ± 1.07 cm in comparison to the native control at 2.83 ± 0.65 cm ($F_{4, 39}$=9.67, p<0.001, Figure 5.2 C). The tuber diameter was also significantly increased in all cases when *K. radicincitans* DSM16656^T cells were presented in comparison to the native control and beads control ($F_{4, 39}$=7.64, p=0.0002). Tuber diameter varied from 24.10 ± 1.14 mm for the native control up to 26.18 ± 0.62 mm for the hydroxyectoine treatment (Figure 5.2 D, Figure 5.2 E).

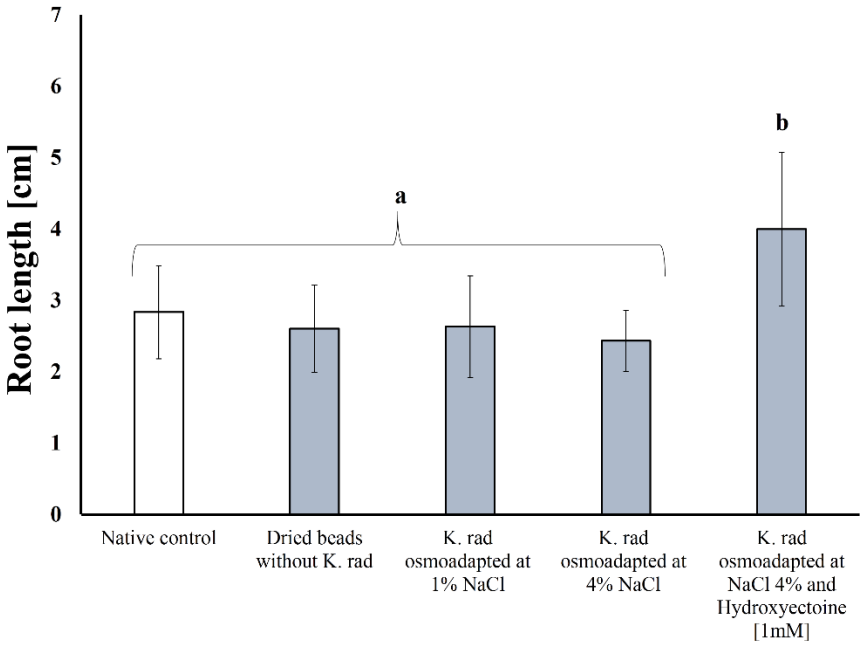
Figure 5-2: Growth promotion in glasshouse radish plants inoculated with amidated pectin dried beads containing pre-conditioning *K. radicincitans* DSM16656^T cells by osmoadaptation at 4% NaCl and by adding hydroxyectoine at 1 mM. **A)** Fresh mass of tubers and leaves. Fresh tuber mass ($F_{4, 39}$=1.47, p=0.2321); leaves fresh mass ($F_{4, 49}$=3.15, p=0.0259) **B)** Dried mass of tubers and leaves**.** Dry tuber mass ($F_{4, 39}$=9.66, p<0.0001) and dry leaves mass ($F_{4, 39}$=7.74, p=0.001). **C).** Root length of radish plants after 8 days of planting ($F_{4, 39}$=9.67, p<0.001). **D).** Tuber diameter after 5 weeks of planting ($F_{4, 39}$=7.64, p=0.0002). **E.** Glasshouse-grown radish plants inoculated with pre-conditioned *K. radicincitans* DSM16656^T cells encapsulated in amidated pectin dried beads. Pre-conditioned in control beads, DM at NaCl 4% and DM at NaCl 4% with the addition of hydroxyectoine [1 mM]. Different letters represent significant differences according to post hoc Dunnett's test. at p<0.05 (means ± SD, n= 8).


Native control
Dried beads without K. rad
K. rad osmoadapted at 1% NaCl
K. rad osmoadapted at 4% NaCl
K. rad osmoadapted at 4% NaCl and Hydroxyectoine [1 mM]
Fresh mass [g]
120
100
80
60
40
20
0
a
a
b
Tuber
Leaves

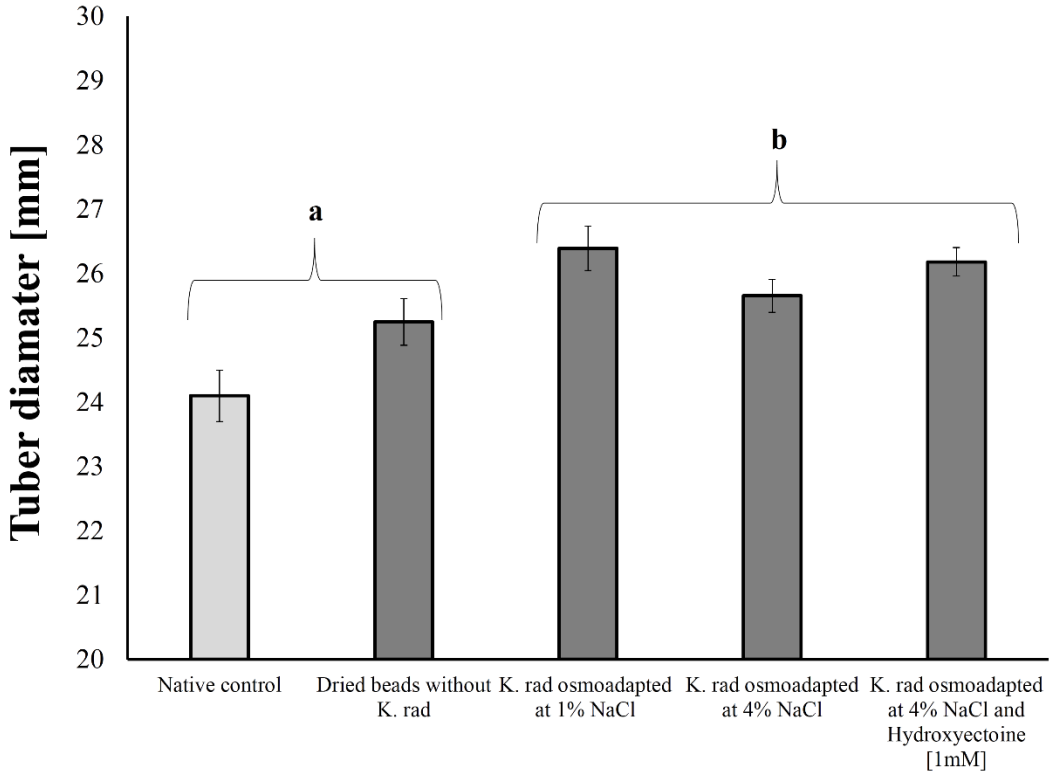
A


Native control
Dried beads without K. rad
K. rad osmoadapted at 1% NaCl
K. rad osmoadapted at 4% NaCl
K. rad osmoadapted at 4% NaCl and HIydroxyectoine [1 mM]
Dry mass [g]
5
4
3
2
1
0
a
b
a
b
Tuber
Leaves

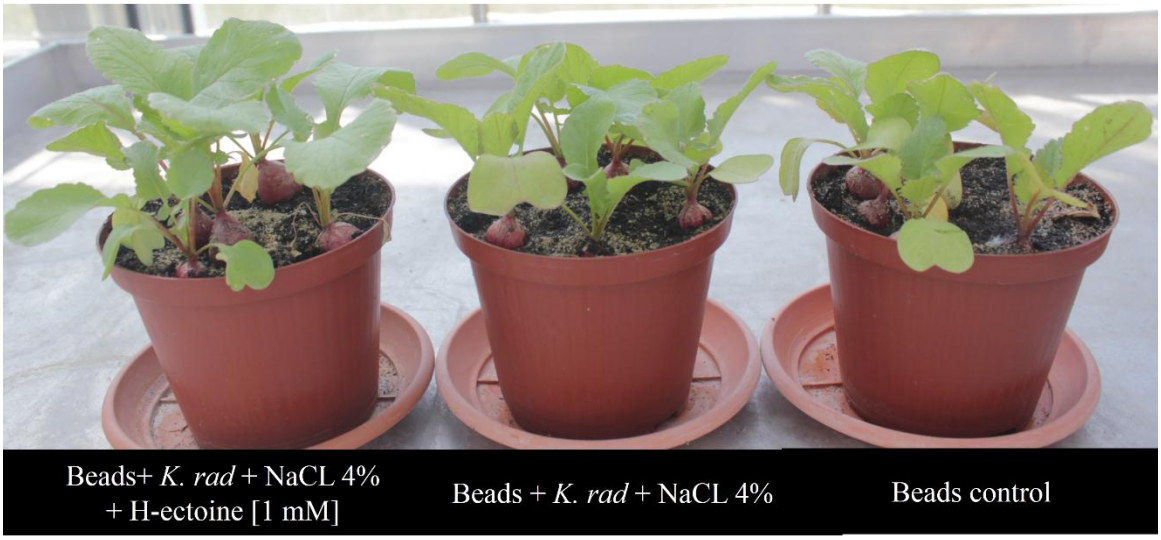
B

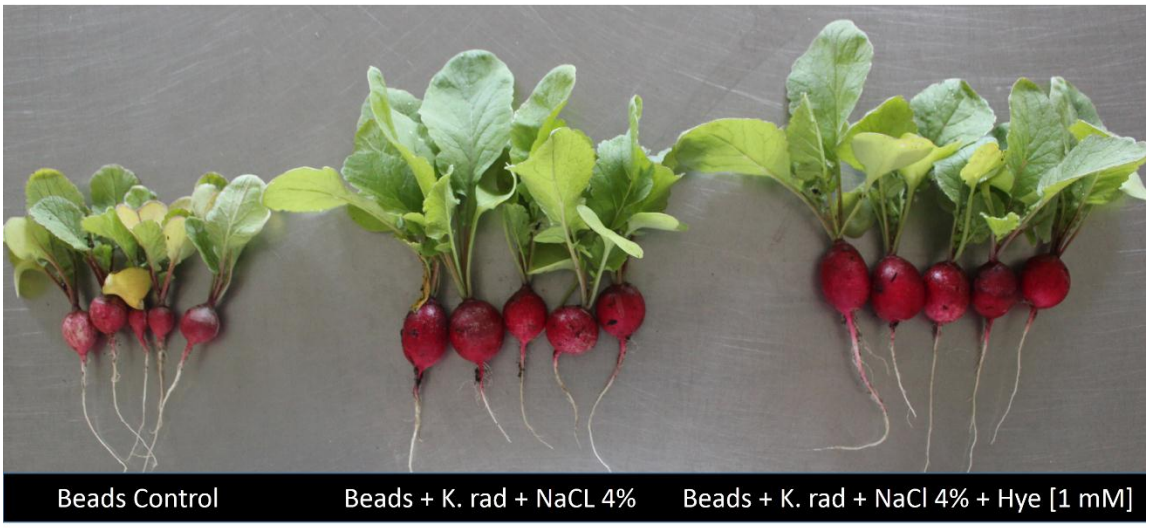

Root length [cm]
7
6
5
4
3
2
1
0
a
b
Native control
Dried beads without K. rad
K. rad osmoadapted at 1% NaCl
K. rad osmoadapted at 4% NaCl
K. rad osmoadapted at NaCl 4% and Hydroxyectoine [1mM]

C


Tuber diamater [mm]
30
29
28
27
26
25
24
23
22
21
20
a
b
Native control
Dried beads without K. rad
K. rad osmoadapted at 1% NaCl
K. rad osmoadapted at 4% NaCl
K. rad osmoadapted at 4% NaCl and Hydroxyectoine [1mM]

D





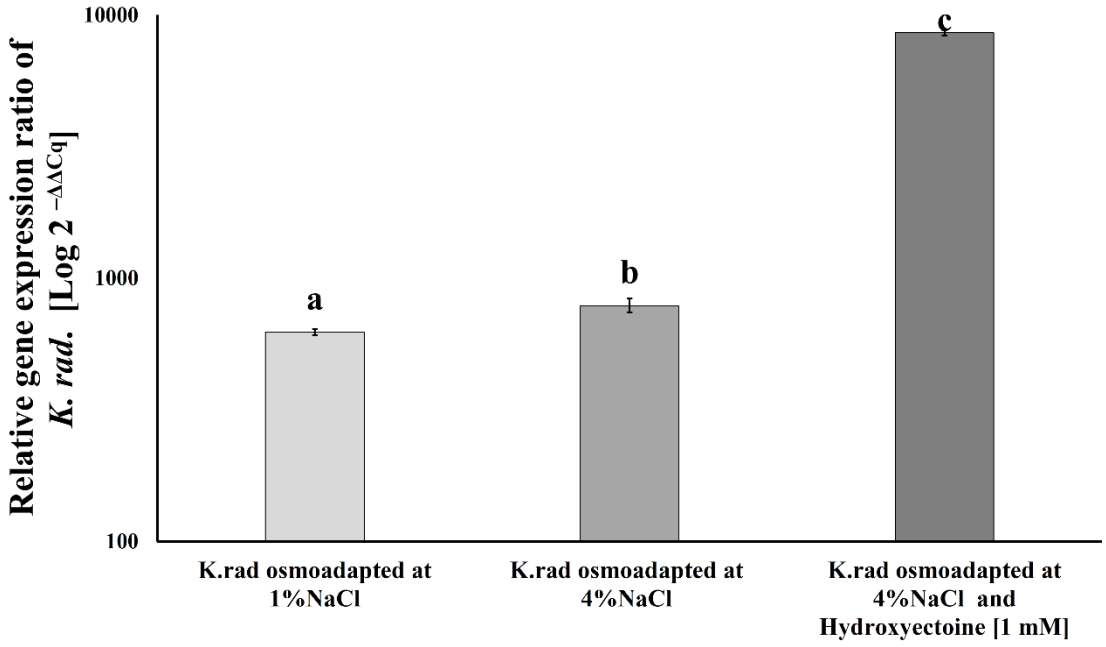
E

5.3.4 Effects of pre-conditioned cells in combination with the amidated pectin encapsulation on plant colonization

In general, *K. radicincitans* cells encapsulated in dried amidated pectin beads were able to colonize plant tissue. Thereby, bacterial cells could leave the beads and settle into the eight days seedlings (Figure 5.3). Thus, regarding relative gene expression response, two dry amidated pectin beads per seed containing pre-conditioned bacteria cells with 4% NaCl

significantly enhanced the plant tissue colonization, in comparison to non-pre-conditioned immobilized cells at 1% NaCl ($F_{2, 11}$=2460.71; p<0.05, Figure 5.3). Noteworthy, consistent with biomass production of radish plants, the endophytismus was stronger with the presence of intracellular hydroxyectoine in *K. radicincitans* DSM16656^T cells osmoadapted at 4% NaCl, which the roughly 10-fold increment was significantly under the evaluated conditions ($F_{3, 15}$=10477.33; p<0.0001, Figure 5.3).

Figure 5-3: Accumulation of *K. radicincitans* DSM16656^T DNA in inoculated radish plants with amidated pectin dried beads. Effect of pre-conditioning of *K. radicincitans* DSM16656^T in culture media by osmoadaptation at 1% NaCl, at 4% NaCl, and 4% NaCl + hydroxyectoine [1 mM], on relative gene expression in plant tissue. Different letters above bars indicate significant differences of treatments according to Tukey post hoc test at p<0.05, (means ± SD, n= 4).



5.3.5 Encapsulated bacteria and radish seedlings interaction: GFP-tagged bacteria approach

Multiple samples were checked under fluorescent microscopy and nearly all inoculated plants were colonized. *In vitro* assessments suggested that the immobilization in amidated pectin beads allowed the successful colonization of plant tissues by the endophyte through

firstly the bead adhesion to the roots and the chemotaxis events, and further establishments onto root surface, root hairs and secondary roots for slow release (Figure 5.4, Figure 5.6). In Figure 5.4, the mapping of chemotaxis of encapsulated *K. radicincitans* DSM16656^T cells was evident, since GFP fluorescence images demonstrated the activation of beads, the localization of high bacterial density and the cells movement towards radish seedlings roots. Therefore, without a chemical gradient, flagella rotates with no overall directional movement. Upon chemical gradient or attractant existence, the length of run is extended (Figure 5.5).

The early bacterial colonization stage on epidermis plant tissue was ubiquitous and evident in Figure 5.7 and Figure 5.10. In general, the bacterium utilizes cell junctures and intracellular spaces as microcompartments for colonization.

The formation of bacterial aggregates within the beads, either moist or dried (Figure 5.8 B-D) along plant interaction is quite impressive, since these aggregates of around 10 µm were also observed in both colonization stages, forming biofilms after seed sprouting in either roots hairs (Figure 5.8 A-B) and upon emerging secondary roots (Figure 5.9). At 48 h post-inoculation, bacteria released from the beads formed a biofilm on the root surface, near root hairs and onto cell maturation regions (Figure 5.6 B, Figure 5.8 C-D, Figure 5.9 D). Similar plant colonization patterns were observed in previous investigations, using poplar endophyte in maize seedlings (Kandel *et al*. 2017)

Figure 5-4: Mapping of the chemoattraction effect of *K. radicincitans* DSM16656^T GFP-tagged cells encapsulated in pectin beads. Left column represents blue reflectance images. Right column represents GFP fluorescence images **A)** Control beads without *K. radicincitans*. **B)** Beads with *K. radicincitans* DSM 16656^T pre-conditioning at NaCl 1% **C)** Beads with *K. radicincitans* pre-conditioning at NaCl 4%**. D)** Beads with *K. radicincitans* DSM16656^T pre-conditioning at NaCl 4% + hydroxyectoine [1mM]**.** Color bar indicates emitted light intensity for both image types.

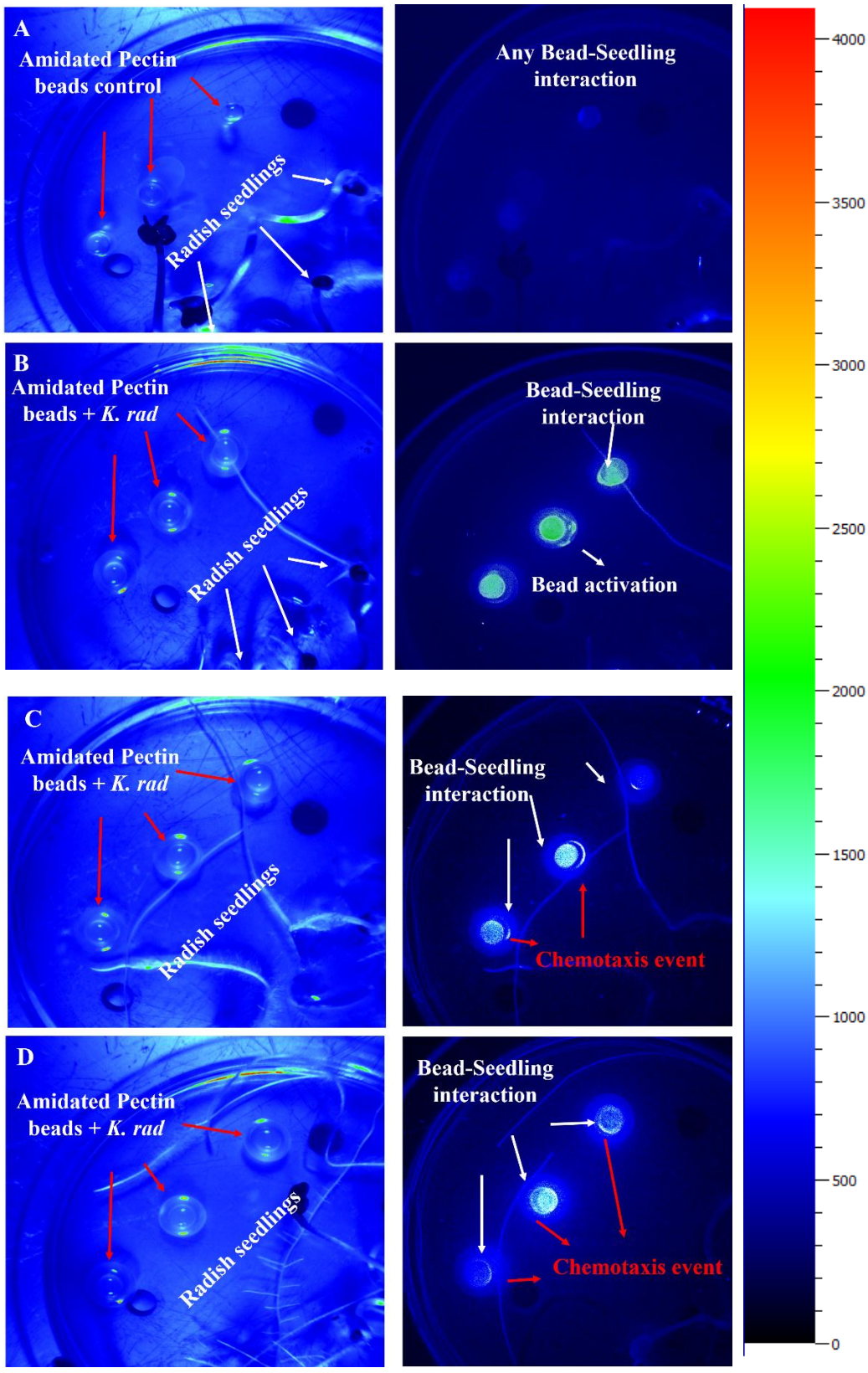

A
Amidated Pectin beads control
Radish seedlings
Any Bead-Seedling interaction
B
Amidated Pectin beads + K. rad
Radish seedlings
Bead-Seedling interaction
Bead activation
C
Amidated Pectin beads + K. rad
Radish seedlings
Bead-Seedling interaction
Chemotaxis event
D
Amidated Pectin beads + K. rad
Radish seedlings
Bead-Seedling interaction
Chemotaxis event
4000
3500
3000
2500
2000
1500
1000
500
0

Figure 5-5: Transmission electron microscopy (TEM) images of *K. radicincitans* DSM16656^{T}, showing their peritrichous flagellar apparatus and chemotactic behavior.

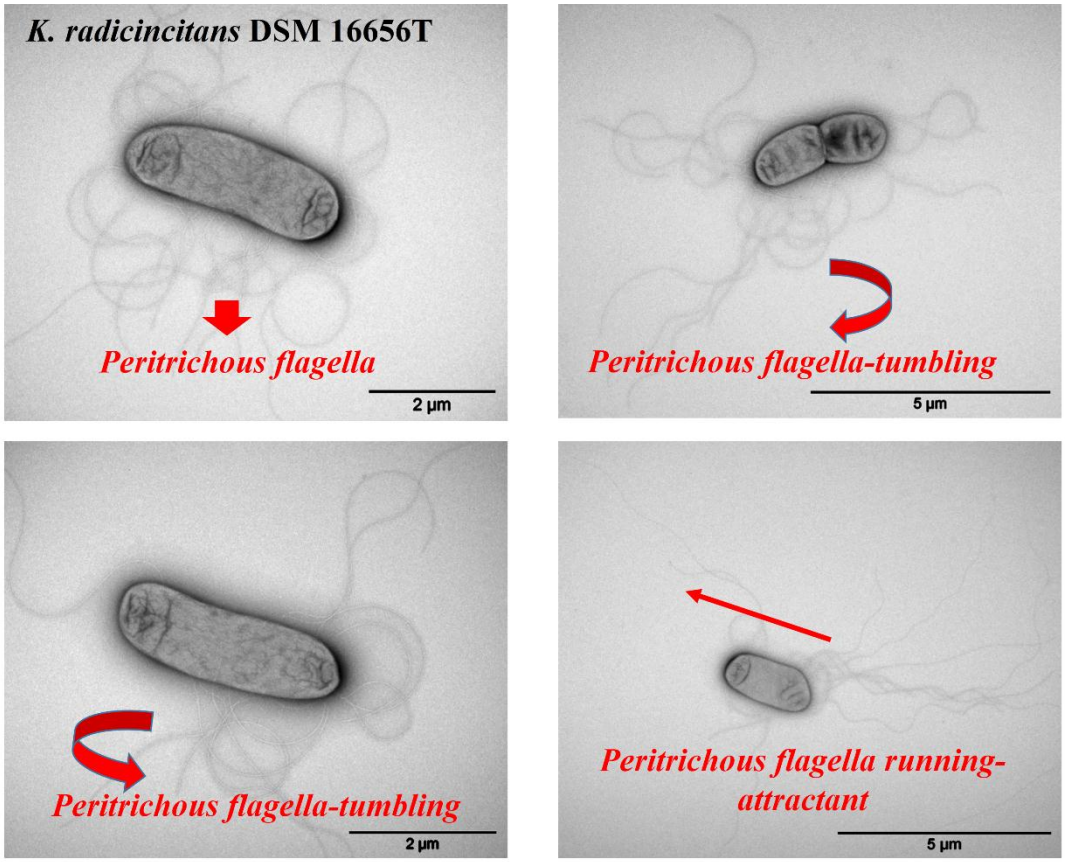
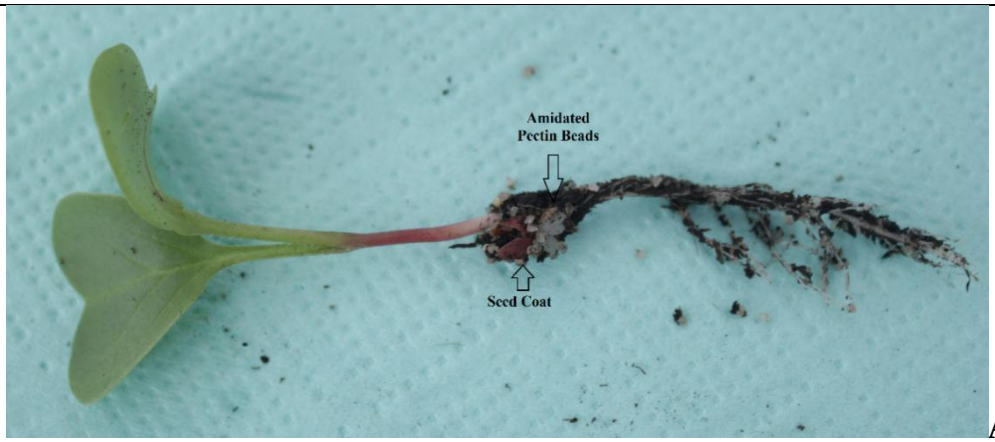
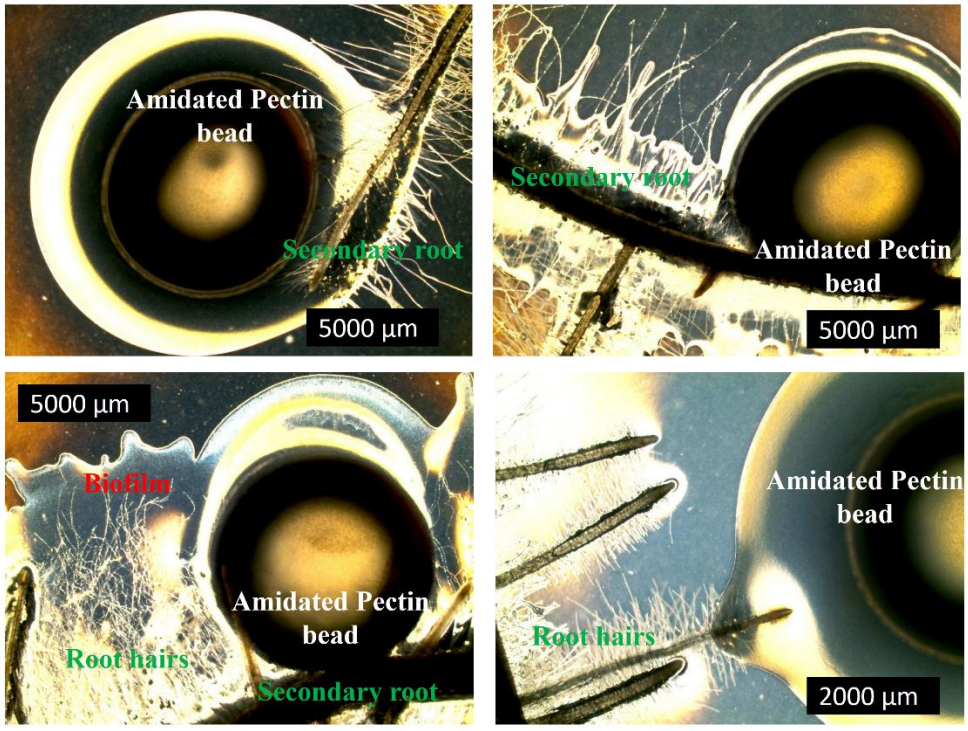


Figure 5-6: A. One-week-old radish seedling interacting with amidated pectin beads from the seed, sampling during the glasshouse assays. **B.** Interaction of encapsulated bacteria cells in amidated pectin dried beads with radish seedlings (gnotobiotic system), phase-contrast microscopy approach (EVOS® XL core, Life Technologies). *K. radicincitans* DSM16656^{T} colonizing root hairs and secondary root surface 4 dpi. Scale bar: 5000 µm.



A



B

Figure 5-7: Radish seedlings (gnotobiotic system) colonization by encapsulated bacteria cells in amidated pectin dried beads. Inverted imaging system for four-color fluorescence and transmitted-light applications approach (EVOS® FL, Life Technologies). GFP-tagged - *K. radicincitans* DSM16656^{T} cells colonizing lateral root surfaces (**A, B, D)** and secondary roots (**C**) 4 dpi. Scale bar: 1000 µm.

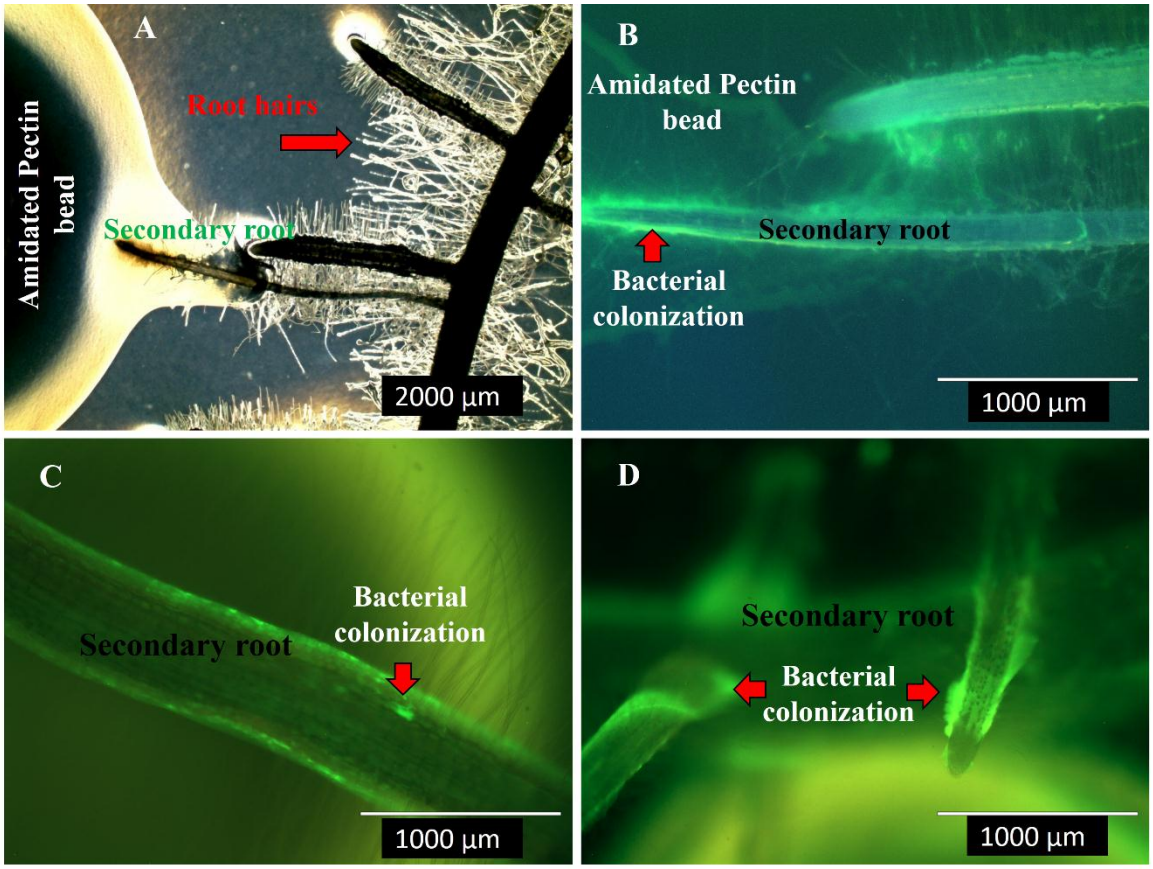


Figure 5-8: Amidated pectin-encapsulated GFP-tagged *K. radicincitans* DSM16656^{T} cells after 24 h of growth. Beads were cut in half [Sliced 20 µm, CM 1800 microtome (Leica Instruments, Nussloch, Germany)], and images were acquired with an inverted microscope (EVOS® FL, Life Technologies). Scale bar: 1000 µm.

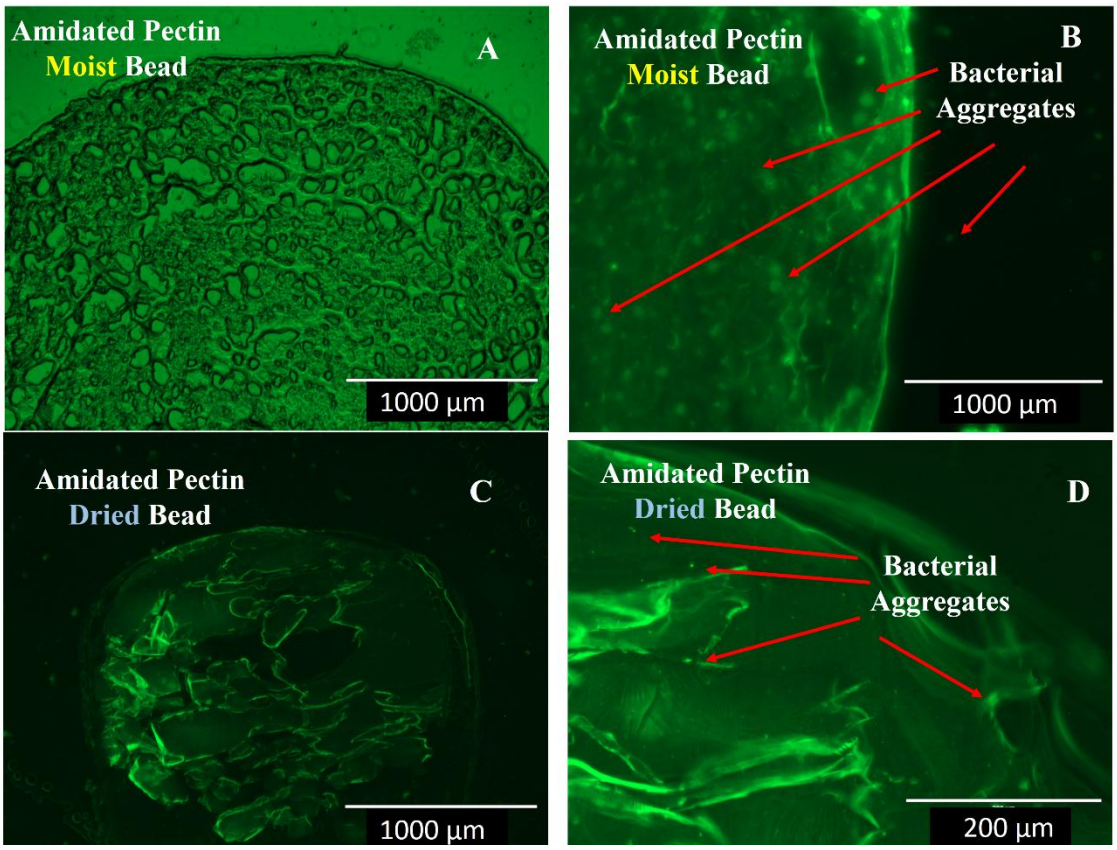
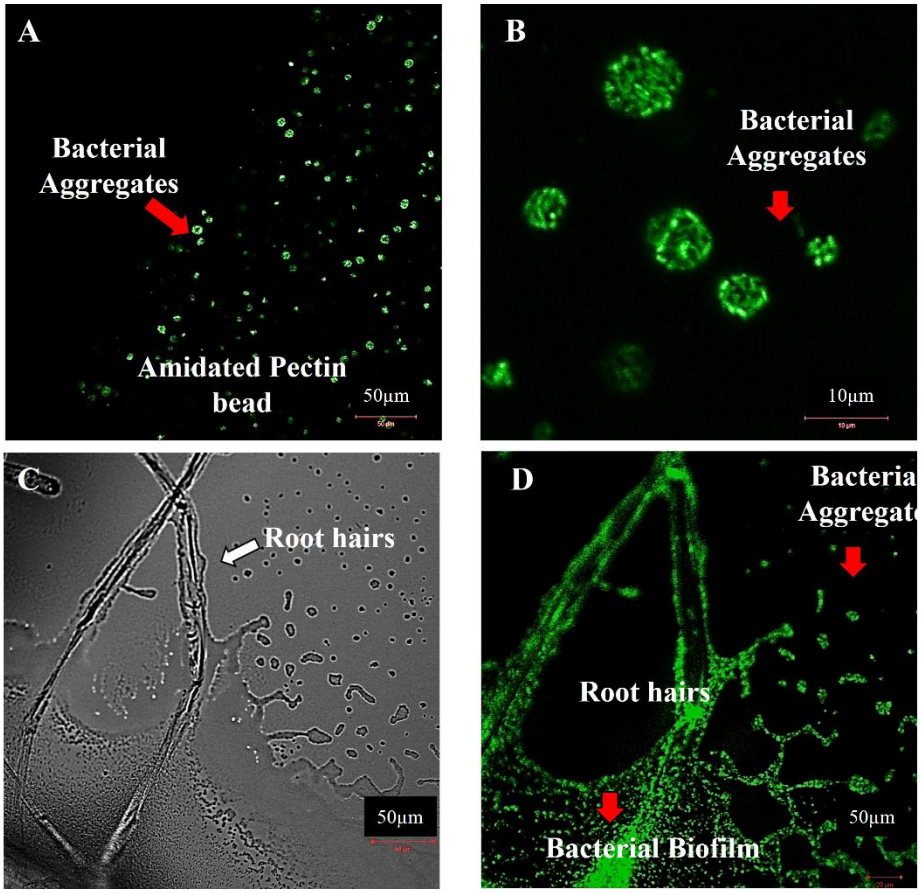


Figure 5-9: Confocal laser scanning micrographs showing the inner colonization of radish seedlings by encapsulated *K. radicincitans* cells expressing eGFP. **A.** Bacterial aggregates formation inside and at the edge of amidated pectin bead [Sliced 20 µm, CM 1800 microtome (Leica Instruments, Nussloch, Germany)]. **B.** Amidated pectin-encapsulated GFP-tagged *K. radicincitans* DSM16656^{T} colonizing root hairs by forming aggregates 4 dpi.



5.4 Discussion

Exploitation and manipulation of beneficial bacterial endophytes can be a sustainable alternative to cope with the demanding eco-friendly and productive agriculture. The formulation of bacterial endophytes candidates can enhance the bio-prospection of this emerging low-input strategy. The potential of formulation approaches of endophytic bacteria as wettable powder (Cheng *et al*. 2015), pellets (Hu *et al*. 2011), gel-based inoculants (da Silva *et al*. 2012) and foliar sprays (Bejarano *et al*. 2017) for applying as plant growth stimulators or biocontrol agents has been demonstrated. However, a few numbers of studies have dealt with the entrapment or encapsulation of endophytic bacteria as proper application technology (Oteino *et al*. 2015; Lally *et al*. 2017), using mainly the coating of seed by a bacteria-calcium alginate mix. Herein, this research section extends the knowledge for applying pre-conditioned cultivation and further cells encapsulation by ionic gelation as an alternative to formulate these endophytes.

Though the evident capability of the endophyte *K. radicincitans* DSM16656^T to promote radish growth, results suggest that the integration of osmoadaptation of bacterial cells during the cultivation and the uptake of advantageous compatible solutes such hydroxyectoine are beneficial to the endophytic activity. Noteworthy, both environment variations during growth may lead to greater phenotypic plasticity (Schulz and Boyle, 2005). Thereby, previous studies discussed the beneficial effects on bacteria endophytes caused by salt stress and the uptake of hydroxyectoine, including metabolic reordering and enhancements of phosphatases activity (Cruz *et al*. 2019).

The amidated pectin-entrapment is a promising alternative to support plant colonization by bacterial endophytes. Hence, encapsulated *K. radicincitans* DSM16656^T cells in dried amidated pectin beads maintain the capability to colonize radish plants. Nevertheless, the negative influence of beads number on endophytic capabilities could be due to the higher stress than the beads would exert on seed, which would limit its space to germinate. Indeed, the relative high bead hygroscopicity (see Chapter 4, Figure 4.6) may cause a dehydration phenomenon, by sequestrating the water located immediately in the vicinity of the seed. These findings are valuable knowledge for the successfully endophytic-bead formula application.

Regarding plant growth-promoting performance, entrapped cells provided outstanding positive radish-yields. These findings are comparable to Berger *et al* (2015), who reported weight increments from 20 to roughly 50 % in tuber and leaves, applying either seed-inoculated or two-leaf sprayed plants with fresh cultivated cells. Here, it is important to point out that these results were achieved by using dried beads, since it is clear that encapsulation of cells and drying intrinsically depressed motility, which is highly implied with the plant interaction and colonization. Besides, dried beads may require a longer time to establish to swell and to uptake water for the surroundings, providing porous within the gel-matrix facilitating the increment of internal a_w for *K. radicincitans* DSM16656^T cells multiplication (Young *et al*. 2006).

Osmoadaptation as sub-lethal pre-conditioning procedure as well as the addition of osmolytes could modify the identity and functioning of metabolites that bacteria cells produce, supporting the competitiveness and the establishment in the rhizosphere before colonizing the plant endosphere (see Chapter 6). Hence, osmotic unbalanced can modify mechanisms of quorum sensing (QS), quenching (QQ) or extracellular polymeric substances (EPS) during the formation of biofilm and further signaling steps with the plant, during the microbiome and endophytic establishment (Dulla and Lindow, 2008; Vu *et al*. 2009; Bacon and White, 2016). Thus, small groups of cells aggregates and biofilm formation, mediated through QS provide advantages to capitalize on favorable environments or withstand stressful conditions (Dulla and Lindow, 2008).

The successful establishment of the embedded endophytic bacterium could also be due to the indirect mechanism provided by the additives into the bead. Since pectin is one of the major components in plant cells walls and it is suggested that it might serve as an environmental factor in the stimulation of bacterial biofilm formation during plant colonization, mimicking natural conditions (Deboy *et al*. 2008; Wu *et al*. 2015). In Chapter 4, it was demonstrated that the amidated pectins with a high content of galacturonic acid could provide an advantageous nutrient source for endophytic bacteria, supporting the early establishment in the soil. Indeed, *K. radicincitans* DSM16656^T can encode for pectinases secretion and utilizes D-galactose precursor of D-galacturonic acid as sole carbon source (Kampfer *et al.* 2005a; Becker *et al*. 2018). Moreover, other studies in spruce provide evidence of D-

galacturonic acid and D-sorbitol utilization as a trait for contributing to the endophytic lifestyle and proliferation in highly reducing microsites in plants thereafter (Shishido *et al*. 1999; Yang *et al*. 2017). Besides, amidated pectin beads have also successfully used as a carrier for delivering fungi endophyte in potato plants (Krell, Jakobs-Schoenwandt, Vidal *et al.* 2018).

Pectin as biopolymer for entrapping bacteria could trigger the enzymatic arsenal of the bacterium, since the early barrier encountered of this polysaccharide may mimic natural conditions and activate the plant cell wall-degrading apparatus, including the secretion of endopolygalacturonase and pectin esterases to degrade the backbone of α-D-galacturonic acid, which are considered as physiological traits in endophytes (Walitang *et al*. 2017). Likewise, other critical enzymes that *K. radicincitans* DSM16656^T could encode for facilitating plant colonization are glycoside hydrolases (>127 proteins), β- glucosidases, mannosidases, galactosidases and glucanases (Deboy *et al*. 2008; Yang *et al*. 2017; Becker *et al*. 2018).

Althoght there were not significant differences in radish yields by applying control beads, considering the other components within the bead, the monosodium glutamate can serve as a nutrient source for plant bio-stimulation, enhancing soil microbial activity and soil respiration (Singh *et al.* 2009). For bacterial cells, glutamate may act as an alternative electron acceptor in beads, facilitating the growth in oxygen-depleted zones and further the whole activation into pectin networks. On the other hand, the cross-linker calcium gluconate could convert to gluconic acid under low pH, which is one of the most essential acids involved in the phosphorus solubilization by PGPBs (Lin *et al.* 2006; de Werra *et al*. 2009), including *Pseudomonas* endophytes (Oteino *et al*. 2015). Taking into account that bead components may modify the nutrient niche of root surfaces and subsequent soil microbiome, they could influence the switch of *K. radicincitans* DSM16656^T from bead- rhizosphere to endophytic lifestyles, altering radish carbon metabolism including secondary metabolites such glucosinolates and inducing priming of defense responses (Brock *et al*. 2013; Brader *et al*. 2014; Saminathan *et al.* 2018).

In line with biomass production of radish plants, the plant colonization by entrapped cells increased with the presence of intracellular hydroxyectoine in *K. radicincitans* DSM16656^T

cultivation, in which the roughly 10-fold increment was significantly under the evaluated conditions. Similar results are described with free-living cells in Chapter 3. This finding indicates that plant colonization improved by synergistic effects of pre-conditioned cells by osmoadaptation and formulation performance. The amassing of hydroxyectoine during cultivation may support the formation and persistence of the endophyte in plant tissue, since this osmolyte may confer protection from osmotic stress during biofilms formation (Mosier *et al.* 2013). Noteworthy, as elucidated in this research (Chapter 3), the hydroxyectoine incorporation provides to bacterial cells advantages such as the up-regulation of acid phosphatases and an acidic proteome.

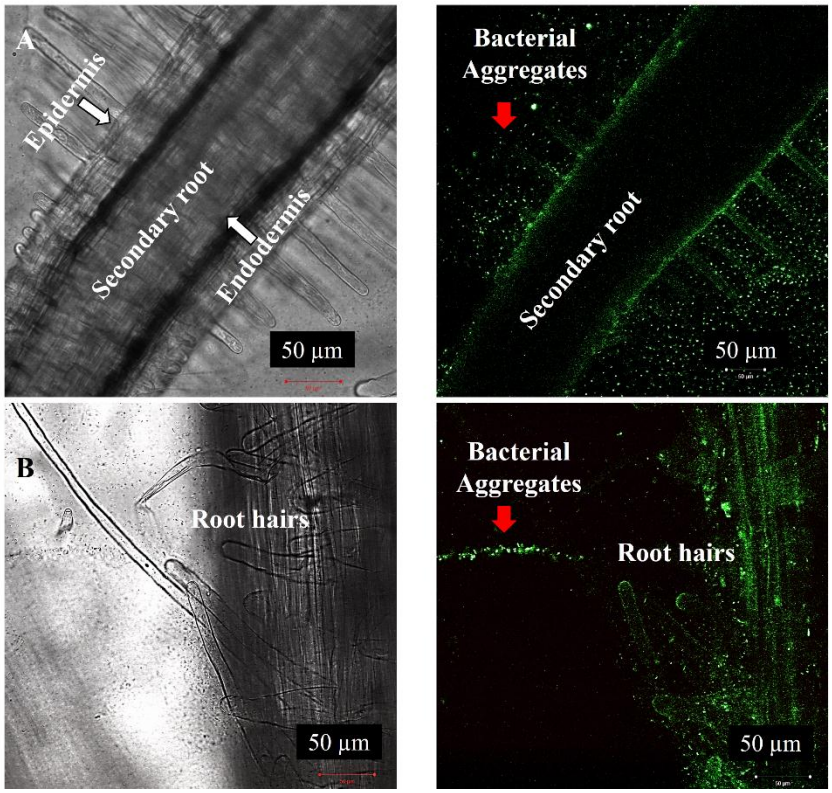
Imaging microscopy and multispectral fluorescence imaging show the colonization patterns of encapsulated GFP-tagged *K. radicincitans* DSM16656^T strain in radish seedlings. Thus, bacterial cells entrapped in pectin beads can colonize early plant tissue and show endophytic life-style by two main ubiquitous pathways. First through root hairs during the first stage of root development: in contact to beads, root hairs establish mainly nearby the frontiers of growth out the bead and eventually into the bead by multiple adhesions entrance events (Figure 5.7 B). In these zones, *K. radicincitans* DSM16656^T cells are predominant planktonic cells and forming aggregates thereafter. The epidermis-root cracks events potentiate colonizing sites for bacterial proliferation (Figure 5.10 C).

Secondly, entrapped *K. radicincitans* DSM16656^T cells are capable of colonizing the secondary roots, either the region of cell maturation (the basis) or the root cap (the tip) reaching the cortical tissue (Figure 5.10). This mechanism occurs during the second stage of root development. Interestingly, secondary roots penetrate the bead matrix and they can establish into it. These advantageous sites for colonization are facilitated by the junction where lateral root emerges through the endodermis, the cortex and the epidermis (Figure 5.10 D) (Witzel *et al*. 2017). Surprisingly, *K. radicincitans* DSM16656^T aggregates-like colonize endosphere compartments as xylem, indicating a wider cells-plasticity for interacting within the variable plant tissues micro-environments (Figure 5.11 A-B).

From the plant side, at one-week-old seedlings stage, the metabolism dominance of radish included amino acids, the terpenoids, nucleotides and the biosynthesis of secondary metabolites. These molecules and the glucosinolates (GLS) accumulation may drive the gate

opening for *K. radicincitans* DSM16656^T cells entrance and persistence (Mitsui *et al*. 2015). Unlike, carbohydrate metabolism is not enriched before 14 days during root thickening. In addition, genes related to stress and stimulus responses, are most enriched at the early seedling stage (Mitsui *et al*. 2015).

Figure 5-10: Confocal laser scanning micrographs showing the inner colonization of radish seedlings by encapsulated *K. radicincitans* cells expressing eGFP. **A.** *K. radicincitans* DSM16656^T colonizing secondary root epidermis 4 dpi. **B.** *K. radicincitans* DSM16656^T colonizing root hairs 4 dpi. **C**. The endophytismus is facilitating by root cracks in secondary roots. **D.** *K. radicincitans* DSM16656^T colonizing junction generated by the lateral root emergence. Scale bar: 20 µm.



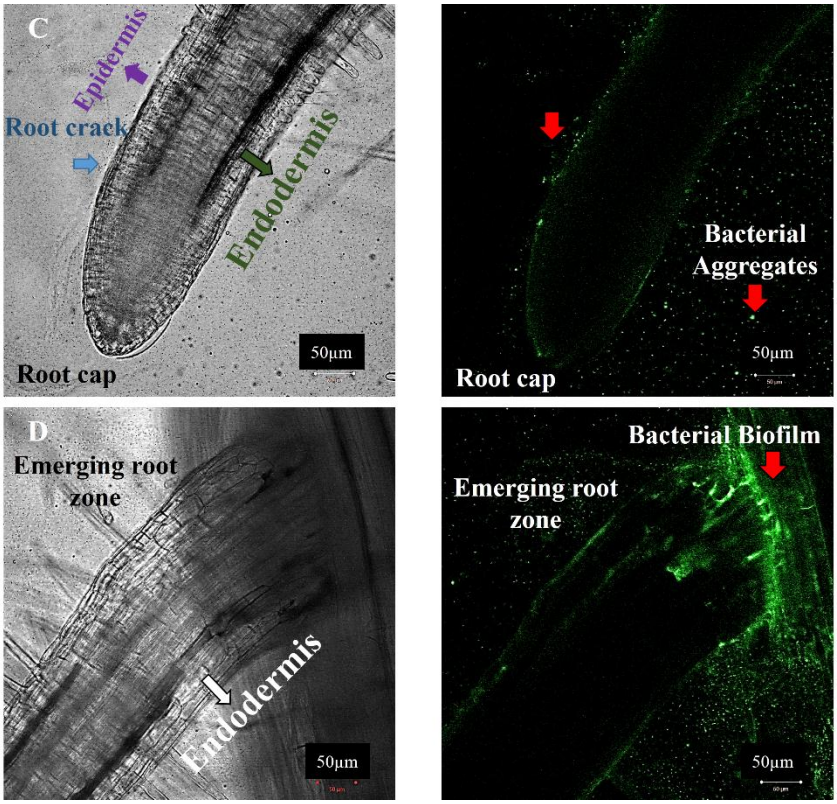
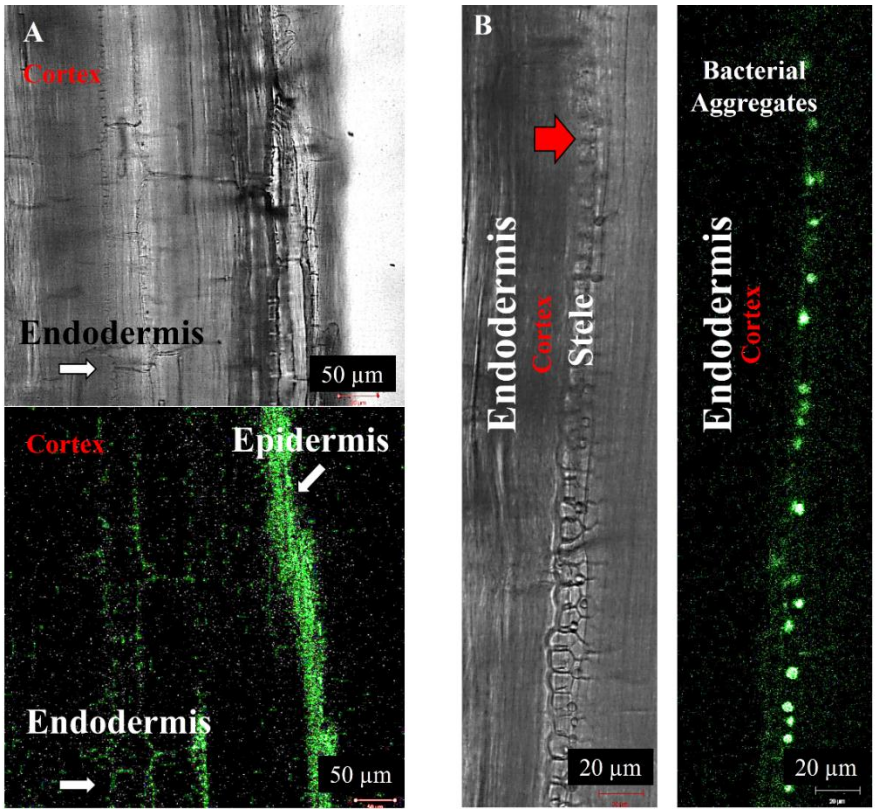


Figure 5.11A illustrates the second stage on bacterial colonization, which involves the migration and proliferation from the high populated epidermis into the endodermis and further reaching the xylem. The bacterial aggregates observation into xylem and stele tissues are surprising, since this is the first report that revealed this aspect from previously entrapped cells (Figure 5.11B).

Figure 5-11: Confocal laser scanning micrographs showing the inner compartments colonization of radish seedlings by encapsulated *K. radicincitans* DSM16656^T cells expressing eGFP. **A.** Bacterial aggregates forming biofilm to colonizing epidermis and moving towards endodermis tissue. **B.** *K. radicincitans* DSM16656^T colonizing stele-xylem tissue within the plant endosphere, and the presence of bacterial aggregates. 4 dpi **C**. Scale bar: 20 µm.



Here, it is observed that *K. radicincitans* DSM16656^T aggregates embedded into the bead proliferated and migrated through the bead material, colonizing the secondary root. Surprisingly, since groups of cells were detected at depths > 100 µm, bacterial aggregates localization were not restricted to the bead borders, where the oxygen concentration would be higher (Sonderholm *et al.* 2017). This suggests that the facultative capability of this endophyte allowed the anoxic growth and the entire exploitation of bead structure, favoring the proliferation through the redox gradient between anoxic sites and the microaerobic arenchymatic tissue environment (Sessitsch *et al*. 2012). Altogether, pectin hydrogels can be considered as an *in-vivo*-like biofilm system, diffusion-limited, wherein bacterial endophytes

growth exhibits central features of *in-vivo* biofilms showed during plant colonization (Figure 5.9A-B).

Since the fluorescence images showed higher GFP intensities in the beads frontiers pointed to the radish roots, it seems to be that the driven force of entrapped *K. radicincitans* DSM16656^T cells to swim towards seedling tissue is the root exudates that diffuse to the neighborhood of the bead. Thus, the induction of chemotactic response of bacterial endophytes to root exudates has been reported (Bacilio-Jimenez *et al.* 2003) and is presented as the first step for colonizing the rhizoplane region (Ray *et al.* 2018). Although all beads containing bacteria endophyte showed chemotactic activity, the images of pre-osmoadapted cells with hydroxyectoine suggested that these cells might have advantages for sensing the roots exudates.

Questions remain regarding this hypothesis, to delve into the capability of osmotic stress to modify flagellar apparatus, metabolite profile and quorum sensing in bacteria endophytes (Sessitsch *et al.* 2012; van Kessel *et al*. 2015). Noticeably, with this study, the use of multispectral-kinetic fluorescence imaging emerges as an applicable methodology for targeted analysis of GFP-tagged bacterial endophytes and plant host interaction.

5.5 Conclusions

Analysis of the data provides evidence that physiological modifications by osmotic stress, the accumulation of compatible solutes during cultivation and the entrapment of these pre-conditioned cells in pectin-base hydrogel networks enclosed a feasible strategy to improve bacterial endophyte-host interactions. For the first time, a successful endophytic activity of *K. radicincitans* DSM16656^T cells encapsulated in amidated dry pectin beads was demonstrated. Besides, there is a gain of knowledge considering the elucidation of entrapped bacterial cells capability to proliferate as aggregates and migrate through the biopolymer matrix, colonizing plant tissues and promoting radish growth under glasshouse conditions. The phenotypic plasticity of *K. radicincitans* DSM16656^T triggered by osmoadaptation and exogenously provided hydroxyectoine during cultivation persists in entrapment cells for

increasing plant bio-stimulation and endophytic performance. These findings give advances in plant growth-promoting bacterial endophytes inoculant technology.

6. CHAPTER 6. METABOLIC PROFILING RESPONSE OF *K. radicincitans* DSM16656^{T}: TO OSMOTIC STRESS AND HYDROXYECTOINE, ASSOCIATED WITH DRYING SURVIVAL AND PLANT COLONIZATION

6.1 Introduction

System biology science embraces four key technologies such as genomics, transcriptomics, proteomics, and metabolomics. All the approaches are in a state of expeditious expansion in Gram-negative bacteria endophytes (Kaul *et al.* 2016). The metabolite profiling and multivariate data mining is a robust way to categorize samples and to reveal critical metabolites responsible for specific processes. Here, this technology was applied to verify the hypothesis that *K. radicincitans* DSM16656^T cells with and without osmoadaptation and hydroxyectoine addition have different metabolic signatures, and those metabolic alterations are involved directly and indirectly on cell drying survival and endophytic colonization capability as was shown in chapters 3, 4 and 5.

Though *K. radicincitans* DSM16656^T can fix atmospheric nitrogen (Ruppel and Merbach, 1995), solubilize rock phosphate (Schilling *et al.* 1998) and even product quality (Berger *et al.* 2017), the potential use of this endophyte as a commercial bio-stimulator is under significant challenges. Hence, this Gram-negative bacterium presents a low resistance for drying, and it is still unknown how the organism adapts to high osmotic environments. Noteworthy, metabolomics regarding the response of endophytes to osmotic stress and compatible solutes is an unknown topic.

So far, few studies have dealt with anhydrobiotic engineering in PGPBE to confer tolerance for drying (Berninger *et al*. 2018). Thereby, there is currently no study on the influence of exogenously compatible solutes during the osmoadaptation in bacterial endophytes and ensuing drying survival. Besides, few studies on metabolic profiling responses by exogenous addition of compatibles solutes in bacteria have been carried out, including the addition of L-proline on *Tetragenococcus halophilus* for revealing differences in TCA cycle and amino acids profile (He *et al.* 2017), and the insights into metabolic osmoadaptation of the ectoine producer *Chromohalobacter salexigens* (Piubeli *et al*. 2018). However, there are no studies regarding Gram-negative bacterial endophytes and metabolic profiling upon salt stress and exogenously supplied hydroxyectoine. Furthermore, the protective mechanism of osmolytes such as hydroxyectoine upon salt stress is partly understood. Therefore, to elucidate the

desiccation protective effects and the re-routing of metabolic flux upon hydroxyectoine addition and salt stress, the metabolic responses of hydroxyectoine-added *K. radicincitans* DSM16656^T cells were analyzed, using a high-throughput analytical gas chromatography-mass spectrometry (GC-MS) approach.

Plant colonization by endophytic bacteria is a complex process that involved five distinct stages: (1) recognizing root exudates and motility towards the plant, (2) attachment to the roots surface, (3) biofilm formation, (4) root surface penetration and (5) plant endosphere colonization (Kandel *et al.* 2017). Each multidimensional interaction is mediated by specific biomolecules. In order to determine how these biomolecules are acting, it is crucial the '*omics*' integration approaches (Kaul *et al.* 2016), since the majority of the research treated with the physiological alteration responses in plants (Sessitsch *et al*. 2012; Brader *et al*. 2014; Becker *et al*. 2018). There is a knowledge gap within revealing the metabolic alterations in osmoadapted-endophytic cells that support plant establishment. Besides, some studies on endophytic plant biostimulant bacteria revealed contradictory outcomes suggesting strain dependence background.

Physiological machinery involved in the multidimensional mechanism for colonizing plants include, the amassing of favorable carbon and nitrogen sources, the chemotaxis proteins (MCPs), the amino acids associated with the membrane-bound histidine-kinase, cell-wall enzymes, and the regulation of specialized proteins for biofilm formation, among others (Borland *et al.* 2016; Pinski *et al.* 2019).

In this research, previous results demonstrated that osmoadaptation and the hydroxyectoine uptake provide to *K. radicincitans* DSM16656^T cells a higher drying tolerance and stimulate the endophytic colonization capability in radish plants. Altogether, is tentative to hypothesize that pre-conditioning of bacteria cells by growing them at high salinities and providing exogenously hydroxyectoine during the adaptation in culture media protects bacterial cells could shift metabolic profiling, increasing the drying survival and supporting plant colonization performance. Thus, by metabolic profiling and data mining analysis, this study section aimed at determining the influence of cells pre-conditioning procedures in *K.*

radicincitans DSM16656^T on its metabolic signatures associated with drying survival and physiological endophytic machinery.

6.2 Materials and Methods

6.2.1 Biomass samples preparation for metabolic profiling

The relative levels of metabolites in *K. radicincitans* DSM16656^T cells during the exponential phase were assessed in an untargeted approach. Intracellular metabolites extraction and gas chromatography-mass spectrometry (GC-MS) analyses were conducted as follows: a culture volume of 2 mL of bacteria at exponential phase at OD_{600} ~0.6 were harvested by fast centrifugation for 1 min at 15000 rpm (Mikro HT 200R, Hettich GmbH & Co. KG, Tuttlingen, Germany) and the pellet was rapidly quenched in liquid nitrogen until further processing. After quenching, samples were freeze-dried (Christ GmbH, Osterode am Harz, Germany) overnight. Metabolites extraction was conducted according to the procedure described by Plassmeier *et al.* 2007. In particular, ~ 5 mg of dried biomass was added to 0.5 g zirconia/silica beads (0.5 mm diameter, BioSpec Inc., OK, USA). Further, 1 ml 80% MeOH was added with ribitol (10 µmol L^{-1}) as an internal standard. Disruption of biomass was performed for 3 × 60 s at 6200 min^{-1} in a homogenizer (Precellys 24, Bertin instruments, Montigny-le-Bretonneux, France). The obtained lysate was centrifuged at 19000x*g* for 5 min (Centrifuge 5424, Eppendorf AG, Wesseling- Berzdorf, Germany) and 650 µL of the supernatant was transferred to 1 mL micro reaction vessels (Supelco Inc., CA, USA). In parallel, one vessel containing 1 mL 80% MeOH/10 µM Ribitol was used as a blank (see Figure 2.2).

Evaporation of the solvent in samples was ensured at 37°C and nitrogen gas contact for 80 min (Reacti Therm heating and stirring module, Thermo Fisher Scientific Inc., MA, USA). The derivatization of dried extracts was carried out with the addition of 75 µL of methoxyamine (20 mg mL^{-1} in pyridine) for 90 min at 37 °C [Sigma- Aldrich GmbH (VWR International GmbH, Darmstadt, Germany)]. Later, 75 µL *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) (Macherey Nagel GmbH & Co. KG, Dueren, Germany) was added for a second derivatization step and the reaction was stirred for another 30 min. Finally,

the derivatization reaction into the vessels was centrifuged at room temperature at 4000 rpm for 5 min, (Centrifuge 5810R, Eppendorf AG) and the supernatant fractions were transferred to HPLC vials with 100 µL inlays (VWR International GmbH) before loading into the GC-MS autosampler.

6.2.2 GC-MS assessments

GC–MS analysis was conducted on a TraceGC gas chromatograph connected with a PolarisQ ion trap mass spectrometer and an AS2000 autosampler (Thermo Finnigan GmbH, Dreieich, Germany) (Plassmeier *et al.* 2007; Krell, Jakobs-Schoenwandt, Persicke *et al*. 2018). Briefly, supernatant fractions were injected at 1 µL volume and 250°C through a 30 m × 0.25 mm Equity-5 column with a 0.25 µm 5% diphenyl/95% dimethylsiloxane coating (Supelco Inc.). The temperature was maintained at 80°C for 3 min. Further, the temperature profile was settled at 5°C min^{-1} to 325 °C, acquiring the mass spectra at 4 scans s^{-1} with a range of 50–550 m/z. Previous to the next injection procedure, the temperature was adjusted to 80°C and maintained for 5 min. The integration of peaks in chromatograms was performed using Xcalibur 2.0 (Thermo Finnigan GmbH). Samples were inspected for the existence of a ribitol peak (m/z: 217). Metabolite relative contents are expressed in arbitrary units (semi-quantitative determination). Moreover, metabolite verification was performed via commercially available standards and the NIST 98 database (NIST, MD, USA). Peak integration was conducted automatically and normalized to dry biomass weight and ribitol area.

6.2.3 Metabolome data processing

To obtain deeper insights into the physiological adaptations of *K. radicincitans* DSM16656^T to sustained hyperosmotic salt stress, the intracellular metabolite levels were measured. Metabolic data processing and statistical analysis were performed by following the workflow of MetaboAnalyst 4.0, a web-based comprehensive tool suite for metabolic data analysis (http://www.metaboanalyst.ca). The data were found to follow normal distribution after data processing and normalization (internal standard ribitol). These data were used for two exploratory multivariate analysis techniques, unsupervised Principal Component Analysis

(PCA) and supervised Partial Least Square-Discriminant Analysis (PLS-DA) for selecting essential features. Intrinsic variation of data and outliers detection was performed using the 2D score plot of the PCA, and the class discrimination was analyzed using score plot of PLS-DA. Performance of PLS-DA model was measured using two parameters, R2 and Q2 and cross-validated by permutation test (Xia *et al.* 2009; Xia *et al.* 2012; Xia *et al.* 2015). R2 is defined as the proportion of variance in the data explained by the model. It represents the total explained variation in the data and indicates goodness of fit. Q2 is defined as the proportion of variance in the data predictable by the model. It describes the extent of separation between the classes and indicates the predictability of the model. In the case of an ideal model, R2 and Q2 should reach 1 (Joghee and Jayaraman, 2014). Significantly contributing metabolites were identified using the variable importance in projection (VIP), which was calculated on the weighted sum of the squares for the partial least squares (PLS-DA) loadings (Farres *et al*. 2015).

For metabolic pathway analysis, *Kosakonia radicincitans* GXGL-4A (krd 283686-Kyoto Encyclopedia of Genes and Genomes- KEGG) was selected as a model. *Escherichia coli K-12 MG1655* was selected for pathway enrichment and pathway topological analysis in MetaboAnalyst 4.0 platform. The p-value (from pathway enrichment analysis) indicates the statistical significance of association of the altered metabolites with the pathway and pathway impact value (from pathway topological analysis) is calculated as the sum of the importance measures of the matched metabolites normalized by the sum of the importance measures of all metabolites in the pathway (Joghee and Jayaraman, 2014).

Statistical analysis

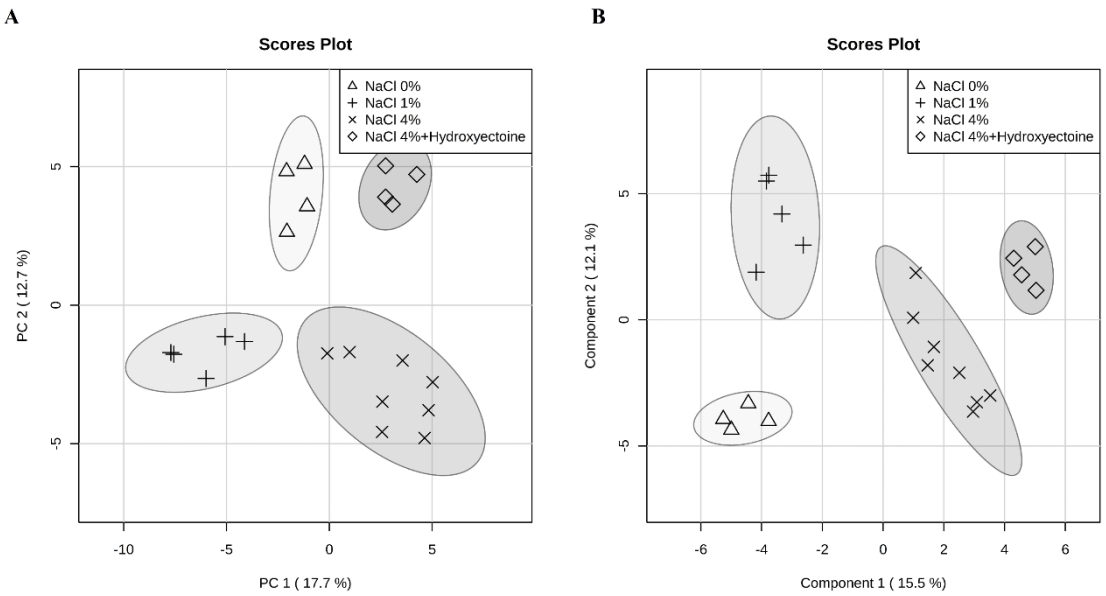
Data were analyzed using the SPSS Statistics v.22 software (SPSS, Chicago, IL). Data were inspected for normality and homogeneity of variance using Shapiro-Wilk's and Levene's test, respectively. Means were tested for significant differences by one-way analysis of variance (ANOVA) followed by a Tukey post hoc test. The level of significance was set at $p<0.05$. Percentage data on drying survival were arcsine transformed before statistical analysis.

6.3 Results

6.3.1 Metabolome response to hyperosmotic salt stress

To elucidate the physiological changes conferring enhancement on drying survival in bacterial cells after osmotic stress, a non-targeted metabolomics to investigate the global metabolic responses of the PGPR endophyte *K. radicincitans* DSM16656^T to salt stress were performed. More than 70 metabolites were identified by comparing to their corresponding mass spectra in the user-generated database and their retention time values. The spectrum of metabolites identified comprised sugars, organic acids, sugar alcohols, polyamines, and amino acids. Principal components analysis (PCA) for the intracellular metabolites showed a significant alteration caused by the osmotic stress (Figure 6.1 A). No outliers were detected by PCA at 95% confidence. The PLS-DA scores for each replicates disclosed three well-separated clusters, where the unstressed conditions at 0% NaCl controls had negative t1 scores and the cells subjected to extended osmotic stress at 4% NaCl had positive t1 scores as illustrated in Figure 6.1 B. The model was significant with R^2 =0.9817; Q^2=0.84722 indicating substantial changes in the metabolic pools under salt stress. Metabolic profiling analyses revealed that prolonged exposure of *K. radicincitans* DSM16656^T to osmotic stress at 4% NaCl resulted in substantial changes in cytoplasmic energy metabolism-associated metabolites, including pyruvate and organic acids such as fumaric acid and L-malic acid, as well as sugars and polyols such as galactose, trehalose, mannitol and *myo*-inositol (Figure 6.2 A).

Figure 6-1: A. PCA scores plot and **B.** Partial least squares discriminant analysis PLS-DA score plot for the detected metabolites during osmoadaptation and by adding hydroxyectoine [1mM]**.**

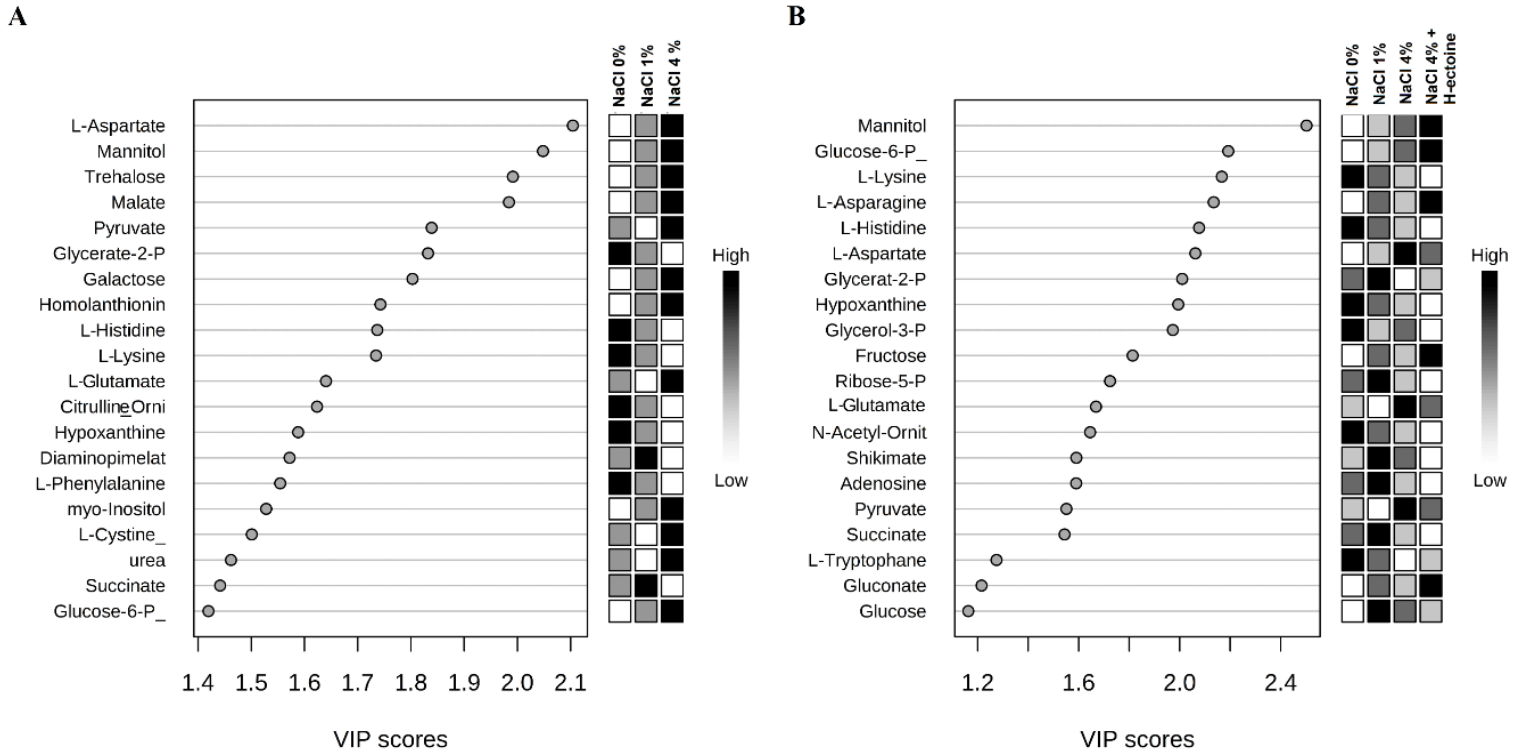


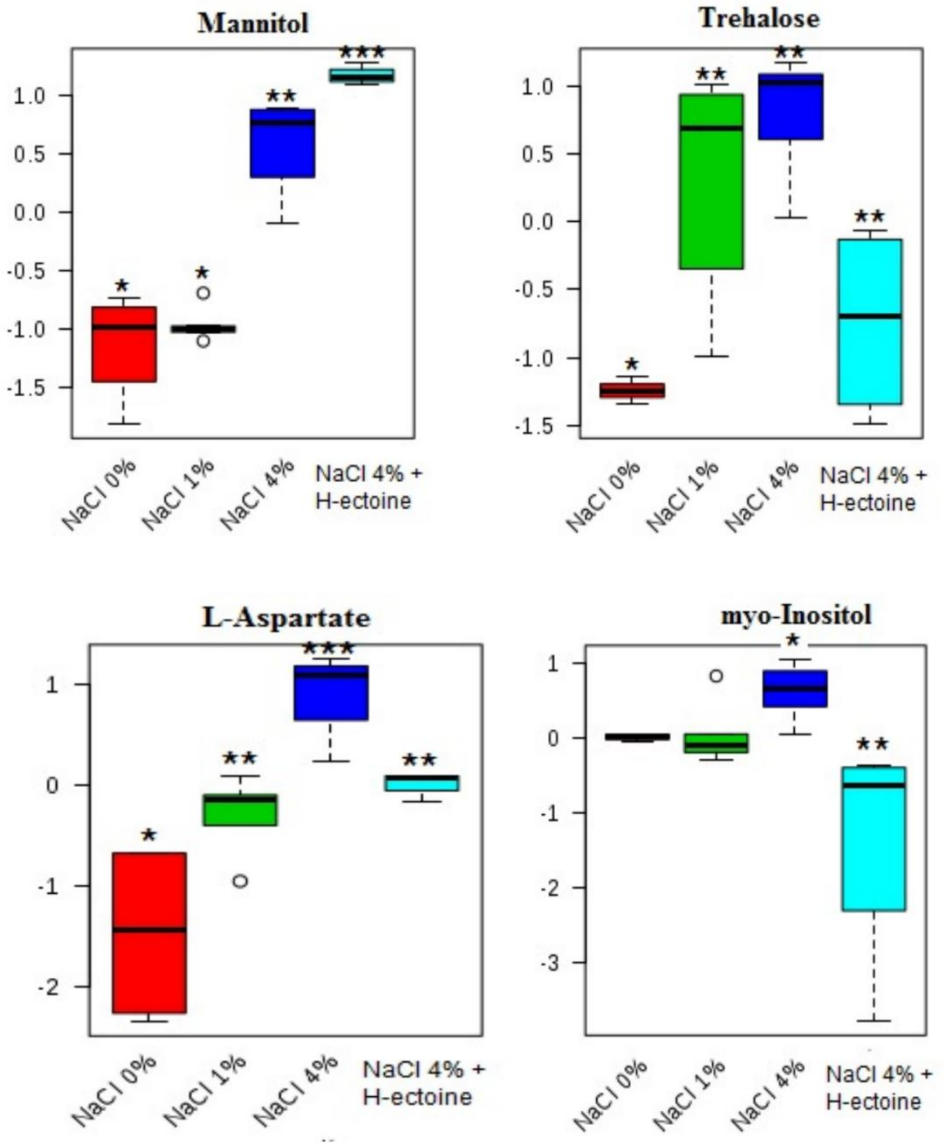
The contribution of the variables was determined by interpreting the variable importance in projection (VIP) score, which is estimated from the weighted sum of the square for each PLS-DA loadings for each component. Within the twenty most important variables identified by VIP scores > 1.9, L-aspartate, malate, trehalose and mannitol were established as metabolites that contributed significantly to the class separation of osmotic stress levels (Figure 6.2 A).

The alterations in the cellular levels of central metabolic-pathway metabolites were analyzed for all *K. radicincitans* DSM16656^T cultivations to elucidate a unified response to osmotic stress. The trend observed for these putative altered metabolites levels and related pathways during salt-stress is summarized in Table 6.1. Particular attention was paid in investigating the levels of compatible solutes and their precursors concerning the salt concentration. Thus, following the increased extracellular osmolarity, the relative abundance of L-aspartate, mannitol and trehalose increased significantly (p <0.05) to 11.07, 66.84 and 65.12-fold respectively, in the *K. radicincitans* DSM16656^T cytoplasm (Figure 6.2 C). Interestingly, the relative abundance of TCA cycle intermediates such as pyruvate, fumarate, malate and citrate

tends to increase upon salt stress (Figure 6.3). Besides, at high salt concentrations, elevated pool contents of L-asparagine and L-glutamine with 3.49 and 2.98-fold change were measured. Unlike, for some aromatic amino acids such as L-histidine, L-tyrosine, L-phenylalanine and L-tryptophan a considerable drop in the relative abundance at DM 4% NaCl was detected.

Figure 6-2: Variable importance in projection (VIP) plot displays the top 20 most important metabolite features identified by PLS-DA. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each group under study. **A**. During osmoadaptation from no amended media DM to 4% NaCl. **B.** Upon addition of hydroxyectoine [1mM] at 4% NaCl amended media. **C.** Box-whisker plots of relative abundance concentrations of important metabolites in PLS-DA model: L-aspartate, mannitol, trehalose and *myo*-inositol. Asterisks indicate the level of statistical significance ($p < 0.05$; n >4).




Mannitol
Trehalose
L-Aspartate
myo-Inositol
NaCl 0%
NaCl 1%
NaCl 4%
NaCl 4% +
H-ectoine

C

Table 6-1: Intracellular metabolites of central metabolic pathways up-regulated or down-regulated with increasing salt concentration (NaCl) in *K. radicincitans* DSM16656^T

Putative metabolites		Related pathway
Up- regulated		
Amino acids		
	L-Aspartate	Alanine, aspartate and glutamate metabolism
	L-Glutamate	Glutathione degradation and glutamate metabolism
Sugars and polyols		
	Fructose-6P	Phosphate pathway
	Glucose- 6P	Phosphate pathway
	Trehalose	Trehalose biosynthesis, D-glucose 1P => trehalose
	Mannitol	Mannitol biosynthesis
	myo-Inositol	*myo*-inositol metabolism
Organic acids	Pyruvate	Pyruvate metabolism, tricarboxylic acid
		Glycolysis (Embden-Meyerhof pathway), glucose => pyruvate
	Malate	Tricarboxylic acid metabolism
		Glyoxylate metabolism
	Citrate	Tricarboxylic acid metabolism
		Glyoxylate metabolism

	Fumarate	Pyruvate metabolism
		Glyoxylate metabolism
Down- regulated		
Amino acids	L-Histidine	Histidine biosynthesis, PRPP => histidine
		Histidine degradation, histidine => N-formiminoglutamate => glutamate
	L-Methionine	Methionine biosynthesis, aspartate => homoserine => methionine
		Methionine degradation
	L-Lysine	Lysine biosynthesis, succinyl-DAP pathway, aspartate => lysine
	L-Phenylalanine	L-Phenylalanine biosynthesis
Others	Citrulline-Ornithine-Arginine	Arginine and proline metabolism
		Urea cycle

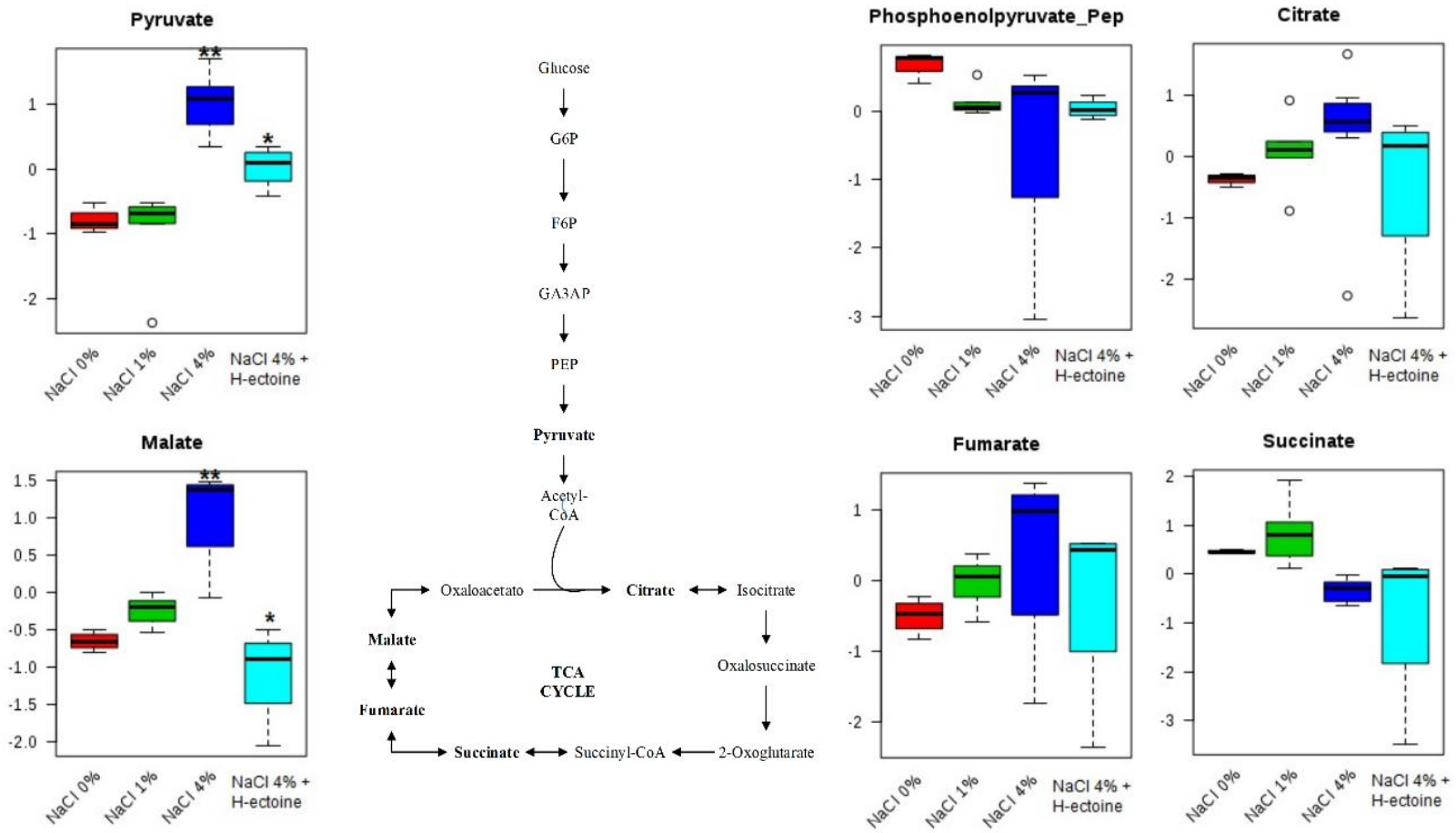


Figure 6-3: Comparison of levels of glycolysis and TCA cycle pathway intermediates in *K. radicincitans* DSM16656^T under salt stress and upon addition of hydroxyectoine. Asterisks indicate the level of statistical significance ($p < 0.05$, $n = 8$) in comparison with the control DM amended at 4% NaCl. GA3P, glyceraldehyde 3-phosphate; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; PEP, phosphoenolpyruvate

6.3.2 Metabolome response to hyperosmotic salt stress upon hydroxyectoine uptake

The presence of hydroxyectoine during the osmoadaptation altered the abundances of metabolites throughout their metabolic networks. PCA plot detected no outliers at 95% confidence (Figure 6.1 A). The addition of hydroxyectoine led to a new cluster within this PCA plot (4% NaCl + hydroxyectoine), which is distinguished from the cluster with 4% NaCl mainly by positive PC2 scores. Similarly, the PLS-DA plot revealed four separated clusters, corresponding to the media amended with NaCl [0, 1, 4%] and the additional treatment with hydroxyectoine. Here relative to 4% NaCl, the direction of separation by addition of hydroxyectoine is along with component 1, suggesting that cells with the presence of the osmolyte were metabolically different (Figure 6.1 B). Looking at the VIP scores (Figure 6.2 B), mannitol, L-lysine, L-asparagine and L-histidine were identified as the main metabolites contributing to class separation. Remarkably, L-aspartate showed a high VIP score in combination with a significant reduction in relative abundance with the inclusion of hydroxyectoine to the medium at 4% NaCl.

The uptake of hydroxyectoine during bacteria growth at DM 4% influenced the relative abundance of the majority of TCA intermediates, such as pyruvate, malate, succinate and citrate (Figure 6.3). Besides, significant changes ($p<0.05$) in most of the detected intracellular amino acids in *K. radicincitans* DSM16656^{T} cells were observed upon hydroxyectoine addition. Hence, exogenously supplied hydroxyectoine led to a significant fold change in the pools of L-asparagine, L-proline, L-serine, L-glutamate, L-homoserine, L-methionine, L-leucine and the urea cycle intermediates citrulline-ornithine-arginine (Figure 6.4; Figure 6.5 A-B). However, the addition of hydroxyectoine induced a drop in relative abundance levels of aromatic amino acids such as L-phenylalanine and L-tryptophan, as well as the reduction of the arginine-proline metabolism intermediate N-acetylornithine. Likewise, it was notable that levels of L-lysine, L-aspartate and L-glutamine decreased in the hydroxyectoine-added cells (Figure 6.4; Figure 6.5 A-B).

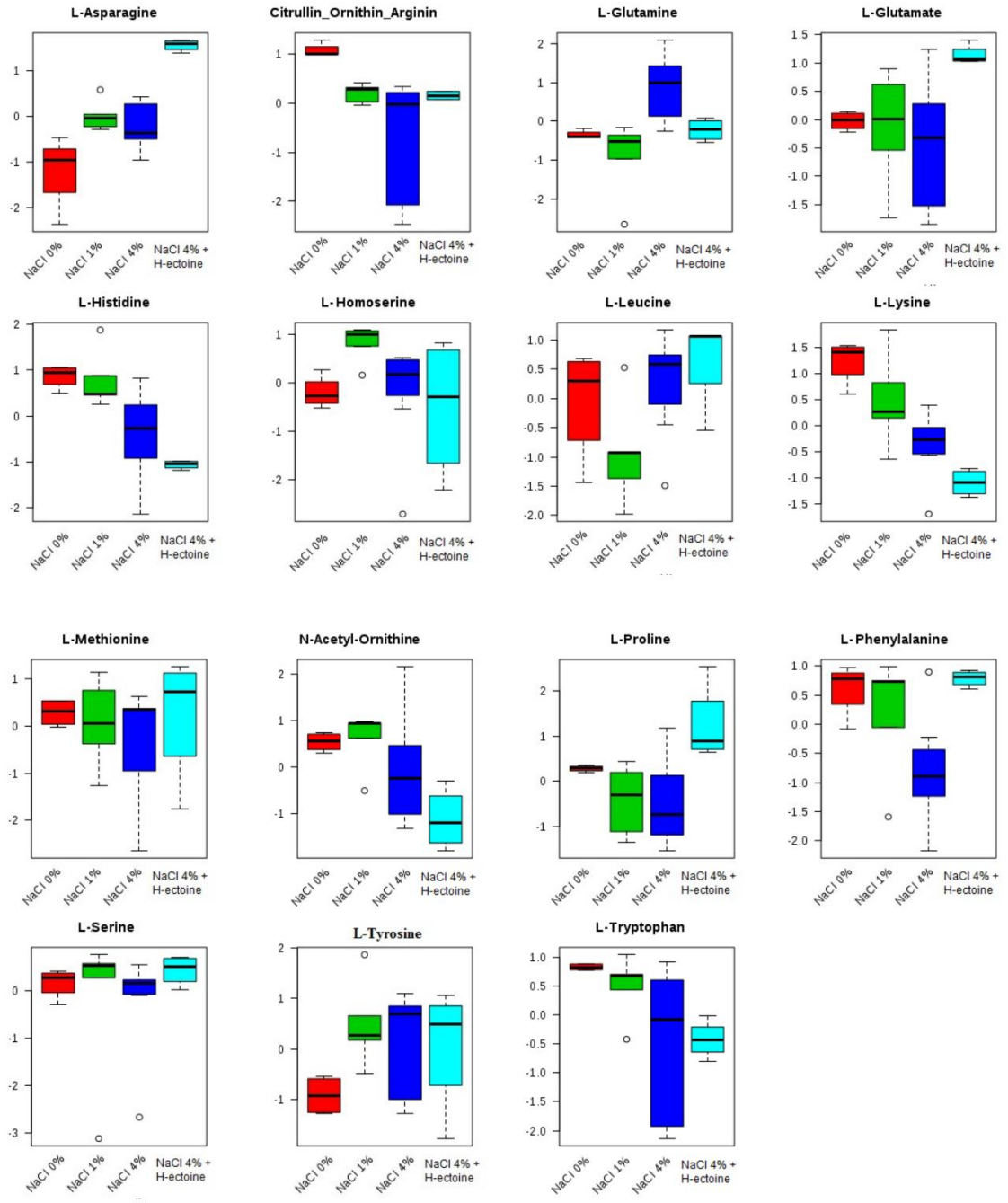
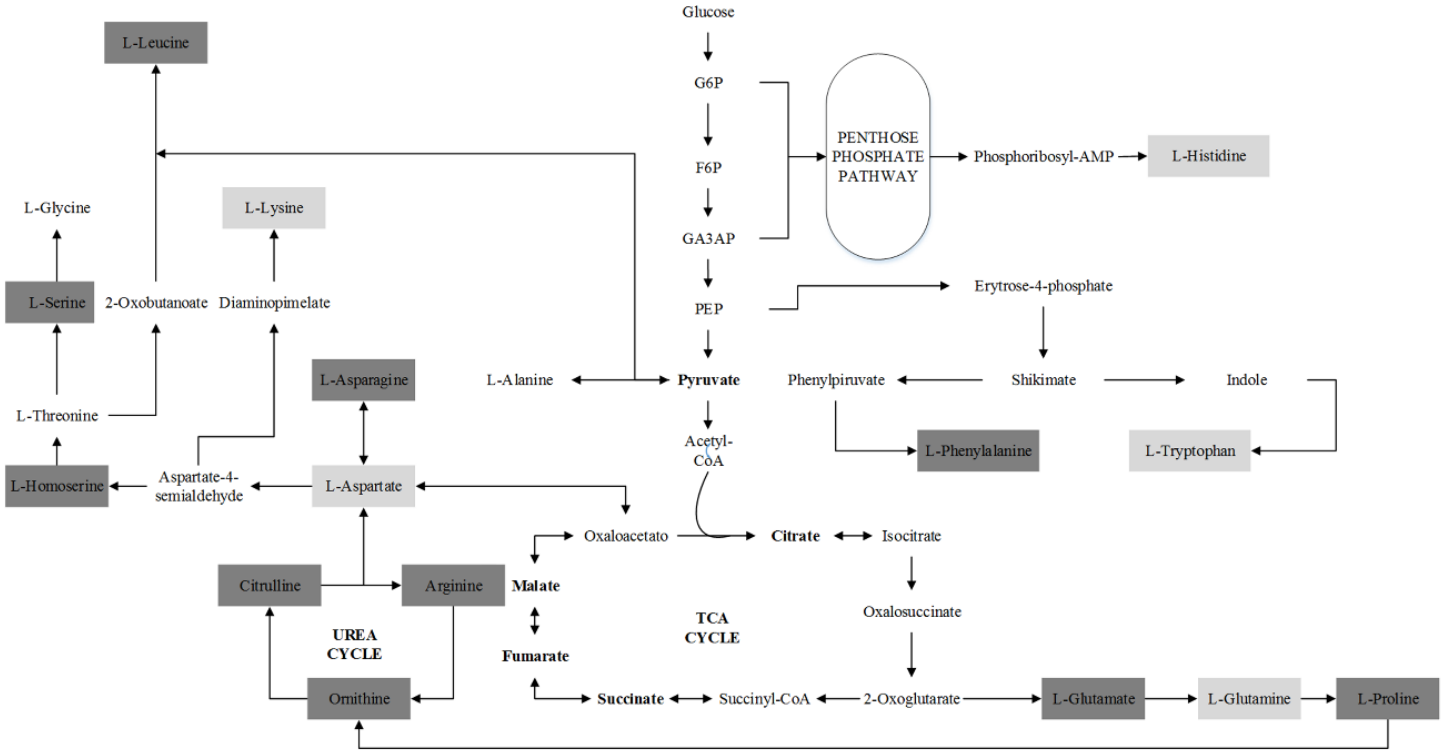


Figure 6-4: Box-whisker plots of selected amino acids with significant changes along with salt stress and the addition of hydroxyectoine in *K. radicincitans* DSM16656^T cells

Figure 6-5: Effect of exogenous hydroxyectoine addition on amino acid metabolism in *K. radicincitans* DSM16656^T under salt stress. **A.** Schematic diagram of amino acid metabolism observed in *K. radicincitans* DSM16656^T, dark and light grey boxes indicate positive and negative fold changes after the addition of hydroxyectoine respectively **B.** Metabolic profile of amino acids under salt stress. Fold change represents the ratio of amino acid content in the hydroxyectoine-added cells and control cells in DM at 4% NaCl, (p <0.05; n >4).



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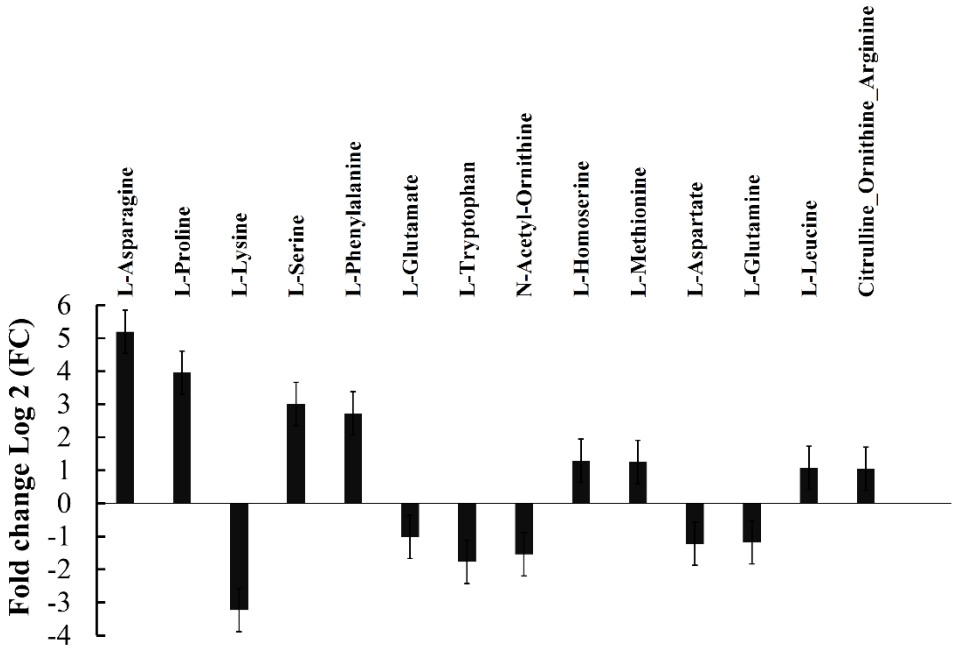
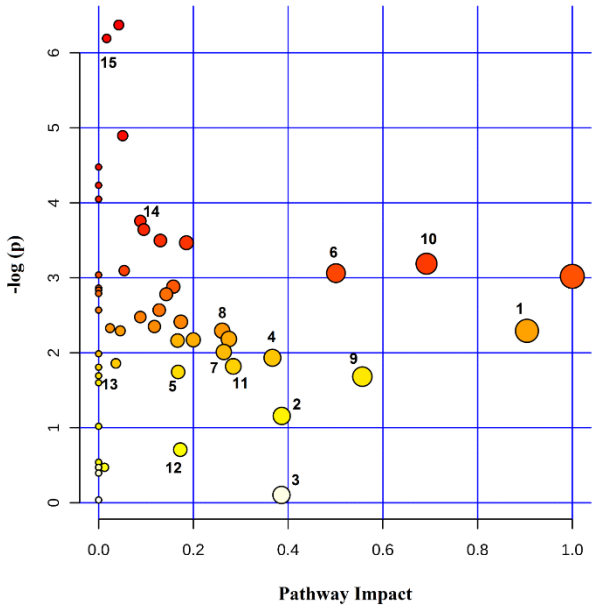


B

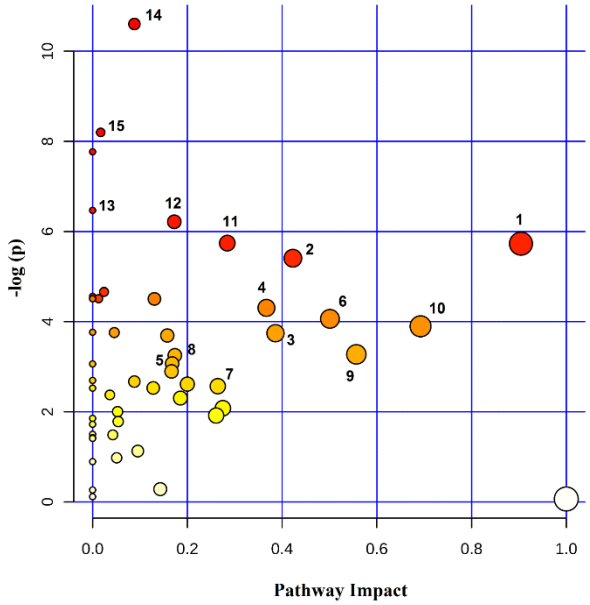
6.3.3 Metabolomic pathway analysis

Pathways altered during salt-stress were identified using MetaboAnalyst platform. The summary of the pathway analysis is shown in Figure 6.6. This topology analysis estimates the importance of a metabolite within a metabolic network. Node color and size are indicative of p-value significance and pathway impact, respectively. Pathways with an impact value >0.4 which is the cut-off that satisfied the condition of $p < 0.05$ were identified as essential for analysis (Du *et al.* 2016). Based on this resolution, the pathways altered during salt-stress were alanine, aspartate and glutamate metabolism, beta-alanine metabolism, cysteine and methionine metabolism and fructose and mannose metabolism (Figure 6.6 A). In contrast, in comparison to the hydroxyectoine-added cells, arginine and proline metabolism, D-glutamine and D-glutamate metabolism and the pentose phosphate pathway were down-regulated (Figure 6.6 B).

Figure 6-6: Putative metabolic pathways associated with the salt-stress response of *K. radicincitans* DSM16656^T cells grown in **A.** NaCl 4%, **B.** NaCl 4% + hydroxyectoine [1mM]. All the matched pathways are represented as nodes. Node colour indicates the significance based on p-value and node size indicates significance of pathway impact. Key: 1. Alanine, aspartate and glutamate metabolism, 2. Arginine and Proline metabolism, 3. Pentose phosphate pathway, 4. Pyruvate metabolism, 5. Glycolysis/gluconeogenesis metabolism, 6. Cysteine and methionine metabolism, 7. Citrate cycle (TCA), 8. Sulfur metabolism, 9. Glycine-serine and threonine metabolism, 10. Beta-alanine metabolism, 11. Butanoate metabolism, 12. D-glutamine and D-glutamate metabolism, 13. Nitrogen metabolism, 14. Fructose and mannose metabolism, 15. Selenoamino acid metabolism.



A



B

6.4 Discussion

Fluctuations in environmental osmolarity and drought tolerance are ubiquitous stress factors encountered by microorganisms during industrial fermentation and further formulation approaches. Therefore, it is necessary to develop and understand efficient adaptation strategies to mitigate the harmfulness of these stress conditions. Among these strategies, the accumulation of compatible solutes and the addition of exogenous osmolytes to protect cells against highly osmolar environments are valuable and effective (He *et al.* 2017; Czech *et al.* 2018). Molecular and physiological processes that allow bacterial endophytes such as *Kosakonia* sp. to withstand salt stress are unknown. Herein, it was used a metabolic profiling approach to investigate systematically the causes of the positive effects on drying survival, mediated by grown cells at high salinities and the inclusion of hydroxyectoine in amended media.

6.4.1 Changes in metabolic profiling in *K. radicincitans* DSM16656^T associated with a higher drying survival

K. radicincitans DSM16656^T cells grown in low a_w-media altered ionically with NaCl, pose superior drying tolerance in comparison to cells grown in an unmodified basal medium (see 4.3.1 section). In Chapter 4, apart from the salt-in and salt-out strategies (Kempf and Bremer, 1998; Czech *et al*. 2018), it was hypothesized that osmoadapted cells undergoing intra-metabolic shifts, which may confer to the cells higher drying survival. This explanation is here supported by the metabolic profiling findings, where high levels of osmolytes such as mannitol, trehalose, *myo*-inositol, L-glutamate and L-aspartate were detected at elevated salinities.

Beyond the accumulation of trehalose, an effective intracellular drying protector in bacteria (Tunnacliffe *et al.* 2001; Reina-Bueno *et al.* 2012), the accumulation of the polyol mannitol as an osmoprotectant in *K. radicincitans* DSM16656^T was rather surprising, since formerly it was found only in a few bacteria to cope with osmotic stress (Sand *et al.* 2015; He *et al*. 2017). Thus, mannitol can protect encapsulated bacteria as in *Bifidobacterium animalis* cells envelopes against drying stress, by interacting with head groups of lipids, phospholipid bilayers and protecting secondary proteins (Dianawati *et al.* 2012). According to the *K. radicincitans* DSM16656^T genome (Becker *et al.* 2018), mannitol is probably synthesized by the reduction of fructose 6-phosphate (F6P) via mannitol-1-phosphate 5-dehydrogenase. Thereby, we found that the fructose and mannose metabolism and levels of F6P tend to increase along with osmotic stress and by the addition of hydroxyectoine, boosting the relative abundance of mannitol.

L-aspartate was found to be the dominant amino acid under regular conditions whose levels along with L-glutamate increased at higher salinities. This was also disclosed in the pathway analysis, demonstrating alanine, aspartate and glutamate as one of the major pathways impacted by osmotic unbalance (Figure 6.6). L-aspartate accumulation was also surprising since only a few studies upon Gram-negative bacteria indicated this feature (Joghee and Jayaraman, 2014; Yin *et al.* 2017). High levels of L-aspartate may confer desiccation

tolerance to bacteria cells by inserting it in the interfacial region of the bacterial plasma membrane increasing membrane fluidity (Martos *et al.* 2007). Moreover, L-aspartate is a powerful chelation agent playing a role in controlling concentrations of cations such as Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+} and Cu^{2+}, for the bacterial survival during extended exposure to osmotic stress (Sajadi, 2010). Evidence indicates that these cations are involved in the regulation of a range of courses in Gram-negative bacteria, including cell division and gene expression as a reaction to external stimuli (Malek *et al.* 2012). Altogether, the increment of L-aspartate levels could benefit *K. radicincitans* DSM16656^T cells desiccation tolerance.

It is noticeably the increase in the TCA-cycle organic acids levels, like pyruvate, malate, and fumarate. These findings are in line with the impact observed upon salt-stress to the pyruvate metabolism as shown by the metabolic pathway analysis (Figure 6.6). Similar results in TCA cycle alteration were found in halophilic bacterial isolates (Joghee and Jayaraman, 2014). This can be explained by a high-energy demand required to preserve cell homeostasis at high osmotic pressure, where the cells had to prioritize cell homeostasis over growth, then the higher energy demand in cells the higher metabolism via TCA cycle. It has been reported that under stress conditions, energy pool management is the first concern of cells (Roessler and Muller, 2001). The increment in oxygen consumption in DOT curves also demonstrates the high-energy demand required to survive at high salinities.

At high salt concentrations, occurred a significant drop in the relative abundance of aromatic amino acids such as L-histidine, L-tyrosine, L-phenylalanine and L-tryptophan. Since levels of basic amino acids such as L-lysine and L-histidine drop along with high salinities and acidic amino acids such as L-aspartate and L- glutamate increased, it is suggested that *K. radicincitans* DSM16656^T possess intracellular acidic proteins functional at high salinities (Oren, 2013). The potential acidic proteome in *K. radicincitans* DSM16656^T may be considered to be correlated with the amassing of KCl to contribute to the intracellular osmotic balance (Oren, 2013).

The superior drying survival of *K. radicincitans* DSM16656^T cells upon adding hydroxyectoine during the osmoadaptation procedure probably occurred due to the

physiochemical features of hydroxyectoine as drying protectant. Thus, hydroxyectoine decreases water activity coefficients (Held *et al.* 2010) and provides superior glass-forming properties and redox stability (Tanne *et al.* 2014). Besides, hydroxyectoine is more efficient than ectoine by increasing the hydration and mobility in lipid membranes, giving an advantage for cell membranes to withstand severe surrounding environments like osmotic unbalance, accelerating cellular repair mechanisms (Harishchandra *et al.* 2010). These properties may confer to Gram-negative bacterial cells more extended life stability, higher desiccation tolerance and protection against drying (Manzanera *et al*. 2002; Manzanera *et al.* 2004; Pastor *et al*. 2010). However, beyond these findings that gave scarce insights into the metabolic response of hydroxyectoine added-cells, this study presents a different role of hydroxyectoine as the trigger of a metabolic shift for providing advantages to cells. Hence, the impact of osmotic stress and hydroxyectoine on bacteria was explored in more detail on the levels of carbon metabolites.

The levels of intermediates involved in glycolysis and the tricarboxylic acid (TCA) cycle were monitored under salt stress. At high salinities, higher levels of glycolytic intermediates (glyceraldehyde 3-phosphate, PEP and pyruvate) and higher contents of TCA cycle intermediates (fumarate, citrate and malate) were detected. A more dynamic central carbon metabolism may provide salt-tolerant bacteria cells with the decisive energy in the form of ATP and precursors constructing bricks to fuel courses conveying salt tolerance, such as the biosynthesis of compatible solutes (Sevin *et al*. 2016b). Conversely, hydroxyectoine possess stress-relieving properties for alleviating the energy requirements for living at high salinities, since the levels of TCA intermediates tend to decrease. Though osmoadaptation is an energy-demanding process in bacteria, generally at higher salinities the enrichment of compatible solutes in the cytoplasm is energetically substantially less demanding than their *novo*-synthesis production (Oren, 2011; Czech *et al*. 2018). Indeed, the intracellular levels of trehalose, L-glutamate, L-aspartate and *myo*-inositol decreased as a response to exogenous hydroxyectoine in amended media.

The function of hydroxyectoine as osmotic stress protectants does not seem to be based exclusively on the extensive intracellular amassing of this compound. Thereby, the hydroxyectoine uptake by *K. radicincitans* DSM16656^T leads to an increase in the relative abundance of amino acids such as L-leucine, L-asparagine, L-serine, L-methionine and the aromatic amino acid L-phenylalanine significantly. The increment of amino acid pools may contribute to counteracting the osmotic unbalanced and further desiccation tolerance. Interestingly, an advantageous amino acid such L-proline increased 15.5-fold in the hydroxyectoine-added cells, enabled by the contraction of L-glutamate accumulation requirements, which may lead in cells the glutamic acid-glutamic semialdehyde-proline pathway. The intracellular increment of L-proline may contribute to the desiccation tolerance in bacteria, since this amino acid may provide protein stability and thermodynamic advantages to cells, such as reducing and increasing the entropy and free energy of thermal of unfolding respectively (Prajapati *et al.* 2007; Mosier *et al*. 2013).

6.4.2 Changes in metabolic profiling in *K. radicincitans* DSM16656^T associated with a higher plant colonization ability

Upon osmoadaptation and hydroxyectoine addition, intracellular metabolites in *K. radicincitans* DSM16656^T could drive the complex plant colonization process. Biomolecules involved into the bacterial cells proliferation, sensing root-chemotaxis and biofilm formation could up-regulate, facilitating the entering the plant.

In Chapter 2 (Figure 2.3), the phenotypic fingerprint in Biolog test revealed that the endophyte utilizes mannitol, trehalose and galactose as advantageous sole C-source. Hence, in the presence of host plant and upon sensing favorable environmental surrounding bacteria cells can activate for proliferating. For the halophytic phenotypes at 4% NaCl, the high relative abundance of metabolites such as mannitol, trehalose, malate, galactose, and pyruvate can be used by cells to proliferate and activate the machinery towards the multidimensional interactions within the plant. Thereby, the incidental trehalose accumulation is described as a competitive feature during the *Sinorhizobium meliloti*-alfalfa interactions, acting as an essential carbon reservoir (Jensen *et al.* 2005). Galactose

metabolism is also reported as a trait in bacterial root endophytes associated with brown sarson (*Brassica rapa L*.) (Padder *et al*. 2017).

Interestingly, N-sources amassing upon high salinities was in agreement with the nutrient preferences of the endophyte, thus L-aspartate and L-glutamate relative levels increased. Apart from the metabolites mentioned above, the hydroxyectoine-added cells increased glucose-6-P, fructose and gluconate, providing another advantageous C-sources for the bacterium, that inject additional nutrients during the early activation, the substrate utilization and colonization stages (Pinski *et al.* 2019). Regarding favorable N-sources, L-proline and L-serine concentration increased in osmoadapted (4% NaCl) hydroxyectoine-added cells concerning the cells without the osmolyte addition.

Enrichment bacterial cells upon osmotic stress, including the intracellular accumulation of selected amino acids and C-sources, may drive the host-plant interaction, since plants require essential amino acids, such as L-proline, L-asparagine, L- glutamate, L-serine among others for their metabolic networks. Then, it is tentative to theorize that high levels of these biomolecules that eventually bacteria cells may secrete for metabolic exchange, can enhance bacteria-plant interaction through plant-nitrogen assimilation mechanisms and amino acid cycling. Thereby, studies in poplar plants suggest that this host tend to amass amino acids such as L-asparagine and urea along with endophyte interaction (Scherling *et al.* 2009).

Bacterial endophyte establishment starts with the chemotaxis of free-living cells towards the roots exudates gradients followed by adhesion to the rhizoplane. Methyl-accepting chemotaxis proteins (MCPs) play a major role in these first stages (Pinski *et al*. 2019). These proteins are linked to a cytoplasmic histidine-aspartate phosphorylating system. According to the endophyte genome, *K. radicincitans* DSM16656^{T} possess methyl-accepting chemotaxis protein I (serine sensor receptor) and protein II (aspartate sensor receptor). Interestingly, the metabolomics data revealed that both amino acids abundance within sensor receptors increase upon osmotic stress. Besides, low levels of L-histidine in salt-stressed cells may suggest a high histidine-kinase activity, which is the enzyme that forms a sensory

complex with MCPs associated with flagellar motors, and it is related to bacterial homeostasis and stimuli sensing (Borland *et al*. 2016).

The early attachments on roots are mediated by flagella and pili as well as by specialized proteins such as curli and hemagglutinins (Mitter *et al.* 2013; Pinski *et al*. 2019). Both proteins are involved within the adhesion of cells on surfaces, cell aggregation and biofilm formation. Among these, *K. radicincitans* DSM16656^{T} can produce filamentous hemagglutinin and it is hypothesized that hemagglutinin activity under 420 mOsmo could not change by osmotic strength, according to vaccine influenza studies (Choi *et al*. 2015).

Mannitol-relative high levels could provide indirect benefits to bacteria for plant colonization. Hence, comparative genomics studies (Wu *et al*. 2011), revealed that the enzyme mannitol dehydrogenase has a beneficial role in several bacterial endophytes, which effectiveness of the plant defense response via the conversion of pathogen-secreted mannitol into mannose gives endophytes a competitive advantage in the endosphere. Interestingly, a gene encoding for mannitol dehydrogenase was found in *K. radicincitans* DSM16656^{T} species. The important outcome here is the metabolic re-ordering triggered by osmotic stress and hydroxyectoine addition, which may indirectly benefit endophyte-host plant interaction via mannitol biosynthesis.

Pre-conditioning by osmotic stress increase the relative abundance of urea, suggesting an increment of amino acid catabolism, which lead to the production of ammonia, that tend to the up-regulation of the urea cycle (Scherling *et al*. 2009). Additionally, an excessive L-proline and L-asparagine concentrations into the hydroxyectoine-added cells pools may lead to catabolism mechanism which triggered additional ammonia boost. Ammonia not only provides a portion of the nitrogen demand but also potentially stimulates plant defense against pathogens. Noteworthy, ammonia by Gram-negative bacterial endophytes is an essential trait for indirect plant-growth promotion (Hassan, 2017; Brigido *et al*. 2019). Considering the potential ammonia increments into the colonization microenvironments, questions remain about how the diazotrophic nature of the endophyte is affecting along with the cells pre-conditioning.

Metabolomics approach confirms the proposed metabolic reordering in endophytic cells exerted at high salinities discussed in Chapter 3. Hence, this research help to decipher the metabolic traits that are involved in the osmoadaptation and hydroxyectoine uptake of *K. radicincitans* DSM16656^T. These essential results extend the knowledge regarding the biological response of this endophyte to these stressors and provide insights into the mechanism for facilitating the plant growth promotion and the endophytic establishment of pre-conditioned cells.

Questions remain regarding how conservative the acidic proteome in *Kosakonia* sp. at high salinities can be, and which genes are involved in such adaptation. Then, further studies along with other alternative *omics* would widen the understanding of the underlying cellular mechanisms in osmoadaptation as a pre-conditioning strategy for enhancing drying survival, plant growth-promoting effects and endophytic establishment.

6.5 Conclusions

To conclude, substantial alterations in endogenous metabolites pools upon exposure to high salinity, including elevated levels of mannitol and L-aspartate, play a crucial role in confering desiccation tolerance to the endophyte *K. radicincitans* DSM16656^T. Metabolic approaches indicate that bacteria cells adapt to prolonged osmotic stress by altering their amino acid and TCA cycle pools. Thus, to maintain the osmotic bacteria cells accumulates hydroxyectoine and/or novo-synthesize compatible solutes and increase intracellular acidic amino acids pools. These meaningful alterations in metabolite pools and eventual acidic proteome induce a phenotypic shift as an osmoadaptation mechanism for conferring survival under desiccation stress and eventually advantages for plant colonization. Metabolic traits include mannitol, trehalose and galactose accumulation as a competitive feature during radish plant-interactions. Besides, it was found the amino acid and urea up-regulation for supporting nitrogen fixing. Finally, this study will encourage anhydrobiotic engineering in Gram-negative bacteria endophytes, supporting the exploitation of compatible solutes for developing dryable and more active biological active formulations.

7. Conclusions and recommendations

7.1 Conclusions

An essential step for implementing PGPBEs within eco-friendly agriculture is the development of sustainable cultivation and delivery systems. To accelerate this labor-intensive process, it is necessary to employ novel formulation-processing strategies. Furthermore, it is critical the understanding of the mode of action and the interaction between the endophytic bacteria and the formulation-plant system. This doctoral research pursued the capacity enhancement of the PGPBE *Kosakonia radicincitans* DSM16656^{T} strain to colonize plant seedlings, solubilize phosphate and support the drying survival during formulation processing. This research represents the first comprehensive study for integrating the pre-conditioning bacteria endophyte cells through osmoadaptation, hydroxyectoine accumulation and the formulation in an amidated pectin-based network.

K. radicincitans DSM16656^{T} phenotypic characterization revealed its affinity for polyols as advantageous C-sources, including glycerol and sorbitol. On the other hand, L-alanine, L-proline and glutamic acid as the most favorable N-sources. After the systematic application of microfermentation, the obtained cost-effective and glycerol-based liquid media permitted 32-fold increment of cells concentration in comparison to the typically used standard nutrient broth.

Data evidence showed that physiological alterations in *K. radicincitans* DSM16656^{T} by osmotic stress at 4% NaCl and by the accumulation of hydroxyectoine during cultivation increased phosphate-solubilization ability, particularly acid phosphatases secretion, and to promote the endophytic root colonization.

The maintenance of bacterial viability during drying is a critical challenge during formulation. This research provided proofs that endogenous metabolites pools alterations upon growing at high salinity, including higher levels of mannitol, L-aspartate and acidic proteome, support desiccation tolerance to the endophyte *K. radicincitans* DSM16656^T. Besides, after encapsulation in pectin-calcium gluconate hydrogel networks, the osmoadapted at 4% NaCl and hydroxyectoine-added cells resisted stronger drought conditions, in comparison to calcium chloride cross-linking.

This work demonstrated for the first time that physiological modifications triggered by osmotic stress at 4%NaCl and hydroxyectoine-amassing events during cultivation, in combination with the entrapment of these pre-conditioned cells in amidated pectin beads enclosed a feasible and innovative strategy, to improve bacterial endophyte-host interactions. Besides, their unknown capability to proliferate and migrate as aggregates through the biopolymer matrix and endodermis plant tissue was elucidated.

Osmoadapted cells increase mannitol, trehalose and galactose levels, which according to their C-source preference could support the activation and further proliferation, boosting plant colonization events. In the same line, increments on favorable N-sources including amino acids such as L-proline, L-glutamate, L-serine, and the urea up-regulation could drive nitrogen-fixing and plant promoting features.

As hypothesized, the integration of cultivation and formulation proceedings target a higher endophytic capability of *K. radicincitans* DSM16656^T. Through the metabolic re-ordering upon growing at high salinities, the amassing of hydroxyectoine and the entrapment in amidated pectin hydrogel beads, the endophyte resist significantly drying stress and increase its plant colonization ability. These data provide evidence that the phenotypic plasticity of *K. radicincitans* DSM16656^T persists in entrapment cells, for increasing plant bio-stimulation and endophytic performance.

Finally, this study opens new perspectives to Gram-negative bacteria endophytes, supporting their exploitation for developing efficient polymeric dryable formulations. These findings comprise a valuable knowledge upon the inoculant technology can integrate the cultivation and formulation procedures, to take the step from the laboratory to the field.

7.2 Recommendations

For future studies, other *omics* approaches are necessary for integrating the metabolomics insights found in this research. Genomic, proteomics and transcriptomics can address a step forward to elucidate more mechanism involved in pre-conditioned bacterial endophytes. Thus, it would be necessary to generate *K. radicincitans* DSM16656^T mutants impaired in synthesis and accumulation of compatible solutes to demonstrate their importance into the formulation-drying process and further endosphere colonization. Besides, to decipher the genic response of pre-treated *K. radicincitans* DSM16656^T cells during the interaction with plant host, under free-living cells and formulated conditions.

Since enzymatic activity in endophytic microorganisms is essential for entering the plant host, further research is required in order to elucidate the effect of pre-conditioning and compatible solutes on enzyme secretion. It is recommended specific experiments to demonstrate the effectiveness of the delivery system strategy for the endophyte under high salinities in soils. In addition, the shelf life test is required to assay the potentiality of the pectin-based formulate under different storages conditions. Besides, the influence of osmoadapted cells on this feature is necessary to establish. Finally, future investigations could validate the integrated strategy of osmoadaptation and formulation in pectin hydrogel beads with another Gram-negative bacterium endophyte, to investigate whether this approach can facilitate the step from the laboratory to the field scale.

7.3 Publications and scientific output

[1] Cruz B, Schoenwandt D, Gomez M, Becker M, Patel A, Ruppel S. 2019. Salt stress and hydroxyectoine enhance phosphate solubilisation and plant colonization capacity of *Kosakonia radicincitans*. *Journal of Advanced Research*. Doi: https://doi.org/10.1016/j.jare.2019.03.012

[2] Cruz B, Schoenwandt D, Gomez M, Patel A, Ruppel S. 2019. Anhydrobiotic engineering for the endophyte bacterium *Kosakonia radicincitans* by osmoadaptation and providing exogenously hydroxyectoine. *World Journal of Microbiology and Biotechnology*. (Under review)

[3] Cruz B, Schoenwandt D, Gomez M, Serrato J, Patel A, Ruppel S. 2019. A new formulation strategy for bacterial endophytes: pre-conditioning of cells and the immobilization in amidated pectin beads. *Journal of Biotechnology*. (Under review)

International scientific meetings

Poster Presentations

[1] Endophytic plant growth promoting bacterium *Kosakonia radicincitans*: An integrated fermentation and formulation approach. Conference: XIV Meeting of the IOBC-WPRS Working Group Biological Control of Fungal and Bacterial Plant Pathogens. September 2016, Berlin, Germany.

[2] Entwicklung eines integrierten Fermentations- und Formulierungsverfahrens für das endophytische Bakterium *Kosakonia radicincitans* als neuartiges Düngemittel/Poster. Conference: Pflanzenschutz: Effizienz und Vielfalt At: September 2016, Halle Saale, Germany

[3] Compatible Solutes Accumulation Capacity of the Endophytic Bacteria *Kosakonia radicincitans*. Conference: Tropentag-"Future Agriculture: Socio-ecological transitions and bio-cultural shifts". September 2017, Bonn, Germany

[4] Encapsulation of pre-conditioned endophytic bacteria in amidated pectin beads. 26th International Conference on Bioencapsulation. August 2019, Strasbourg, France.

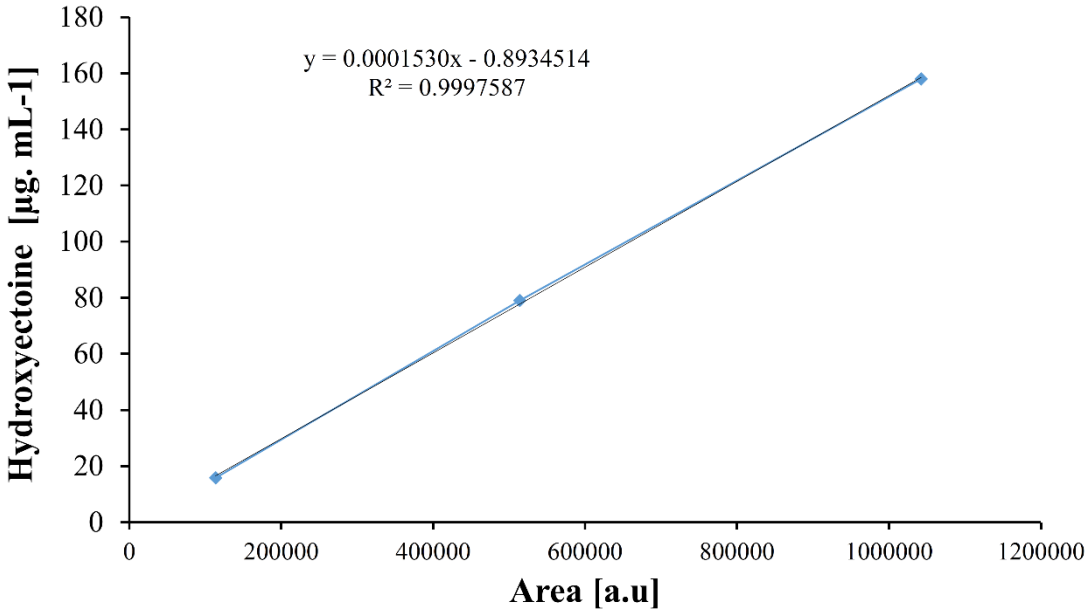
Oral Presentations in International Conferences

[1] Osmoadaptation and compatible solutes accumulation by bacterium *Kosakonia radicincitans* enhance its capacity as endophyte in radish plants. Conference: 61. Deutsche Pflanzenschutztagung. September 2018, Universität Hohenheim, Germany.

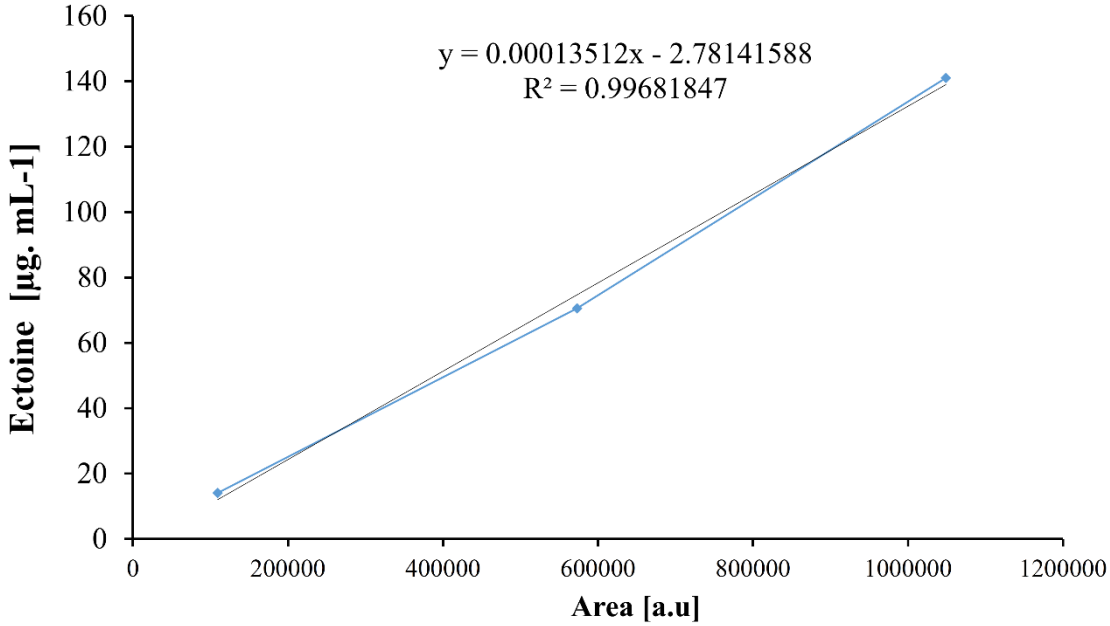
[2] Osmoadaptation and hydroxyectoine accumulation enhance endophytism of encapsulated *Kosakonia radicincitans* in radish plants. Conference: The Plant Microbiome, Exploration of Plant-Microbe Interactions for improving agricultural productivity. November 2018, TU-Berlin, ElGouna, Hurghada, Egypt.

Appendix 1: HPLC calibration curves for ectoine and hydroxyectoine quantification

A. EC 150/4.6 NUCLEODUR® 100-5 NH_2-RP column and a UV-detector at 215 nm, at a flow rate of 1 mL min^{-1} at 30°C accompanied by a column heater and using a solvent gradient established between eluents A and B (80% ACN in HPLC water).



B.



C.

Appendix 2: Biolog GEN III microplate outcome

D. The **"Phenotypic Fingerprint" of *K. radicincitans* DSM 16656^T** obtaining with the Biolog GEN III MicroPlate analyses. The microwells contain 94 phenotypic tests: 71 carbon source utilization assays and 23 chemical sensitivity assays.

A2	**Dextrin**	+	C9	**Inosine**	+++	F3	**L-Galactonic Acid Lactone**	+++	H4	**β-Hydroxy-D,Lbutyric Acid**	-
A3	**D-Maltose**	+++	C10	**1% Sodium Lactate**	+++	F4	**D-Gluconic Acid**	+++	H5	**α-Keto-Butyric Acid**	-
A4	**D-Trehalose**	+++	C11	**Fusidic Acid**	+++	F5	**D-Glucuronic acid**	+++	H6	**Acetoacetic Acid**	+
A5	**D-Cellobiose**	+++	C12	**D-Serine**	+++	F6	**Glucuronamide**	++	H7	**Propionic Acid**	+
A6	**Gentiobiose**	+++	D1	**D-Sorbitol**	+++	F7	**Mucic Acid**	+++	H8	**Acetic Acid**	+++
A7	**Sucrose**	+++	D2	**D-Mannitol**	+++	F8	**Quinic Acid**	-	H9	**Formic Acid**	+
A8	**D-Turanose**	++	D3	**D-Arabitol**	-	F9	**D-Saccharic Acid**	+++	H10	**Aztreonam**	+++
A9	**Stachyose**	+	D4	**myo-Inositol**	-	F10	**Vancomycin**	+++	H11	**Sodium Butyrate**	+++
A10	**Positive Control**	+++	D5	**Glycerol**	+++						
A11	**pH 6**	+++									

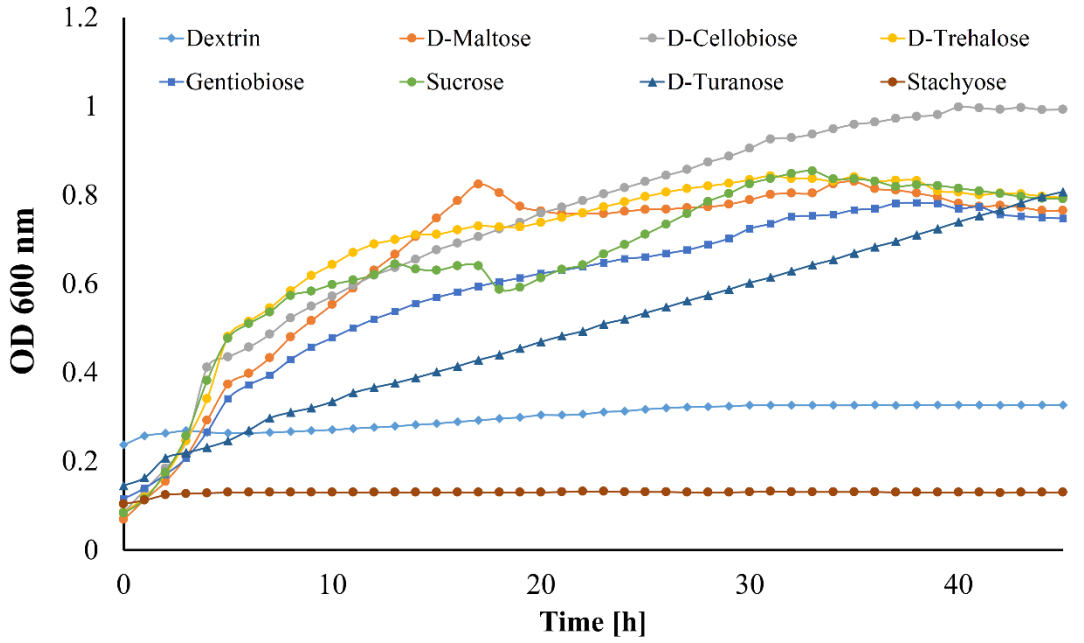
A12	**pH 5**	+++		D6	**D-Glucose-6-PO4**	+++		F11	**Tetrazolium Violet**	-	
B1	**D-Raffinose**	-									
B2	**α-D-Lactose**	++		D7	**D-Fructose-6-PO4**	+++		F12	**Tetrazolium Blue**	+++	
B3	**D-Melibiose**	-									
B4	**β-Methyl-D-Glucoside**	+++		D8	**D-Aspartic Acid**	+		G1	**p-Hydroxy-Phenylacetic v**	-	
				D9	**D-Serine**	+					
B5	**D-Salicin**	+++		D10	**Troleandomycin**	++					
B6	**N-Acetyl-DGlucosamine**	+++		D11	**Rifamycin SV**	+		G2	**Methyl Pyruvate**	+	
B7	**N-Acetyl-β-D-Mannosamine**	++		D12	**Minocycline**	+		G3	**D-Lactic Acid Methyl Ester**	+++	
				E1	**Gelatin**	-					
B8	**N-Acetyl-Dgalactosamine**	-		E2	**Glycyl-L-Proline**	+++		G4	**L-Lactic Acid**	+++	
B9	**N-Acetyl Neuraminic Acid**	-		E3	**L-Alanine**	+++		G5	**Citric Acid**	+++	
				E4	**L-Arginine**	+		G6	**α-Keto-Glutaric acid**	-	
B10	**1% NaCl**	+++		E5	**L-Aspartic Acid**	+++					
B11	**4% NaCl**	+++		E6	**L-Glutamic Acid**	+++		G7	**D-Malic Acid**	-	
B12	**8% NaCl**	+		E7	**L-Histidine**	-		G8	**L-Malic Acid**	-	
C1	**α-D-Glucose**	+		E8	**L-Pyroglutamic Acid**	-		G9	**Bromo-SuccinicAcid**	-	

H12	**Sodium Bromate**	+

C2	**D-Mannose**	+						G10	**Nalidixic Acid**	-
C3	**D-Fructose**	+		E9	**L-Serine**	+++		G11	**Lithium Chloride**	+++
C4	**D-Galactose**	++		E10	**Lincomycin**	+++		G12	**PotassiumTellurite**	-
C5	**3-Methyl Glucose**	-		E11	**Guanidine HCl**	+++		H1	**Tween 40**	-
C6	**D-Fucose**	-		E12	**Niaproof 4**	+++		H2	**γ-Amino-Butryric Acid**	-
C7	**L-Fucose**	-		F1	**Pectin**	+				
C8	**L-Rhamnose**	++		F2	**D-Galacturonic Acid**	+++		H3	**α-Hydroxy-Butyric Acid**	-

More details of GEN III microplate test

http://208.106.130.253/pdf/teach_portal/MI/Universal_GEN_III_Protocol_07172013.pdf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D-Maltose
D-Cellobiose
D-Trehalose
Gentiobiose
Sucrose
D-Turanose
Stachyose
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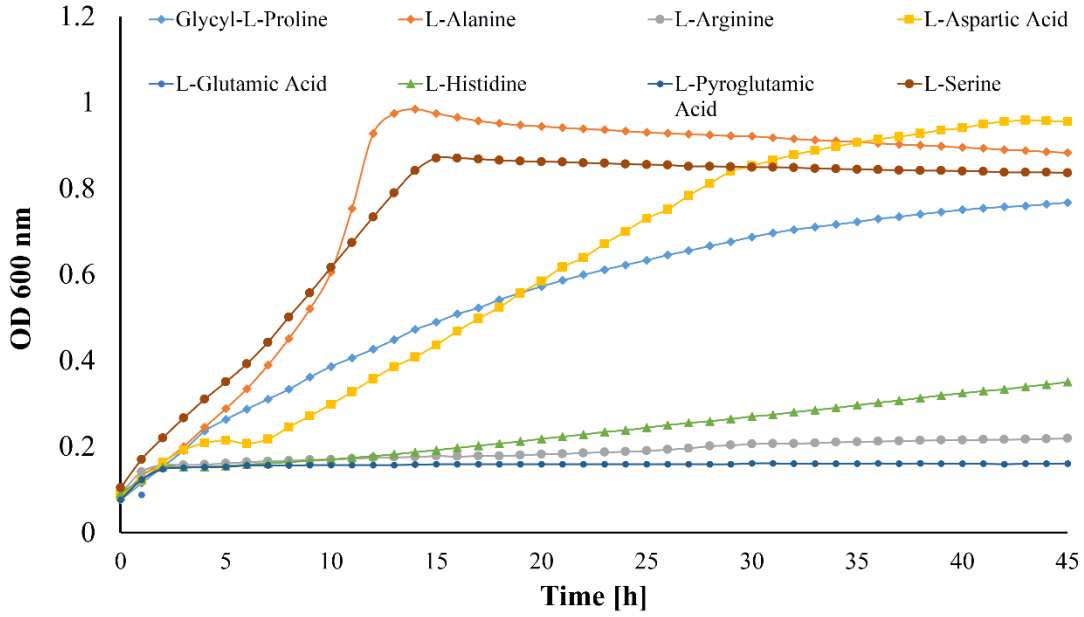
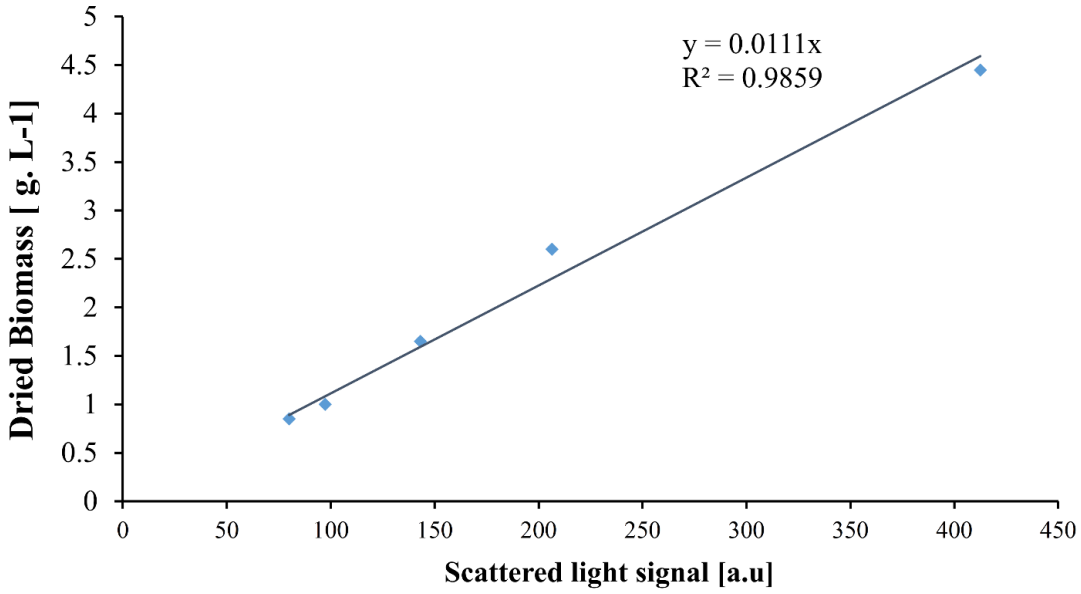
0.4
0.6
0.8
1
1.2
0
10
20
30
40

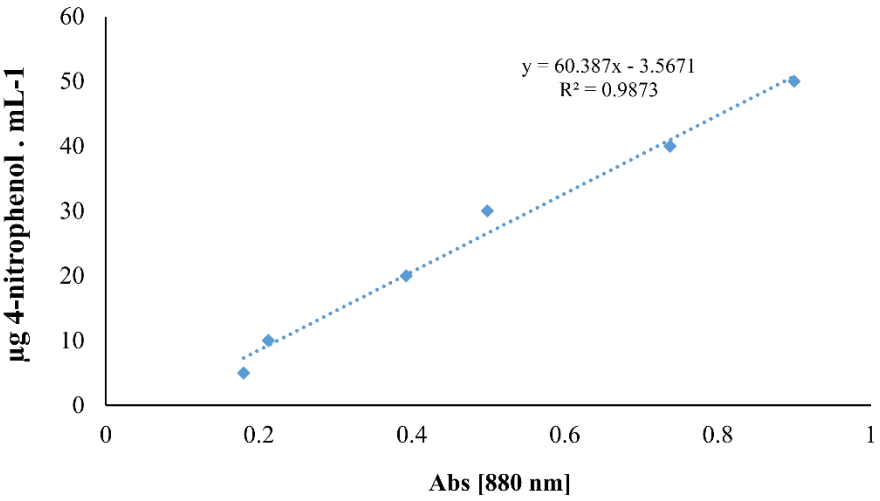

Glycyl-L-Proline
L-Alanine
L-Arginine
L-Aspartic Acid
L-Glutamic Acid
L-Histidine
L-Pyroglutamic Acid
L-Serine
OD 600 nm
Time [h]
0
0.2
0.4
0.6
0.8
1
1.2
0
5
10
15
20
25
30
35
40
45

Appendix 3: Correlation of scattered light intensities and biomass concentrations in BioLector®

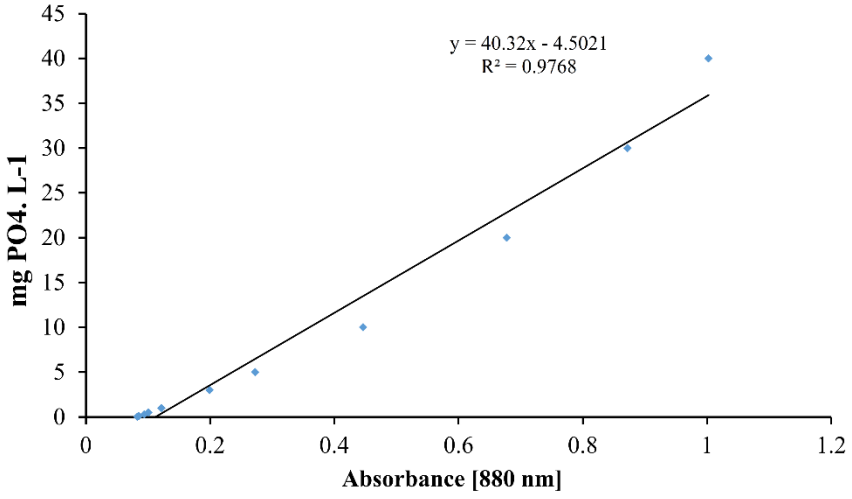


Appendix 4: Quantitative spectrophotometric analysis: A. Calibration curve for orthophosphate quantificacion. B. calibration curve for phosphatase enzyme activity

A

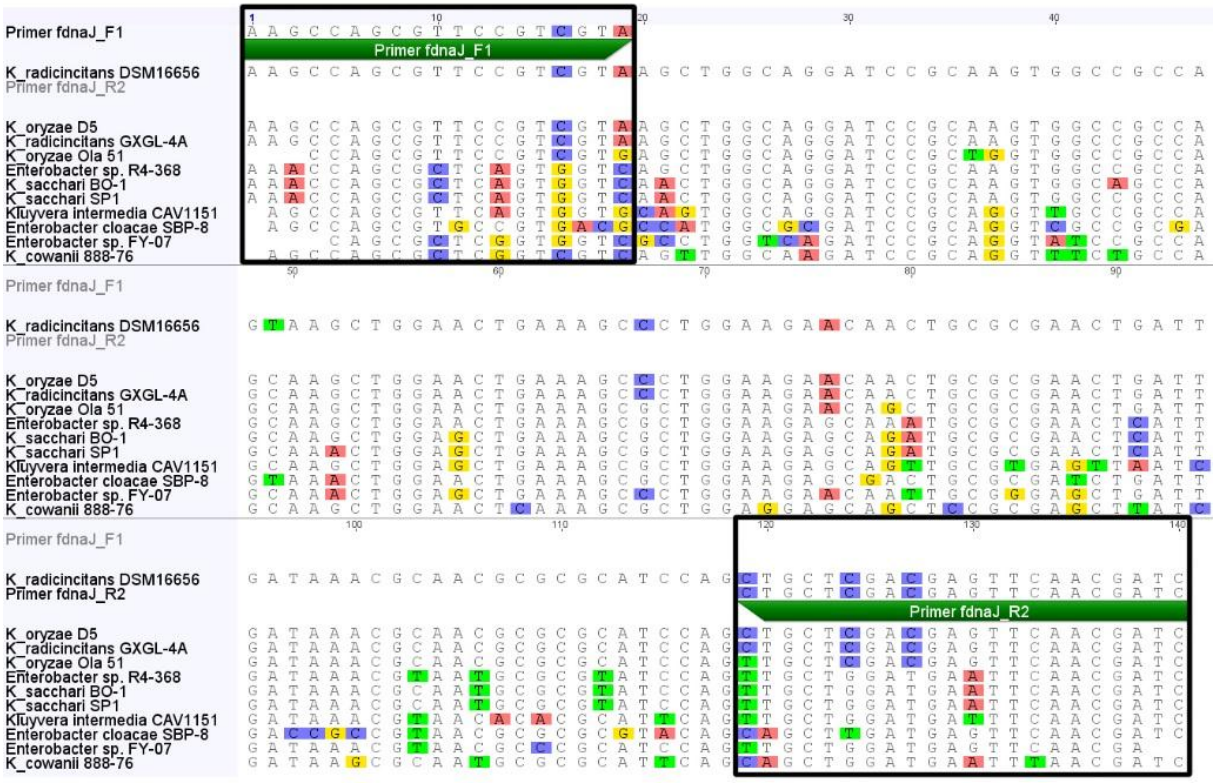


B



Appendix 5: Gene sequence used for qPCR analysis

Alignment of the ten best NCBI blastn search hits to a 140 bp gene sequence of a DNA repair protein used for qPCR analysis of *Kosakonia radicincitans* DSM16656^{T}. Position and length of primer sites are indicated by green bars; DNA sequences of other strains corresponding to primer sites of DSM 16656 ^{T} are framed in black; nucleotides differing from the consensus are highlighted in color (Witzel *et al.* 2017).



Appendix 6: Preparation of Electro Competent bacteria cells

REFERENCE

Adapted by Tetsuya Chujo

REAGENTS: LB medium (**g L^{-1}):** 1% Bacto Tryptone (10), 0.5% Bacto Yeast extract (5), 85 mM NaCl (5).

Make up to 1 L with Milli-Q water. pH to 7-7.5 with 0.5 mL of 5 M NaOH

Autoclave 600 ml in 2 L flasks and aliquot the remainder into Universals.

SOB medium (**g L^{-1})**: 2.0 % Bacto Tryptone (20), 0.5% Bacto Yeast extract (5), 5 M NaCl 2 mL [final: 10 mM], 2 M KCl 1.25 ml [final: 2.5 mM], Milli-Q water 990 mL.

2M Mg^{2+} solution	**g/100 mL**
1 M $MgSO_4.7H_2O$	24.65
1 M $MgCl_2.6H_2O$	20.33

Autoclave, and add 10 mL sterile 2M Mg^{2+} solution (1M $MgSO_4.7H_2O$ + 1M $MgCl_2.6H_2O$)

SOC medium

Add 1/100 vol. sterile 2 M Glucose solution into SOB medium plus 10% Glycerol.

Autoclave, and store at 4°C

2× Sterile Culture tubes with lids

6× Sterile GSA Bottles

4× Sterile SS34 tubes

2L Sterile water (ice cold)

50 ml 10% Glycerol (ice cold)

Sterile 1.5 mL Eppendorf Tubes

METHOD

Day 1

1. Streak *K. radicincitans* onto 2 LB plates, and incubate overnight at 37°C.

Day 2

2. Transfer a single colony into a test tube with 10 mL LB medium. Incubate overnight at 37°C.

Day 3

3. Transfer 6 mL of pre-cultured *K. radicincitans* into 2L flask containing 600 mL LB medium. Shake at 250 rpm at 37°C for 4-5 h or until mid-log phase i.e. OD_{600}=0.4~0.5.
4. Store the flasks on ice for 10 min, then transfer cells to GSA bottles. Harvest by centrifuging at 3000 rpm for 15 min at 4°C.
5. Pour off the supernatant and resuspend the cells in 600 mL of ice-cold sterile MQ water.
6. Centrifuge at 3000 rpm for 15 min at 4°C. Thoroughly remove the supernatant.
7. Resuspend the pellet in 300 mL ice-cold sterile MQ water.
8. Centrifuge at 3000 rpm for 15 min at 4°C. Thoroughly remove the supernatant.
9. Resuspend the pellet in 12 mL of ice-cold sterile 10% glycerol.
10. Centrifuge at 3000 rpm for 15 min at 4°C. Thoroughly remove the supernatant.
11. Resuspend the pellet in 1.2 mL of ice-cold sterile 10% glycerol.
12. Aliquot 50µl of cells into sterile 1.5 mL Eppendorf tubes. Flash-freeze in liquid N_2
13. Store at -80°C

NOTES

1. It is very important that the *K. radicincitans* cells are kept cold all the time, use cold GSA tubes and 1.5 mL tubes, as well as cold buffers.
2. The final concentration of the cells should be at least 10^{10} ml^{-1}.
3. At step 3, monitor Absorbance from 3 h as it is important that cell density does not exceed mid-log phase. Use LB media to blank spectrophotometer.

References

[1] Adams PD, Kloepper JW. 2002. Effect of host genotype on indigenous bacterial endophytes of cotton (Gossypium hirsutum L.). Plant and Soil 240:181-189.

[2] Adinarayana K, Ellaiah P. 2002. Response surface optimization of the critical medium components for the production of alkaline protease by a newly isolated Bacillus sp. Journal of Pharmacy and Pharmaceutical Sciences 5:281-287.

[3] Alain K, Querellou J. 2009. Cultivating the uncultured: limits, advances and future challenges. Extremophiles 13:583-594.

[4] Ali S, Duan J, Charles TC, Glick BR. 2014. A bioinformatics approach to the determination of genes involved in endophytic behavior in Burkholderia spp. Journal of Theoretical Biology 343:193-198.

[5] Amidon GE, Houghton ME. (Amidon1995 co-authors). 1995. The Effect of Moisture on the Mechanical and Powder Flow Properties of Microcrystalline Cellulose. Pharmaceutical Research 12:923-929.

[6] Argandona M, Nieto JJ, Iglesias-Guerra F, Calderon MI, Garcia-Estepa R, Vargas C. 2010. Interplay between Iron Homeostasis and the Osmotic Stress Response in the Halophilic Bacterium Chromohalobacter salexigens. Applied and Environmental Microbiology 76:3575-3589.

[7] Arku B, Mullane N, Fox E, Fanning SA, Jordan K. 2008. Enterobacter sakazakii survives spray drying. International Journal of Dairy Technology 61:102-108.

[8] Aziz MZ, Yaseen M, Naveed M, Shahid M. 2018. Alginate-Entrapped Enterobacter spp. MN17 Coated Diammonium Phosphate Improves Growth, Yield and Phosphorus Use Efficiency of Wheat. International Journal of Agriculture and Biology 20:2401-2407.

[9] Bacilio-Jimenez M, Aguilar-Flores S, Ventura-Zapata E, Perez-Campos E, Bouquelet S, Zenteno E. 2003. Chemical characterization of root exudates from rice (Oryza sativa) and their effects on the chemotactic response of endophytic bacteria. Plant and Soil 249:271-277.

[10] Bacon CW, White JF. 2016. Functions, mechanisms and regulation of endophytic and epiphytic microbial communities of plants. Symbiosis 68:87-98.

[11] Badyakina AO, Koryakina YA, Suzina NE, Nesmeyanova MA. 2003. Membrane phospholipid composition of Escherichia coli affects secretion of periplasmic alkaline phosphatase into the medium. Biochemistry-Moscow 68:752-759.

[12] Barbosa J, Borges S, Teixeira P. 2015. Influence of sub-lethal stresses on the survival of lactic acid bacteria after spray-drying in orange juice. Food Microbiology 52:77-83.

[13] Barra PJ, Inostroza NG, Acuna JJ, Mora ML, Crowley DE, Jorquera MA. 2016. Formulation of bacterial consortia from avocado (Persea americana Mill.) and their effect on growth, biomass and superoxide dismutase activity of wheat seedlings under salt stress. Applied Soil Ecology 102:80-91.

[14] Bashan Y, de-Bashan LE, Prabhu SR, Hernandez JP. 2014. Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1998-2013). Plant and Soil 378:1-33.

[15] Bashan Y, Hernandez JP, Leyva LA, Bacilio M. 2002. Alginate microbeads as inoculant carriers for plant growth-promoting bacteria. Biology and Fertility of Soils 35:359-368.

[16] Bashan Y, Trejo A, De-Bashan LE. 2011. Development of two culture media for mass cultivation of Azospirillum spp. and for production of inoculants to enhance plant growth. Biology and Fertility of Soils 47:963-969.

[17] Bazilah ABI, Sariah M, Abidin MAZ, Yasmeen S. 2011. Effect of Carrier and Temperature on the Viability of Burkholderia sp (UPMB3) and Pseudomonas sp (UPMP3) during Storage. International Journal of Agriculture and Biology 13:198-202.
[18] Beales N. 2004. Adaptation of microorganisms to cold temperatures, weak acid preservatives, low pH, and osmotic stress: A review. Comprehensive Reviews in Food Science and Food Safety 3:1-20.
[19] Becker M, Patz S, Becker Y, Berger B, Drungowski M, Bunk B, Overmann J, Spröer C, Reetz J, Tchuisseu Tchakounte GV, et al. 2018. Comparative Genomics Reveal a Flagellar System, a Type VI Secretion System and Plant Growth-Promoting Gene Clusters Unique to the Endophytic Bacterium Kosakonia radicincitans. Frontiers in Microbiology 9.
[20] Bejarano A, Sauer U, Preininger C. 2017. Design and development of a workflow for microbial spray formulations including decision criteria. Applied Microbiology and Biotechnology 101:7335-7346.
[21] Berg G. 2009. Plant-microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. Applied Microbiology and Biotechnology 84:11-18.
[22] Berger B, Baldermann S, Ruppel S. 2017. The plant growth-promoting bacterium Kosakonia radicincitans improves fruit yield and quality of Solanum lycopersicum. J Sci Food Agric.
[23] Berger B, Brock AK, Ruppel S. 2013. Nitrogen supply influences plant growth and transcriptional responses induced by Enterobacter radicincitans in Solanum lycopersicum. Plant and Soil 370:641-652.
[24] Berger B, Patz S, Ruppel S, Dietel K, Faetke S, Junge H, Becker M. 2018. Successful Formulation and Application of Plant Growth-Promoting Kosakonia radicincitans in Maize Cultivation. Biomed Research International.
[25] Berger B, Wiesner M, Brock AK, Schreiner M, Ruppel S. 2015. K-radicincitans, a beneficial bacteria that promotes radish growth under field conditions. Agronomy for Sustainable Development 35:1521-1528.
[26] Bergottini VM, Filippidou S, Junier T, Johnson S, Chain PS, Otegui MB, Zapata PD, Junier P. 2015. Genome Sequence of Kosakonia radicincitans Strain YD4, a Plant Growth-Promoting Rhizobacterium Isolated from Yerba Mate (Ilex paraguariensis St. Hill.). Genome Announc 3.
[27] Berninger T, Lopez OG, Bejarano A, Preininger C, Sessitsch A. 2018. Maintenance and assessment of cell viability in formulation of non-sporulating bacterial inoculants. Microbial Biotechnology 11:277-301.
[28] Berninger T, Mitter B, Preininger C. 2017. Zeolite-based, dry formulations for conservation and practical application of Paraburkholderia phytofirmans PsJN. Journal of Applied Microbiology 122:974-986.
[29] Bisutti IL, Hirt K, Stephan D. 2015. Influence of different growth conditions on the survival and the efficacy of freeze-dried Pseudomonas fluorescens strain Pf153. Biocontrol Science and Technology 25:1269-1284.
[30] Bonaterra A, Cabrefiga J, Camps J, Montesinos E. 2007. Increasing survival and efficacy of a bacterial biocontrol agent of fire blight of rosaceous plants by means of osmoadaptation. FEMS Microbiology Ecology 61:185-195.
[31] Boniolo FS, Rodrigues RC, Delatorre EO, da Silveira MM, Flores VMQ, Berbert-Molina MA. 2009. Glycine Betaine Enhances Growth of Nitrogen-Fixing Bacteria Gluconacetobacter diazotrophicus PAL5 Under Saline Stress Conditions. Current Microbiology 59:593-599.

[32] Borland S, Prigent-Combaret C, Wisniewski-Dye F. 2016. Bacterial hybrid histidine kinases in plant-bacteria interactions. Microbiology-Sgm 162:1715-1734.
[33] Boza Y, Barbin D, Scamparini ARP. 2004. Survival of Beijerinckia sp microencapsulated in carbohydrates by spray-drying. Journal of Microencapsulation 21:15-24.
[34] Brader G, Compant S, Mitter B, Trognitz F, Sessitsch A. 2014. Metabolic potential of endophytic bacteria. Current Opinion in Biotechnology 27:30-37.
[35] Brady C, Cleenwerck I, Venter S, Coutinho T, De Vos P. 2013a. Taxonomic evaluation of the genus Enterobacter based on multilocus sequence analysis (MLSA): Proposal to reclassify E. nimipressuralis and E. amnigenus into Lelliottia gen. nov as Lelliottia nimipressuralis comb. nov and Lelliottia amnigena comb. nov., respectively, E. gergoviae and E. pyrinus into Pluralibacter gen. nov as Pluralibacter gergoviae comb. nov and Pluralibacter pyrinus comb. nov., respectively, E. cowanii, E. radicincitans, E. oryzae and E. arachidis into Kosakonia gen. nov as Kosakonia cowanii comb. nov., Kosakonia radicincitans comb. nov., Kosakonia oryzae comb. nov and Kosakonia arachidis comb. nov., respectively, and E. turicensis, E. helveticus and E. pulveris into Cronobacter as Cronobacter zurichensis nom. nov., Cronobacter helveticus comb. nov and Cronobacter pulveris comb. nov., respectively, and emended description of the genera Enterobacter and Cronobacter. Systematic and Applied Microbiology 36:309-319.
[36] Brady C, Cleenwerck I, Venter S, Coutinho T, De Vos P. 2013b. Taxonomic evaluation of the genus Enterobacter based on multilocus sequence analysis (MLSA): Proposal to reclassify E. nimipressuralis and E. amnigenus into Lelliottia gen. nov. as Lelliottia nimipressuralis comb. nov. and Lelliottia amnigena comb. nov., . Systematic and Applied Microbiology 36:309-319.
[37] Brigido C, Singh S, Menendez E, Tavares MJ, Glick BR, Felix MD, Oliveira S, Carvalho M. 2019. Diversity and Functionality of Culturable Endophytic Bacterial Communities in Chickpea Plants. Plants-Basel 8.
[38] Brock AK, Berger B, Mewis I, Ruppel S. 2013. Impact of the PGPB Enterobacter radicincitans DSM 16656 on Growth, Glucosinolate Profile, and Immune Responses of Arabidopsis thaliana. Microbial Ecology 65:661-670.
[39] Brock AK, Berger B, Schreiner M, Ruppel S, Mewis I. 2018. Plant growth-promoting bacteria Kosakonia radicincitans mediate anti-herbivore defense in Arabidopsis thaliana. Planta 248:1383-1392.
[40] Broeckx G, Vandenheuvel D, Claes IJJ, Lebeer S, Kiekens F. 2016. Drying techniques of probiotic bacteria as an important step towards the development of novel pharmabiotics. International Journal of Pharmaceutics 505:303-318.
[41] Brown AD. 1978. Compatible solutes and extreme water stress in eukaryotic micro-organisms. Adv Microb Physiol 17:181-242.
[42] Buchenauer A, Hofmann MC, Funke M, Buchs J, Mokwa W, Schnakenberg U. 2009. Micro-bioreactors for fed-batch fermentations with integrated online monitoring and microfluidic devices. Biosensors & Bioelectronics 24:1411-1416.
[43] Bull H, Murray PG, Thomas D, Fraser AM, Nelson PN. 2002. Acid phosphatases. Journal of Clinical Pathology-Molecular Pathology 55:65-72.
[44] Bursy J, Kuhlmann AU, Pittelkow M, Hartmann H, Jebbar M, Pierik AJ, Bremer E. 2008. Synthesis and Uptake of the Compatible Solutes Ectoine and 5-Hydroxyectoine by Streptomyces coelicolor A3(2) in Response to Salt and Heat Stresses. Applied and Environmental Microbiology 74:7286-7296.
[45] Cabrefiga J, Frances J, Montesinos E, Bonaterra A. 2014. Improvement of a dry formulation of Pseudomonas fluorescens EPS62e for fire blight disease biocontrol by combination of

culture osmoadaptation with a freeze-drying lyoprotectant. Journal of Applied Microbiology 117:1122-1131.
[46] Cabrefiga J, Frances J, Montesinos E, Bonaterra A. 2011. Improvement of Fitness and Efficacy of a Fire Blight Biocontrol Agent via Nutritional Enhancement Combined with Osmoadaptation. Applied and Environmental Microbiology 77:3174-3181.
[47] Calvo P, Nelson L, Kloepper JW. 2014. Agricultural uses of plant biostimulants. Plant and Soil 383:3-41.
[48] Card SD, Hume DE, Roodi D, McGill CR, Millner JP, Johnson RD. 2015. Beneficial endophytic microorganisms of Brassica - A review. Biological Control 90:102-112.
[49] Carvalho AS, Silva J, Ho P, Teixeira P, Malcata FX, Gibbs P. 2002. Survival of freeze-dried Lactobacillus plantarum and Lactobacillus rhamnosus during storage in the presence of protectants. Biotechnology Letters 24:1587-1591.
[50] Cassidy MB, Lee H, Trevors JT. 1996. Environmental applications of immobilized microbial cells: A review. Journal of Industrial Microbiology 16:79-101.
[51] Compant S, Clement C, Sessitsch A. 2010. Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. Soil Biology & Biochemistry 42:669-678.
[52] Cornelis P, Wei Q, Andrews SC, Vinckx T. 2011. Iron homeostasis and management of oxidative stress response in bacteria. Metallomics 3:540-549.
[53] Costa E, Usall J, Teixido N, Garcia N, Vinas I. 2000. Effect of protective agents, rehydration media and initial cell concentration on viability of Pantoea agglomerans strain CPA-2 subjected to freeze-drying. J Appl Microbiol 89:793-800.
[54] Croes S, Weyens N, Janssen J, Vercampt H, Colpaert JV, Carleer R, Vangronsveld J. 2013. Bacterial communities associated with Brassica napusL. grown on trace element-contaminated and non-contaminated fields: a genotypic and phenotypic comparison. Microbial Biotechnology 6:371-384.
[55] Crowe JH, Crowe LM, Oliver AE, Tsvetkova N, Wolkers W, Tablin F. 2001. The trehalose myth revisited: introduction to a symposium on stabilization of cells in the dry state. Cryobiology 43:89-105.
[56] Cruz B, Schoenwandt D, Gomez M, Becker M, Patel A, Ruppel S. 2019. Salt stress and hydroxyectoine enhance phosphate solubilisation and plant colonisation capacity of Kosakonia radicincitans. Journal of Advanced Research 19: 91-97.
[57] Csonka LN. 1989. Physiological and genetic responses of bacteria to osmotic stress. Microbiological reviews 53:121-147.
[58] Czech L, Hermann L, Stoveken N, Richter AA, Hoppner A, Smits SHJ, Heider J, Bremer E. 2018. Role of the Extremolytes Ectoine and Hydroxyectoine as Stress Protectants and Nutrients: Genetics, Phylogenomics, Biochemistry, and Structural Analysis. Genes 9.
[59] Chan ES, Lee BB, Ravindra P, Poncelet D. 2009. Prediction models for shape and size of ca-alginate macrobeads produced through extrusion-dripping method. Journal of Colloid and Interface Science 338:63-72.
[60] Chan ES, Wong SL, Lee PP, Lee JS, Ti TB, Zhang ZB, Poncelet D, Ravindra P, Phan SH, Yim ZH. 2011. Effects of starch filler on the physical properties of lyophilized calcium-alginate beads and the viability of encapsulated cells. Carbohydrate Polymers 83:225-232.
[61] Chan SY, Choo WS, Young DJ, Loh XJ. 2017. Pectin as a rheology modifier: Origin, structure, commercial production and rheology. Carbohydrate Polymers 161:118-139.
[62] Chandrangsu P, Rensing C, Helmann JD. 2017. Metal homeostasis and resistance in bacteria. Nature Reviews Microbiology 15:338-350.

[63] Cheng H, Li LL, Hua J, Yuan HH, Cheng SY. 2015. A Preliminary Preparation of Endophytic Bacteria CE3 Wettable Powder for Biological Control of Postharvest Diseases. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 43:159-164.
[64] Choi HJ, Song JM, Bondy BJ, Compans RW, Kang SM, Prausnitz MR. 2015. Effect of Osmotic Pressure on the Stability of Whole Inactivated Influenza Vaccine for Coating on Microneedles. PLoS One 10.
[65] da Costa MS, Santos H, Galinski EA. 1998. An overview of the role and diversity of compatible solutes in Bacteria and Archaea. Adv Biochem Eng Biotechnol 61:117-153.
[66] da Silva MF, Antonio CD, de Oliveira PJ, Xavier GR, Rumjanek NG, Soares LHD, Reis VM. 2012. Survival of endophytic bacteria in polymer-based inoculants and efficiency of their application to sugarcane. Plant and Soil 356:231-243.
[67] da Silva SF, Olivares FL, Canellas LP. 2017. The biostimulant manufactured using diazotrophic endophytic bacteria and humates is effective to increase sugarcane yield. Chemical and Biological Technologies in Agriculture 4:24.
[68] de-Bashan LE, Antoun H, Bashan Y. 2008. Involvement of indole-3-acetic acid produced by the growth-promoting bacterium Azospirillum spp. in promoting growth of Chlorella vulgaris. Journal of Phycology 44:938-947.
[69] De Gregorio PR, Michavila G, Muller LR, Borges CD, Pomares MF, de Sa ELS, Pereira C, Vincent PA. 2017. Beneficial rhizobacteria immobilized in nanofibers for potential application as soybean seed bioinoculants. PLoS One 12:22.
[70] de Werra P, Pechy-Tarr M, Keel C, Maurhofer M. 2009. Role of Gluconic Acid Production in the Regulation of Biocontrol Traits of Pseudomonas fluorescens CHA0. Applied and Environmental Microbiology 75:4162-4174.
[71] Deboy RT, Mongodin EF, Fouts DE, Tailford LE, Khouri H, Emerson JB, Mohamoud Y, Watkins K, Henrissat B, Gilbert HJ, et al. 2008. Insights into plant cell wall degradation from the genome sequence of the soil bacterium Cellvibrio japonicus. Journal of Bacteriology 190:5455-5463.
[72] del Moral A, Severin J, Ramos-Cormenzana A, Trüper HG, Galinski EA. 1994. Compatible solutes in new moderately halophilic isolates. Fems Microbiology Letters 122:165-172.
[73] Dianawati D, Mishra V, Shah NP. 2012. Role of Calcium Alginate and Mannitol in Protecting Bifidobacterium. Applied and Environmental Microbiology 78:6914-6921.
[74] Doty SL. 2008. Enhancing phytoremediation through the use of transgenics and endophytes. New Phytologist 179:318-333.
[75] Du JL, Li ML, Yuan ZF, Guo MC, Song JZ, Xie XZ, Chen YL. 2016. A decision analysis model for KEGG pathway analysis. Bmc Bioinformatics 17.
[76] Dulla G, Lindow SE. 2008. Quorum size of Pseudomonas syringae is small and dictated by water availability on the leaf surface. Proceedings of the National Academy of Sciences of the United States of America 105:3082-3087.
[77] Dutta B, Schneider RW, Robertson CL, Walcott RR. 2016. Embryo Localization Enhances the Survival of Acidovorax citrulli in Watermelon Seeds. Phytopathology 106:330-338.
[78] Eevers N, Gielen M, Sanchez-Lopez A, Jaspers S, White JC, Vangronsveld J, Weyens N. 2015. Optimization of isolation and cultivation of bacterial endophytes through addition of plant extract to nutrient media. Microbial Biotechnology 8:707-715.
[79] Egamberdieva D, Wirth SJ, Shurigin VV, Hashem A, Abd Allah EF. 2017. Endophytic Bacteria Improve Plant Growth, Symbiotic Performance of Chickpea (Cicer arietinum L.) and Induce Suppression of Root Rot Caused by Fusarium solani under Salt Stress. Frontiers in Microbiology 8:13.

[80] Ekandjo LK, Ruppel S, Remus R, Witzel K, Patz S, Becker Y. 2018. Site-directed mutagenesis to deactivate two nitrogenase isozymes of Kosakonia radicincitans DSM16656(T). Canadian Journal of Microbiology 64:97-106.
[81] Estrada GA, Baldani VLD, de Oliveira DM, Urquiaga S, Baldani JI. 2013. Selection of phosphate-solubilizing diazotrophic Herbaspirillum and Burkholderia strains and their effect on rice crop yield and nutrient uptake. Plant and Soil 369:115-129.
[82] Fageria NK, Baligar VC, Li YC. 2008. The role of nutrient efficient plants in improving crop yields in the twenty first century. Journal of Plant Nutrition 31:1121-1157.
[83] Farres M, Platikanov S, Tsakovski S, Tauler R. 2015. Comparison of the variable importance in projection (VIP) and of the selectivity ratio (SR) methods for variable selection and interpretation. Journal of Chemometrics 29:528-536.
[84] Farrow JM, Wells G, Pesci EC. 2018. Desiccation tolerance in Acinetobacter baumannii is mediated by the two-component response regulator BfmR. PLoS One 13:25.
[85] Fernandes PI, Rohr TG, de Oliveira PJ, Xavier GR, Rumjanek NG. 2009. Polymers as carriers for rhizobial inoculant formulations. Pesquisa Agropecuaria Brasileira 44:1184-1190.
[86] Fisher RA. 1992. The Arrangement of Field Experiments. In: Kotz S, Johnson NL, editors. Breakthroughs in Statistics: Methodology and Distribution. New York, NY: Springer New York. p. 82-91.
[87] Fu N, Chen XD. 2011. Towards a maximal cell survival in convective thermal drying processes. Food Research International 44:1127-1149.
[88] Funke M, Buchenauer A, Schnakenberg U, Mokwa W, Diederichs S, Mertens A, Muller C, Kensy F, Buchs J. 2010. Microfluidic BioLector-Microfluidic Bioprocess Control in Microtiter Plates. Biotechnology and Bioengineering 107:497-505.
[89] Funke M, Diederichs S, Kensy F, Muller C, Buchs J. 2009. The Baffled Microtiter Plate: Increased Oxygen Transfer and Improved Online Monitoring in Small Scale Fermentations. Biotechnology and Bioengineering 103:1118-1128.
[90] Gaiero JR, McCall CA, Thompson KA, Day NJ, Best AS, Dunfield KE. 2013. inside the root microbiome: bacterial root endophytes and plant growth promotion. american journal of botany 100:1738-1750.
[91] Gao L, Sun MH, Liu XZ, Che YS. 2007. Effects of carbon concentration and carbon to nitrogen ratio on the growth and sporulation of several biocontrol fungi. Mycological Research 111:87-92.
[92] Garcia-Fraile P, Menendez E, Rivas R. 2015. Role of bacterial biofertilizers in agriculture and forestry. Aims Bioengineering 2:183-205.
[93] Garcia AH. 2011. Anhydrobiosis in bacteria: from physiology to applications. J Biosci 36:939-950.
[94] Garima G, Nath JP. 2015. Screening of potential PGPR candidates as future biofertilizers-A strategic approach from lab to field. Research Journal of Biotechnology 10:48-62.
[95] Ghandi A, Powell IB, Howes T, Chen XD, Adhikari B. 2012. Effect of shear rate and oxygen stresses on the survival of Lactococcus lactis during the atomization and drying stages of spray drying: A laboratory and pilot scale study. Journal of Food Engineering 113:194-200.
[96] Glick BR. 2012. Plant Growth-Promoting Bacteria: Mechanisms and Applications. Scientifica 2012:15.
[97] Glick BR, Bashan Y. 1997. Genetic manipulation of plant growth-promoting bacteria to enhance biocontrol of phytopathogens. Biotechnol Adv 15:353-378.
[98] Gottel NR, Castro HF, Kerley M, Yang ZM, Pelletier DA, Podar M, Karpinets T, Uberbacher E, Tuskan GA, Vilgalys R, et al. 2011. Distinct Microbial Communities within the

Endosphere and Rhizosphere of Populus deltoides Roots across Contrasting Soil Types. Applied and Environmental Microbiology 77:5934-5944.

[99] Grammann K, Volke A, Kunte HJ. 2002. New type of osmoregulated solute transporter identified in halophilic members of the Bacteria domain: TRAP transporter TeaABC mediates uptake of ectoine and hydroxyectoine in Halomonas elongata DSM 2581(T). Journal of Bacteriology 184:3078-3085.

[100] Grobelak A, Kokot P, Swiatek J, Jaskulak M, Rorat A. 2018. Bacterial ACC Deaminase Activity in Promoting Plant Growth on Areas Contaminated with Heavy Metals. Journal of Ecological Engineering 19:150-157.

[101] Gundogdu TK, Deniz I, Caliskan G, Sahin ES, Azbar N. 2016. Experimental design methods for bioengineering applications. Critical Reviews in Biotechnology 36:368-388.

[102] Gupta RS. 2002. Phylogeny of Bacteria: Are we now close to understanding it? Asm News 68:284-+.

[103] Gyaneshwar P, Kumar GN, Parekh LJ, Poole PS. 2002. Role of soil microorganisms in improving P nutrition of plants. Plant and Soil 245:83-93.

[104] Haghshenas B, Abdullah N, Nami Y, Radiah D, Rosli R, Khosroushahi AY. 2015. Microencapsulation of probiotic bacteria Lactobacillus plantarum 15HN using alginate-psyllium-fenugreek polymeric blends. Journal of Applied Microbiology 118:1048-1057.

[105] Hahn MB, Solomun T, Wellhausen R, Hermann S, Seitz H, Meyer S, Kunte HJ, Zeman J, Uhlig F, Smiatek J, et al. 2015. Influence of the Compatible Solute Ectoine on the Local Water Structure: Implications for the Binding of the Protein G5P to DNA. Journal of Physical Chemistry B 119:15212-15220.

[106] Halo BA, Khan AL, Waqas M, Al-Harrasi A, Hussain J, Ali L, Adnan M, Lee IJ. 2015. Endophytic bacteria (Sphingomonas sp LK11) and gibberellin can improve Solanum lycopersicum growth and oxidative stress under salinity. Journal of Plant Interactions 10:117-125.

[107] Hallmann J, Quadt-Hallmann A, Mahaffee WF, Kloepper JW. 1997. Bacterial endophytes in agricultural crops. Canadian Journal of Microbiology 43:895-914.

[108] Hallmann J, QuadtHallmann A, Mahaffee WF, Kloepper JW. 1997. Bacterial endophytes in agricultural crops. Canadian Journal of Microbiology 43:895-914.

[109] Hardoim PR, Hardoim CCP, van Overbeek LS, van Elsas JD. 2012. Dynamics of Seed-Borne Rice Endophytes on Early Plant Growth Stages. PLoS One 7.

[110] Harish S, Kavino M, Kumar N, Saravanakumar D, Soorianathasundaram K, Samiyappan R. 2008. Biohardening with Plant Growth Promoting Rhizosphere and Endophytic bacteria induces systemic resistance against Banana bunchy top virus. Applied Soil Ecology 39:187-200.

[111] Harishchandra RK, Wulff S, Lentzen G, Neuhaus T, Galla HJ. 2010. The effect of compatible solute ectoines on the structural organization of lipid monolayer and bilayer membranes. Biophys Chem 150:37-46.

[112] Hassan SE. 2017. Plant growth-promoting activities for bacterial and fungal endophytes isolated from medicinal plant of Teucrium polium L. Journal of Advanced Research 8:687-695.

[113] Hayat R, Ali S, Amara U, Khalid R, Ahmed I. 2010. Soil beneficial bacteria and their role in plant growth promotion: a review. Annals of Microbiology 60:579-598.

[114] He GQ, Wu CD, Huang J, Zhou RQ. 2017. Effect of Exogenous Proline on Metabolic Response of Tetragenococcus halophilus under Salt Stress. Journal of Microbiology and Biotechnology 27:1681-1691.

[115] Held C, Neuhaus T, Sadowski G. 2010. Compatible solutes: Thermodynamic properties and biological impact of ectoines and prolines. Biophysical Chemistry 152:28-39.

[116] Hernandez-Hernandez L, Coutino-Megchun JT, Rincon-Molina CI, Ruiz-Valdiviezo VM, Culebro-Ricaldi JM, Cruz-Rodriguez RI, Palomeque-Dominguez HH, Rincon-Rosales R. 2018. Endophytic bacteria from root nodules of Ormosia macrocalyx with potential as plant growth promoters and antifungal activity. Journal of Environmental Biology 39:997-1005.
[117] Herrmann L, Lesueur D. 2013. Challenges of formulation and quality of biofertilizers for successful inoculation. Applied Microbiology and Biotechnology 97:8859-8873.
[118] Hu XJ, Roberts DP, Maul JE, Emche SE, Liao X, Guo XL, Liu YY, McKenna LF, Buyer JS, Liu SY. 2011. Formulations of the endophytic bacterium Bacillus subtilis Tu-100 suppress Sclerotinia sclerotiorum on oilseed rape and improve plant vigor in field trials conducted at separate locations. Canadian Journal of Microbiology 57:539-546.
[119] Huber R, Ritter D, Hering T, Hillmer AK, Kensy F, Muller C, Wang L, Buchs J. 2009. Robo-Lector - a novel platform for automated high-throughput cultivations in microtiter plates with high information content. Microbial Cell Factories 8.
[120] Humbert P, Przyklenk M, Vemmer M, Patel AV. 2017. Calcium gluconate as cross-linker improves survival and shelf life of encapsulated and dried Metarhizium brunneum and Saccharomyces cerevisiae for the application as biological control agents. Journal of Microencapsulation 34:47-56.
[121] Humbert P, Przyklenk M, Vemmer M, Schumann M, Vidal S, Patel AV. 2017. Technical scale production of encapsulated Saccharomyces cerevisiae and Metarhizium brunneum attractive to wireworms. Biocontrol Science and Technology 27:1049-1070.
[122] Humbert P, Vemmer M, Giampa M, Bednarz H, Niehaus K, Patel AV. 2017. Co-encapsulation of amyloglucosidase with starch and Saccharomyces cerevisiae as basis for a long-lasting CO2 release. World Journal of Microbiology & Biotechnology 33.
[123] Huynh UTD, Assifaoui A, Chambin O. 2017. Pellets based on polyuronates: Relationship between gelation and release properties. Journal of Food Engineering 199:27-35.
[124] James EK, Olivares FL, de Oliveira ALM, dos Reis FB, da Silva LG, Reis VM. 2001. Further observations on the interaction between sugar cane and Gluconacetobacter diazotrophicus under laboratory and greenhouse conditions. Journal of Experimental Botany 52:747-760.
[125] Janssen PH, Yates PS, Grinton BE, Taylor PM, Sait M. 2002. Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions Acidobacteria, Actinobacteria, Proteobacteria, and Verrucomicrobia. Applied and Environmental Microbiology 68:2391-2396.
[126] Jensen JB, Ampomah OY, Darrah R, Peters NK, Bhuvaneswari TV. 2005. Role of trehalose transport and utilization in Sinorhizobium meliloti - Alfalfa interactions. Molecular Plant-Microbe Interactions 18:694-702.
[127] Jiao LX, Ran JJ, Xu XX, Wang JJ. 2015. Heat, acid and cold stresses enhance the expression of DnaK gene in Alicyclobacillus acidoterrestris. Food Research International 67:183-192.
[128] Joe MM, Devaraj S, Benson A, Sa TM. 2016. Isolation of phosphate solubilizing endophytic bacteria from Phyllanthus amarus Schum & Thonn: Evaluation of plant growth promotion and antioxidant activity under salt stress. Journal of Applied Research on Medicinal and Aromatic Plants 3:71-77.
[129] Joe MM, Karthikeyan B, Chauhan PS, Shagol C, Islam MR, Deiveekasundaram M, Sa T. 2012. Survival of Azospirillum brasilense flocculated cells in alginate and its inoculation effect on growth and yield of maize under water deficit conditions. European Journal of Soil Biology 50:198-206.
[130] Joe MM, Saravanan VS, Islam MR, Sa T. 2014. Development of alginate-based aggregate inoculants of Methylobacterium sp and Azospirillum brasilense tested under in vitro conditions to promote plant growth. Journal of Applied Microbiology 116:408-423.

[131] Joghee NN, Jayaraman G. 2014. Metabolomic characterization of halophilic bacterial isolates reveals strains synthesizing rare diaminoacids under salt stress. Biochimie 102:102-111.
[132] John RP, Tyagi RD, Brar SK, Surampalli RY, Prevost D. 2011. Bio-encapsulation of microbial cells for targeted agricultural delivery. Critical Reviews in Biotechnology 31:211-226.
[133] Kamagata Y, Tamaki H. 2005. Cultivation of Uncultured Fastidious Microbes. Microbes and Environments 20:85-91.
[134] Kamnev AA, Tugarova AV, Antonyuk LP, Tarantilis PA, Polissiou MG, Gardiner PHE. 2005. Effects of heavy metals on plant-associated rhizobacteria: Comparison of endophytic and non-endophytic strains of Azospirillum brasilense. Journal of Trace Elements in Medicine and Biology 19:91-95.
[135] Kampfer P, Ruppel S, Remus R. 2005a. Enterobacter radicincitans sp nov., a plant growth promoting species of the family Enterobacteriaceae. Systematic and Applied Microbiology 28:213-221.
[136] Kampfer P, Ruppel S, Remus R. 2005b. Enterobacter radicincitans sp. nov., a plant growth promoting species of the family Enterobacteriaceae. Syst Appl Microbiol 28:213-221.
[137] Kandel SL, Joubert PM, Doty SL. 2017. Bacterial Endophyte Colonization and Distribution within Plants. Microorganisms 5.
[138] Kaul S, Sharma T, K Dhar M. 2016. "Omics" Tools for Better Understanding the Plant-Endophyte Interactions. Frontiers in Plant Science 7:955-955.
[139] Kempf B, Bremer E. 1998. Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments. Arch Microbiol 170:319-330.
[140] Kennedy M, Krouse D. 1999. Strategies for improving fermentation medium performance: a review. Journal of Industrial Microbiology and Biotechnology 23:456-475.
[141] Kensy F, Zang E, Faulhammer C, Tan RK, Buchs J. 2009. Validation of a high-throughput fermentation system based on online monitoring of biomass and fluorescence in continuously shaken microtiter plates. Microbial Cell Factories 8.
[142] Khaksar G, Treesubsuntorn C, Thiravetyan P. 2017. Euphorbia milii-native bacteria interactions under airborne formaldehyde stress: Effect of epiphyte and endophyte inoculation in relation to IAA, ethylene and ROS levels. Plant Physiol Biochem 111:284-294.
[143] Khalid A, Arshad M, Zahir ZA. 2004. Screening plant growth-promoting rhizobacteria for improving growth and yield of wheat. J Appl Microbiol 96:473-480.
[144] Knapp S, Ladenstein R, Galinski EA. 1999. Extrinsic protein stabilization by the naturally occurring osmolytes beta-hydroxyectoine and betaine. Extremophiles 3:191-198.
[145] Kong X, Liu Y, Gou X, Zhang H, Wang X, Zhang J. 2001. Directed Evolution of Operon of Trehalose-6-phosphate Synthase/Phosphatase from Escherichia coli. Biochemical and Biophysical Research Communications 280:396-400.
[146] Kosaraju SL. 2005. Colon targeted delivery systems: Review of polysaccharides for encapsulation and delivery. Critical Reviews in Food Science and Nutrition 45:251-258.
[147] Kostka-Rick R, Manning WJ. 1993. Radish (Raphanus sativus L.): a model for studying plant responses to air pollutants and other environmental stresses. Environ Pollut 82:107-138.
[148] Krell V, Jakobs-Schoenwandt D, Persicke M, Patel AV. 2018. Endogenous arabitol and mannitol improve shelf life of encapsulated Metarhizium brunneum. World Journal of Microbiology & Biotechnology 34.
[149] Krell V, Jakobs-Schoenwandt D, Vidal S, Patel AV. 2018. Cellulase enhances endophytism of encapsulated Metarhizium brunneum in potato plants. Fungal Biology.

[150] Krey T, Caus M, Baum C, Ruppel S, Eichler-Lobermann B. 2011. Interactive effects of plant growth-promoting rhizobacteria and organic fertilization on P nutrition of Zea mays L. and Brassica napus L. Journal of Plant Nutrition and Soil Science 174:602-613.
[151] Kunin CM, Tong HH, Maher WE. 1993. Naturally-occurring, osmo-remedial variants of Escherichia coli. J Med Microbiol 38:216-221.
[152] Kunte HJ, Galinski EA, Truper HG. 1993. a modified fmoc-method for the detection of amino acid-type osmolytes and tetrahydropyrimidines (ectoines). Journal of Microbiological Methods 17:129-136.
[153] Lacava PT, Azevedo JL. 2013. Endophytic Bacteria: A Biotechnological Potential in Agrobiology System. In: Maheshwari DK, Saraf M, Aeron A, editors. Bacteria in Agrobiology: Crop Productivity. Berlin, Heidelberg: Springer Berlin Heidelberg. p. 1-44.
[154] Lagendijk EL, Validov S, Lamers GEM, de Weert S, Bloemberg GV. 2010. Genetic tools for tagging Gram-negative bacteria with mCherry for visualization in vitro and in natural habitats, biofilm and pathogenicity studies. Fems Microbiology Letters 305:81-90.
[155] Lakshmanan R, Shakila RJ, Jeyasekaran G. 2002. Changes in the halophilic amine forming bacterial flora during salt-drying of sardines (Sardinella gibbosa). Food Research International 35:541-546.
[156] Lally RD, Galbally P, Moreira AS, Spink J, Ryan D, Germaine KJ, Dowling DN. 2017. Application of Endophytic Pseudomonas fluorescens and a Bacterial Consortium to Brassica napus Can Increase Plant Height and Biomass under Greenhouse and Field Conditions. Frontiers in Plant Science 8.
[157] Lara C, Sanes SC, Oviedo LE. 2013. Impact of native phosphate solubilizing bacteria on the growth and development of radish (Raphanus sativus L.) plants. Biotecnología Aplicada 30:276-279.
[158] Le Cocq K, Gurr SJ, Hirsch PR, Mauchline TH. 2017. Exploitation of endophytes for sustainable agricultural intensification. Molecular Plant Pathology 18:469-473.
[159] Lim C-H, Ozkanca R, Flint KP. 1996. The effects of osmotic stress on survival and alkaline phosphatase activity of Aeromonas hydrophila. Fems Microbiology Letters 137:19-24.
[160] Lin T-F, Huang H-I, Shen F-T, Young C-C. 2006. The protons of gluconic acid are the major factor responsible for the dissolution of tricalcium phosphate by Burkholderia cepacia CC-Al74. Bioresource Technology 97:957-960.
[161] Lindow SE, Leveau JHJ. 2002. Phyllosphere microbiology. Current Opinion in Biotechnology 13:238-243.
[162] Lippert K, Galinski EA. (Lippert1992 co-authors). 1992. Enzyme stabilization be ectoine-type compatible solutes: protection against heating, freezing and drying. Applied Microbiology and Biotechnology 37:61-65.
[163] Liu C, Dunaway-Mariano D, Mariano PS. 2017. Rational design of reversible inhibitors for trehalose 6-phosphate phosphatases. European Journal of Medicinal Chemistry 128:274-286.
[164] Liu LS, Fishman ML, Kost J, Hicks KB. 2003. Pectin-based systems for colon-specific drug delivery via oral route. Biomaterials 24:3333-3343.
[165] Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. Methods 25:402-408.
[166] Lodewyckx C, Vangronsveld J, Porteous F, Moore ERB, Taghavi S, Mezgeay M, van der Lelie D. 2002. Endophytic bacteria and their potential applications. Critical Reviews in Plant Sciences 21:583-606.
[167] Lohse R, Jakobs-Schonwandt D, Vidal S, Patel AV. 2015. Evaluation of new fermentation and formulation strategies for a high endophytic establishment of Beauveria bassiana in oilseed rape plants. Biological Control 88:26-36.

[168] Loiret FG, Grimm B, Hajirezaei MR, Kleiner D, Ortega E. 2009. Inoculation of sugarcane with Pantoea sp increases amino acid contents in shoot tissues; serine, alanine, glutamine and asparagine permit concomitantly ammonium excretion and nitrogenase activity of the bacterium. Journal of Plant Physiology 166:1152-1161.
[169] Lugtenberg BJJ, Dekkers L, Bloemberg GV. 2001. Molecular determinants of rhizosphere colonization by Pseudomonas. Annual Review of Phytopathology 39:461.
[170] Mahanty T, Bhattacharjee S, Goswami M, Bhattacharyya P, Das B, Ghosh A, Tribedi P. 2017. Biofertilizers: a potential approach for sustainable agriculture development. Environmental Science and Pollution Research 24:3315-3335.
[171] Mahlangu SG, Serepa-Diamini MH. 2018. First report of bacterial endophytes from the leaves of Pellaea calomelanos in South Africa. South African Journal of Science 114:55-63.
[172] Malek W, Wdowiak-Wrobel S, Targonska M, Kalita M, Gnat S. 2012. Osmoadaptation and potassium transport systems in Gram-negative bacteria. Postepy Mikrobiologii 51:93-98.
[173] Malusa E, Sas-Paszt L, Ciesielska J. 2012. Technologies for Beneficial Microorganisms Inocula Used as Biofertilizers. Scientific World Journal:12.
[174] Manzanera M, de Castro AG, Tondervik A, Rayner-Brandes M, Strom AR, Tunnacliffe A. 2002. Hydroxyectoine is superior to trehalose for anhydrobiotic engineering of Pseudomonas putida KT2440. Applied and Environmental Microbiology 68:4328-4333.
[175] Manzanera M, Vilchez S, Tunnacliffe A. 2004. High survival and stability rates of Escherichia coli dried in hydroxyectoine. Fems Microbiology Letters 233:347-352.
[176] Martos GI, Minahk CJ, de Valdez GF, Morero R. 2007. Effects of protective agents on membrane fluidity of freeze-dried Lactobacillus delbrueckii ssp bulgaricus. Letters in Applied Microbiology 45:282-288.
[177] McIntyre HJ, Davies H, Hore TA, Miller SH, Dufour JP, Ronson CW. 2007. Trehalose biosynthesis in Rhizobium leguminosarum bv. trifolii and its role in desiccation tolerance. Applied and Environmental Microbiology 73:3984-3992.
[178] Mei C, Chretien R, Yan S, Carey J, He Y, Lowman S. 2017. Characterization of Bacterial Endophytes and Their Applications. Phytopathology 107:16-16.
[179] Meza B, de-Bashan LE, Bashan Y. 2015. Involvement of indole-3-acetic acid produced by Azospirillum brasilense in accumulating intracellular ammonium in Chlorella vulgaris. Research in Microbiology 166:72-83.
[180] Minaxi JS. 2011. Efficacy of rhizobacterial strains encapsulated in nontoxic biodegradable gel matrices to promote growth and yield of wheat plants. Applied Soil Ecology 48:301-308.
[181] Mitsui Y, Shimomura M, Komatsu K, Namiki N, Shibata-Hatta M, Imai M, Katayose Y, Mukai Y, Kanamori H, Kurita K, et al. 2015. The radish genome and comprehensive gene expression profile of tuberous root formation and development. Scientific Reports 5.
[182] Mitter B, Petric A, Shin MW, Chain PS, Hauberg-Lotte L, Reinhold-Hurek B, Nowak J, Sessitsch A. 2013. Comparative genome analysis of Burkholderia phytofirmans PsJN reveals a wide spectrum of endophytic lifestyles based on interaction strategies with host plants. Front Plant Sci 4:120.
[183] Miyamoto-Shinohara Y, Sukenobe J, Imaizumi T, Nakahara T. 2008. Survival of freeze-dried bacteria. J Gen Appl Microbiol 54:9-24.
[184] Mocali S, Bertelli E, Di Cello F, Mengoni A, Sfalanga A, Viliani F, Caciotti A, Tegli S, Surico G, Fani R. 2003. Fluctuation of bacteria isolated from elm tissues during different seasons and from different plant organs. Research in Microbiology 154:105-114.
[185] Moreno-Galván A, Rojas-Tapias DF, Bonilla R. 2012. Development and evaluation of an alternative culture medium for mass cultivation of Azospirillum brasilense C16 using sequential statistical designs. Ciencia y Tecnología Agropecuaria 13:201-206.

[186] Mosier AC, Justice NB, Bowen BP, Baran R, Thomas BC, Northen TR, Banfield JF. 2013. Metabolites Associated with Adaptation of Microorganisms to an Acidophilic, Metal-Rich Environment Identified by Stable-Isotope-Enabled Metabolomics. Mbio 4:8.
[187] Munarin F, Tanzi MC, Petrini P. 2012. Advances in biomedical applications of pectin gels. International journal of biological macromolecules 51:681-689.
[188] Murphy J, Riley JP. 1962. A modified single solution method for the determination of phosphate in natural waters. Analytica Chimica Acta 27:31-36.
[189] Murphy JF, Reddy MS, Ryu CM, Kloepper JW, Li RH. 2003. Rhizobacteria-mediated growth promotion of tomato leads to protection against Cucumber mosaic virus. Phytopathology 93:1301-1307.
[190] Nagata S, Sasaki H, Oshima A, Takeda S, Hashimoto Y, Ishida A. 2005. Effect of proline and K+ on the stimulation of cellular activities in Escherichia coli K-12 under high salinity. Bioscience Biotechnology and Biochemistry 69:740-746.
[191] Naghili H, Tajik H, Mardani K, Razavi Rouhani SM, Ehsani A, Zare P. 2013. Validation of drop plate technique for bacterial enumeration by parametric and nonparametric tests. Vet Res Forum 4:179-183.
[192] Nocker A, Fernandez PS, Montijn R, Schuren F. 2012. Effect of air drying on bacterial viability: A multiparameter viability assessment. J Microbiol Methods 90:86-95.
[193] Nour EH, Hamza MA, Fayez M, Monib M, Ruppel S, Hegazi NA. 2012. The crude plant juices of desert plants as appropriate culture media for the cultivation of rhizospheric microorganisms. Journal of Advanced Research 3:35-43.
[194] Ono H, Okuda M, Tongpim S, Imai K, Shinmyo A, Sakuda S, Kaneko Y, Murooka Y, Takano M. 1998. Accumulation of compatible solutes, ectoine and hydroxyectoine, in a moderate halophile, Halomonas elongata KS3 isolated from dry salty land in Thailand. Journal of Fermentation and Bioengineering 85:362-368.
[195] Oren A. 2013. Life at high salt concentrations, intracellular KCl concentrations, and acidic proteomes. Frontiers in Microbiology 4.
[196] Oren A. 2011. Thermodynamic limits to microbial life at high salt concentrations. Environmental Microbiology 13:1908-1923.
[197] Ortiz-Galeana MA, Hernandez-Salmeron JE, Valenzuela-Aragon B, de los Santos-Viilalobos S, Rocha-Granados MD, Santoyo G. 2018. diversity of cultivable endophytic bacteria associated with blueberry plants (vaccinium corymbosum l.) cv. biloxi with plant growth-promoting traits. chilean journal of agricultural & animal sciences 34:140-151.
[198] Ortuno N, Cordoba M, Claros M, Castillo J. 2018. Evaluation of native potatoes in double bacteria and the development of a biofertilizer. Revista Latinoamericana De La Papa 22:12-37.
[199] Oteino N, Lally RD, Kiwanuka S, Lloyd A, Ryan D, Germaine KJ, Dowling DN. 2015. Plant growth promotion induced by phosphate solubilizing endophytic Pseudomonas isolates. Frontiers in Microbiology 6.
[200] Padder S, Dar GH, Bhat A Z, Verma K, Wani A B. 2017. Morphological metabolic and biochemical characterization of bacterial root endophytes associated withh brown sarson (Brassica rapa L). Journal of Pharmacognosy and Phytochemistry. 6(2): 226-232.
[201] Pastor JM, Salvador M, Argandona M, Bernal V, Reina-Bueno M, Csonka LN, Iborra JL, Vargas C, Nieto JJ, Canovas M. 2010. Ectoines in cell stress protection: Uses and biotechnological production. Biotechnology Advances 28:782-801.
[202] Patel JK, Madaan S, Archana G. 2018. Antibiotic producing endophytic Streptomyces spp. colonize above-ground plant parts and promote shoot growth in multiple healthy and pathogen challenged cereal crops. Microbiological Research 215:36-45.

[203] Paul EA. 2015. Chapter 1 - Soil Microbiology, Ecology, and Biochemistry: An Exciting Present and Great Future Built on Basic Knowledge and Unifying Concepts. In. Soil Microbiology, Ecology and Biochemistry (Fourth Edition). Boston: Academic Press. p. 1-14.
[204] Peacock DG, Richardson JF. 2012. Chemical Engineering, Volume 3: Chemical and Biochemical Reactors and Process Control: Elsevier Science.
[205] Peiren J, Buyse J, De Vos P, Lang E, Clermont D, Hamon S, Begaud E, Bizet C, Pascual J, Ruvira MA, et al. 2015. Improving survival and storage stability of bacteria recalcitrant to freeze-drying: a coordinated study by European culture collections. Applied Microbiology and Biotechnology 99:3559-3571.
[206] Pereira G, Magalhaes KT, Lorenzetii ER, Souza TP, Schwan RF. 2012. A Multiphasic Approach for the Identification of Endophytic Bacterial in Strawberry Fruit and their Potential for Plant Growth Promotion. Microbial Ecology 63:405-417.
[207] Pham VHT, Kim J. 2012. Cultivation of unculturable soil bacteria. Trends in Biotechnology 30:475-484.
[208] Pinski A, Betekhtin A, Hupert-Kocurek K, Mur LAJ, Hasterok R. 2019. Defining the Genetic Basis of Plant(-)Endophytic Bacteria Interactions. Int J Mol Sci 20.
[209] Pirhadi M, Enayatizamir N, Motamedi H, Sorkheh K. 2018. impact of soil salinity on diversity and community of sugarcane endophytic plant growth promoting bacteria (saccharum officinarum l. var. cp48). Applied Ecology and Environmental Research 16:725-739.
[210] Pirttila AM, Joensuu P, Pospiech H, Jalonen J, Hohtola A. 2004. Bud endophytes of Scots pine produce adenine derivatives and other compounds that affect morphology and mitigate browning of callus cultures. Physiologia Plantarum 121:305-312.
[211] Piubeli F, Salvador M, Argandona M, Nieto JJ, Bernal V, Pastor JM, Canovas M, Vargas C. 2018. Insights into metabolic osmoadaptation of the ectoines-producer bacterium Chromohalobacter salexigens through a high-quality genome scale metabolic model. Microbial Cell Factories 17:20.
[212] Plassmeier J, Barsch A, Persicke M, Niehaus K, Kalinowski J. 2007. Investigation of central carbon metabolism and the 2-methylcitrate cycle in Corynebacterium glutamicum by metabolic profiling using gas chromatography-mass spectrometry. Journal of Biotechnology 130:354-363.
[213] Prajapati RS, Das M, Sreeramulu S, Sirajuddin M, Srinivasan S, Krishnamurthy V, Ranjani R, Ramakrishnan C, Varadarajan R. 2007. Thermodynamic effects of proline introduction on protein stability. Proteins-Structure Function and Bioinformatics 66:480-491.
[214] Pusey PL, Wend C. 2012. Potential of osmoadaptation for improving Pantoea agglomerans E325 as biocontrol agent for fire blight of apple and pear. Biological Control 62:29-37.
[215] Qurashi AW, Sabri AN. 2011. Osmoadaptation and plant growth promotion by salt tolerant bacteria under salt stress. African Journal of Microbiology Research 5:3546-3554.
[216] Rainey PB, Travisano M. 1998. Adaptive radiation in a heterogeneous environment. Nature 394:69-72.
[217] Rajendran L, Samiyappan R, Raguchander T, Saravanakumar D. 2007. Endophytic bacteria mediate plant resistance against cotton bollworm. Journal of Plant Interactions 2:1-10.
[218] Ray S, Mishra S, Bisen K, Singh S, Sarma BK, Singh HB. 2018. Modulation in phenolic root exudate profile of Abelmoschus esculentus expressing activation of defense pathway. Microbiological Research 207:100-107.
[219] Reed MLE, Glick BR. 2005. Growth of canola (Brassica napus) in the presence of plant growth-promoting bacteria and either copper or polycyclic aromatic hydrocarbons. Canadian Journal of Microbiology 51:1061-1069.

[220] Reina-Bueno M, Argandona M, Nieto JJ, Hidalgo-Garcia A, Iglesias-Guerra F, Delgado MJ, Vargas C. 2012. Role of trehalose in heat and desiccation tolerance in the soil bacterium Rhizobium etli. Bmc Microbiology 12.
[221] Reinhold-Hurek B, Hurek T. 2011. Living inside plants: bacterial endophytes. Current Opinion in Plant Biology 14:435-443.
[222] Reis VM, dos Reis FB, Quesada DM, de Oliveira OCA, Alves BJR, Urquiaga S, Boddey RM. 2001. Biological nitrogen fixation associated with tropical pasture grasses. Australian Journal of Plant Physiology 28:837-844.
[223] Remus R, Ruppel S, Jacob H-J, Hecht-Buchholz C, Merbach W. (Remus2000 co-authors). 2000. Colonization behaviour of two enterobacterial strains on cereals. Biology and Fertility of Soils 30:550-557.
[224] Ren C, Wang T, Xu Y, Deng J, Zhao F, Yang G, Han X, Feng Y, Ren G. 2018. Differential soil microbial community responses to the linkage of soil organic carbon fractions with respiration across land-use changes. Forest Ecology and Management 409:170-178.
[225] Rho H, Hsieh M, Kandel SL, Cantillo J, Doty SL, Kim S-H. 2018. Do Endophytes Promote Growth of Host Plants Under Stress? A Meta-Analysis on Plant Stress Mitigation by Endophytes. Microbial Ecology 75:407-418.
[226] Riggs PJ, Chelius MK, Iniguez AL, Kaeppler SM, Triplett EW. 2001. Enhanced maize productivity by inoculation with diazotrophic bacteria. Australian Journal of Plant Physiology 28:829-836.
[227] Rodriguez-Salazar J, Moreno S, Espin G. 2017. LEA proteins are involved in cyst desiccation resistance and other abiotic stresses in Azotobacter vinelandii. Cell Stress & Chaperones 22:397-408.
[228] Roessler M, Muller V. 2001. Osmoadaptation in bacteria and archaea: common principles and differences. Environmental Microbiology 3:743-754.
[229] Rosenblueth M, Martinez-Romero E. 2006. Bacterial endophytes and their interactions with hosts. Molecular Plant-Microbe Interactions 19:827-837.
[230] Ruiza D, Agaras B, de Werrab P, Wall LG, Valverde C. 2011. Characterization and screening of plant probiotic traits of bacteria isolated from rice seeds cultivated in Argentina. Journal of Microbiology 49:902-912.
[231] Rungin S, Indananda C, Suttiviriya P, Kruasuwan W, Jaemsaeng R, Thamchaipenet A. 2012. Plant growth enhancing effects by a siderophore-producing endophytic streptomycete isolated from a Thai jasmine rice plant (Oryza sativa L. cv. KDML105). Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology 102:463-472.
[232] Ruppel S, Hecht-Buchholz C, Remus R, Ortmann U, Schmelzer R. 1992. Settlement of the diazotrophic, phytoeffective bacterial strain Pantoea agglomerans on and within winter wheat: An investigation using ELISA and transmission electron microscopy. Plant and Soil 145:261-273.
[233] Ruppel S, Merbach W. 1995. Effects of different nitrogen sources on nitrogen fixation and bacterial growth of Pantoea agglomerans and Azospirillum sp. in bacterial pure culture: An investigation using 15N2 incorporation and acetylene reduction measures. Microbiological Research 150:409-418.
[234] Ruppel S, Ruehlmann J, Merbach W. 2006. Quantification and localization of bacteria in plant tissues using quantitative real-time PCR and online emission fingerprinting. Plant and Soil 286:21-35.
[235] Ryan RP, Germaine K, Franks A, Ryan DJ, Dowling DN. 2008. Bacterial endophytes: recent developments and applications. Fems Microbiology Letters 278:1-9.

[236] Saavedra-Leos Z, Leyva-Porras C, Araujo-Diaz SB, Toxqui-Teran A, Borras-Enriquez AJ. 2015. Technological Application of Maltodextrins According to the Degree of Polymerization. Molecules 20:21067-21081.
[237] Sabaratnam S, Traquair JA. 2002. Formulation of a Streptomyces biocontrol agent for the suppression of Rhizoctonia damping-off in tomato transplants. Biological Control 23:245-253.
[238] Sajadi SAA. 2010. Metal ion-binding properties of L-glutamic acid and L-aspartic acid, a comparative investigation. Natural Science Vol.02No.02:6.
[239] Saminathan T, Garcia M, Ghimire B, Lopez C, Bodunrin A, Nimmakayala P, Abburi VL, Levi A, Balagurusamy N, Reddy UK. 2018. Metagenomic and Metatranscriptomic Analyses of Diverse Watermelon Cultivars Reveal the Role of Fruit Associated Microbiome in Carbohydrate Metabolism and Ripening of Mature Fruits. Frontiers in Plant Science 9.
[240] Sand M, Rodrigues M, Gonzalez JM, de Crecy-Lagard V, Santos H, Muller V, Averhoff B. 2015. Mannitol-1-phosphate dehydrogenases/phosphatases: a family of novel bifunctional enzymes for bacterial adaptation to osmotic stress. Environmental Microbiology 17:711-719.
[241] Sansone F, Mencherini T, Picerno P, d'Amore M, Aquino RP, Lauro MR. 2011. Maltodextrin/pectin microparticles by spray drying as carrier for nutraceutical extracts. Journal of Food Engineering 105:468-476.
[242] Santivarangkna C, Kulozik U, Foerst P. 2007. Alternative drying processes for the industrial preservation of lactic acid starter cultures. Biotechnology Progress 23:302-315.
[243] Santoyo G, Moreno-Hagelsieb G, Del Carmen Orozco-Mosqueda M, Glick BR. 2016. Plant growth-promoting bacterial endophytes. Microbiol Res 183:92-99.
[244] Sato ACK, Oliveira PR, Cunha RL. 2008. Rheology of mixed pectin solutions. Food Biophysics 3:100-109.
[245] Shazia Afrasayab and Shahida Hasnain, 2000. Synergistic Growth Stimulatory Effects of Mixed Culture Bacterial Inoculations on the Early Growth of *Triticum aestivum* Var Inqlab 91 under NaCl Stress. *Pakistan Journal of Biological Sciences*, 3: 1016-1023.
[246] Scherling C, Ulrich K, Ewald D, Weckwerth W. 2009. A metabolic signature of the beneficial interaction of the endophyte paenibacillus sp. isolate and in vitro-grown poplar plants revealed by metabolomics. Mol Plant Microbe Interact 22:1032-1037.
[247] Schilling G, Gransee A, Deuhel A, Ležovič G, Ruppel S. 1998. Phosphorus availability, root exudates, and microbial activity in the rhizosphere. Zeitschrift für Pflanzenernährung und Bodenkunde 161:465-478.
[248] Schoebitz M, Ceballos C, Ciamp L. 2013. Effect of immobilized phosphate solubilizing bacteria on wheat growth and phosphate uptake. Journal of soil science and plant nutrition 13:1-10.
[249] Schoebitz M, Lopez MD, Roldan A. 2013. Bioencapsulation of microbial inoculants for better soil-plant fertilization. A review. Agronomy for Sustainable Development 33:751-765.
[250] Schoebitz M, Simonin H, Poncelet D. 2012. Starch filler and osmoprotectants improve the survival of rhizobacteria in dried alginate beads. Journal of Microencapsulation 29:532-538.
[251] Scholz-Seidel C, Ruppel S. 1992. Nitrogenase- and Phytohormone Activities of Pantoea agglomerans in Culture and their Reflection in Combination with Wheat Plants. Zentralblatt für Mikrobiologie 147:319-328.
[252] Schreiner M, Krumbein A, Ruppel S. 2009. Interaction between Plants and Bacteria: Glucosinolates and Phyllospheric Colonization of Cruciferous Vegetables by Enterobacter radicincitans DSM 16656. Journal of Molecular Microbiology and Biotechnology 17:124-135.

[253] Schuck P, Dolivet A, Mejean S, Herve C, Jeantet R. 2013. Spray drying of dairy bacteria: New opportunities to improve the viability of bacteria powders. International Dairy Journal 31:12-17.
[254] Schultz-Jensen N, Aamand J, Sorensen SR. 2016. Bioaugmentation potential of free and formulated 2,6-dichlorobenzamide (BAM) degrading Aminobacter sp MSH1 in soil, sand and water. AMB Express 6:8.
[255] Schulz B, Boyle C. 2005. The endophytic continuum. Mycological Research 109:661-686.
[256] Sessitsch A, Hardoim P, Doring J, Weilharter A, Krause A, Woyke T, Mitter B, Hauberg-Lotte L, Friedrich F, Rahalkar M, et al. 2012. Functional Characteristics of an Endophyte Community Colonizing Rice Roots as Revealed by Metagenomic Analysis. Molecular Plant-Microbe Interactions 25:28-36.
[257] Sessitsch A, Reiter B, Berg G. 2004. Endophytic bacterial communities of field-grown potato plants and their plant-growth-promoting and antagonistic abilities. Canadian Journal of Microbiology 50:239-249.
[258] Sevilla M, Burris RH, Gunapala N, Kennedy C. 2001. Comparison of benefit to sugarcane plant growth and N-15(2) incorporation following inoculation of sterile plants with Acetobacter diazotrophicus wild-type and Nif(-) mutant strains. Molecular Plant-Microbe Interactions 14:358-366.
[259] Sevin DC, Stahlin JN, Pollak GR, Kuehne A, Sauer U. 2016a. Global Metabolic Responses to Salt Stress in Fifteen Species. PLoS One 11:e0148888.
[260] Sevin DC, Stahlin JN, Pollak GR, Kuehne A, Sauer U. 2016b. Global Metabolic Responses to Salt Stress in Fifteen Species. PLoS One 11.
[261] Shao YY, Gao SR, Guo HL, Zhang HP. 2014. Influence of culture conditions and preconditioning on survival of Lactobacillus delbrueckii subspecies bulgaricus ND02 during lyophilization. Journal of Dairy Science 97:1270-1280.
[262] Sharaff M, Kamat S, Archana G. 2017. Analysis of copper tolerant rhizobacteria from the industrial belt of Gujarat, western India for plant growth promotion in metal polluted agriculture soils. Ecotoxicology and Environmental Safety 138:113-121.
[263] Shishido M, Breuil C, Chanway CP. 1999. Endophytic colonization of spruce by plant growth-promoting rhizobacteria. FEMS Microbiology Ecology 29:191-196.
[264] Singh D, Geat N, Rajawat MVS, Prasanna R, Kar A, Singh AM, Saxena AK. 2018. Prospecting endophytes from different Fe or Zn accumulating wheat genotypes for their influence as inoculants on plant growth, yield, and micronutrient content. Annals of Microbiology 68:815-833.
[265] Singh M, Kumar A, Singh R, Pandey KD. 2017. Endophytic bacteria: a new source of bioactive compounds. 3 Biotech 7.
[266] Singh N, Rai V, Tripathi CKM. 2012. Production and optimization of oxytetracycline by a new isolate Streptomyces rimosus using response surface methodology. Medicinal Chemistry Research 21:3140-3145.
[267] Singh R, Arora NK. 2016. Bacterial Formulations and Delivery Systems against Pests in Sustainable Agro-Food Production. In. Reference Module in Food Science: Elsevier.
[268] Singh RK, Malik N, Singh S. 2013. Improved Nutrient Use Efficiency Increases Plant Growth of Rice with the Use of IAA-Overproducing Strains of Endophytic Burkholderia cepacia Strain RRE25. Microbial Ecology 66:375-384.
[269] Singh S, Rekha PD, Arun AB, Young CC. 2009. Impacts of monosodium glutamate industrial wastewater on plant growth and soil characteristics. Ecological Engineering 35:1559-1563.
[270] Singh V, Haque S, Niwas R, Srivastava A, Pasupuleti M, Tripathi CKM. 2017. Strategies for Fermentation Medium Optimization: An In-Depth Review. Frontiers in Microbiology 7.

[271] Sleator RD, Hill C. 2002. Bacterial osmoadaptation: the role of osmolytes in bacterial stress and virulence. Fems Microbiology Reviews 26:49-71.
[272] Smith SA, Tank DC, Boulanger LA, Bascom-Slack CA, Eisenman K, Kingery D, Babbs B, Fenn K, Greene JS, Hann BD, et al. 2008. Bioactive Endophytes Warrant Intensified Exploration and Conservation. PLoS One 3.
[273] Sonderholm M, Kragh KN, Koren K, Jakobsen TH, Darch SE, Alhede M, Jensen PO, Whiteley M, Kuhl M, Bjarnsholt T. 2017. Pseudomonas aeruginosa Aggregate Formation in an Alginate Bead Model System Exhibits In Vivo-Like Characteristics. Applied and Environmental Microbiology 83.
[274] Sprent JI, de Faria SM. 1988. Mechanisms of infection of plants by nitrogen fixing organisms. Plant and Soil 110:157-165.
[275] Sriamornsak P. 2011. Application of pectin in oral drug delivery. Expert Opinion on Drug Delivery 8:1009-1023.
[276] Suhaimi NSM, Yap KP, Ajam N, Thong KL. 2014. Genome sequence of Kosakonia radicincitans UMEnt01/12, a bacterium associated with bacterial wilt diseased banana plant. Fems Microbiology Letters 358:11-13.
[277] Sunnotel O, Nigam P. 2002. Pectinolytic activity of bacteria isolated from soil and two fungal strains during submerged fermentation. World Journal of Microbiology and Biotechnology 18:835-839.
[278] Szymanska S, Borruso L, Brusetti L, Hulisz P, Furtado B, Hrynkiewicz K. 2018. Bacterial microbiome of root-associated endophytes of Salicornia europaea in correspondence to different levels of salinity. Environmental Science and Pollution Research 25:25420-25431.
[279] Tabatabai MA, Bremner JM. 1969. Use of p-nitrophenyl phosphate for assay of soil phosphatase activity. Soil Biology and Biochemistry 1:301-307.
[280] Taghavi S, Garafola C, Monchy S, Newman L, Hoffman A, Weyens N, Barac T, Vangronsveld J, van der Lelie D. 2009. Genome Survey and Characterization of Endophytic Bacteria Exhibiting a Beneficial Effect on Growth and Development of Poplar Trees. Applied and Environmental Microbiology 75:748-757.
[281] Tamosiune I, Staniene G, Haimi P, Stanys V, Rugienius R, Baniulis D. 2018. Endophytic Bacillus and Pseudomonas spp. Modulate Apple Shoot Growth, Cellular Redox Balance, and Protein Expression Under in Vitro Conditions. Frontiers in Plant Science 9.
[282] Tan RX, Zou WX. 2001. Endophytes: a rich source of functional metabolites. Natural Product Reports 18:448-459.
[283] Tanne C, Golovina EA, Hoekstra FA, Meffert A, Galinski EA. 2014. Glass-forming property of hydroxyectoine is the cause of its superior function as a desiccation protectant. Frontiers in Microbiology 5.
[284] Tao P, Li H, Yu YJ, Gu JD, Liu YD. 2016. Ectoine and 5-hydroxyectoine accumulation in the halophile Virgibacillus halodenitrificans PDB-F2 in response to salt stress. Applied Microbiology and Biotechnology 100:6779-6789.
[285] Taurian T, Anzuay MS, Angelini JG, Tonelli ML, Luduena L, Pena D, Ibanez F, Fabra A. 2010. Phosphate-solubilizing peanut associated bacteria: screening for plant growth-promoting activities. Plant and Soil 329:421-431.
[286] Tavares MJ, Nascimento FX, Glick BR, Rossi MJ. 2018. The expression of an exogenous ACC deaminase by the endophyte Serratia grimesii BXF1 promotes the early nodulation and growth of common bean. Letters in Applied Microbiology 66:252-259.
[287] Teixeira P, Castro H, Kirby R. 1996. Evidence of membrane lipid oxidation of spray-dried Lactobacillus bulgaricus during storage. Letters in Applied Microbiology 22:34-38.
[288] Teixido N, Canamas TP, Usall J, Torres R, Magan N, Vinas I. 2005. Accumulation of the compatible solutes, glycine-betaine and ectoine, in osmotic stress adaptation and heat shock

cross-protection in the biocontrol agent Pantoea agglomerans CPA-2. Letters in Applied Microbiology 41:248-252.
[289] Tewari S, Arora NK. 2014. Multifunctional Exopolysaccharides from Pseudomonas aeruginosa PF23 Involved in Plant Growth Stimulation, Biocontrol and Stress Amelioration in Sunflower Under Saline Conditions. Current Microbiology 69:484-494.
[290] Thakur BR, Singh RK, Handa AK, Rao MA. 1997. Chemistry and uses of pectin — A review. Critical Reviews in Food Science and Nutrition 37:47-73.
[291] Timm CM, Campbell AG, Utturkar SM, Jun SR, Parales RE, Tan WA, Robeson MS, Lu TYS, Jawdy S, Brown SD, et al. 2015. Metabolic functions of Pseudomonas fluorescens strains from Populus deltoides depend on rhizosphere or endosphere isolation compartment. Frontiers in Microbiology 6.
[292] Tripathi CKM, Praveen V, Singh V, Bihari V. 2004. Production of antibacterial and antifungal metabolites by Streptomyces violaceusniger and media optimization studies for the maximum metabolite production. Medicinal Chemistry Research 13:790-799.
[293] Trivedi P, Pandey A. 2008. Plant growth promotion abilities and formulation of Bacillus megaterium strain B 388 (MTCC6521) isolated from a temperate Himalayan location. Indian Journal of Microbiology 48:342-347.
[294] Trivedi P, Pandey A, Palni LMS. 2005. Carrier-based preparations of plant growth-promoting bacterial inoculants suitable for use in cooler regions. World Journal of Microbiology & Biotechnology 21:941-945.
[295] Tunnacliffe A, de Castro AG, Manzanera M. 2001. Anhydrobiotic engineering of bacterial and mammalian cells: Is intracellular trehalose sufficient? Cryobiology 43:124-132.
[296] van Kessel JC, Rutherford ST, Cong JP, Quinodoz S, Healy J, Bassler BL. 2015. Quorum Sensing Regulates the Osmotic Stress Response in Vibrio harveyi. Journal of Bacteriology 197:73-80.
[297] Vartoukian SR, Palmer RM, Wade WG. 2010. Strategies for culture of 'unculturable' bacteria. Fems Microbiology Letters 309:1-7.
[298] Vega FE, Pava-Ripoll M, Posada F, Buyer JS. 2005. Endophytic bacteria in Coffea arabica L. Journal of Basic Microbiology 45:371-380.
[299] Vemmer M, Patel AV. 2013. Review of encapsulation methods suitable for microbial biological control agents. Biological Control 67:380-389.
[300] Verma SC, Ladha JK, Tripathi AK. 2001. Evaluation of plant growth promoting and colonization ability of endophytic diazotrophs from deep water rice. Journal of Biotechnology 91:127-141.
[301] Verma SK, Kingsley K, Bergen M, English C, Elmore M, Kharwar RN, White JF. 2018. Bacterial endophytes from rice cut grass (Leersia oryzoides L.) increase growth, promote root gravitropic response, stimulate root hair formation, and protect rice seedlings from disease. Plant and Soil 422:223-238.
[302] Verma VC, Singh SK, Prakash S. 2011. Bio-control and plant growth promotion potential of siderophore producing endophytic Streptomyces from Azadirachta indica A. Juss. Journal of Basic Microbiology 51:550-556.
[303] Vessey JK. 2003. Plant growth promoting rhizobacteria as biofertilizers. Plant and Soil 255:571-586.
[304] Vu B, Chen M, Crawford RJ, Ivanova EP. 2009. Bacterial Extracellular Polysaccharides Involved in Biofilm Formation. Molecules 14:2535-2554.
[305] Wakelin SA, Warren RA, Harvey PR, Ryder MH. 2004. Phosphate solubilization by Penicillium spp. closely associated with wheat roots. Biology and Fertility of Soils 40:36-43.

[306] Walitang DI, Kim K, Madhaiyan M, Kim YK, Kang Y, Sa T. 2017. Characterizing endophytic competence and plant growth promotion of bacterial endophytes inhabiting the seed endosphere of Rice. Bmc Microbiology 17.
[307] Wan LQ, Jiang J, Arnold DE, Guo XE, Lu HH, Mow VC. 2008. Calcium Concentration Effects on the Mechanical and Biochemical Properties of Chondrocyte-Alginate Constructs. Cellular and Molecular Bioengineering 1:93-102.
[308] Wang C, Zhu D, Nagata S. 2006. Supplementation effects of hydroxyectoine on proline uptake of downshocked Brevibacterium sp. JCM 6894. Journal of Bioscience and Bioengineering 101:178-184.
[309] Webb R, Buratini J. 2016. Global Challenges for the 21st Century: the Role and Strategy of the Agri-Food Sector. Animal Reproduction 13:133-142.
[310] Wewetzer SJ, Kunze M, Ladner T, Luchterhand B, Roth S, Rahmen N, Kloss R, Silva ACE, Regestein L, Buchs J. 2015. Parallel use of shake flask and microtiter plate online measuring devices (RAMOS and BioLector) reduces the number of experiments in laboratory-scale stirred tank bioreactors. Journal of Biological Engineering 9.
[311] Wieland G, Neumann R, Backhaus H. 2001. Variation of microbial communities in soil, rhizosphere, and rhizoplane in response to crop species, soil type, and crop development. Applied and Environmental Microbiology 67:5849-5854.
[312] Winter J, Linke K, Jatzek A, Jakob U. 2005. Severe oxidative stress causes inactivation of DnaK and activation of the redox-regulated chaperone Hsp33. Molecular Cell 17:381-392.
[313] Witzel K, Gwinn-Giglio M, Nadendla S, Shefchek K, Ruppel S. 2012. Genome Sequence of Enterobacter radicincitans DSM16656(T), a Plant Growth-Promoting Endophyte. Journal of Bacteriology 194:5469-5469.
[314] Witzel K, Strehmel N, Baldermann S, Neugart S, Becker Y, Becker M, Berger B, Scheel D, Grosch R, Schreiner M, et al. 2017. Arabidopsis thaliana root and root exudate metabolism is altered by the growth-promoting bacterium Kosakonia radicincitans DSM 16656(T). Plant and Soil 419:557-573.
[315] Wolfe J, Bryant G. 1999. Freezing, drying, and/or vitrification of membrane- solute-water systems. Cryobiology 39:103-129.
[316] Wood JM, Bremer E, Csonka LN, Kraemer R, Poolman B, van der Heide T, Smith LT. 2001. Osmosensing and osmoregulatory compatible solute accumulation by bacteria. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 130:437-460.
[317] Wu K, Fang ZY, Guo R, Pan B, Shi W, Yuan SF, Guan HL, Gong M, Shen B, Shen QR. 2015. Pectin Enhances Bio-Control Efficacy by Inducing Colonization and Secretion of Secondary Metabolites by Bacillus amyloliquefaciens SQY 162 in the Rhizosphere of Tobacco. PLoS One 10.
[318] Wu X, Monchy S, Taghavi S, Zhu W, Ramos J, van der Lelie D. 2011. Comparative genomics and functional analysis of niche-specific adaptation in Pseudomonas putida. FEMS Microbiol Rev 35:299-323.
[319] Xia JG, Mandal R, Sinelnikov IV, Broadhurst D, Wishart DS. 2012. MetaboAnalyst 2.0-a comprehensive server for metabolomic data analysis. Nucleic Acids Research 40:W127-W133.
[320] Xia JG, Psychogios N, Young N, Wishart DS. 2009. MetaboAnalyst: a web server for metabolomic data analysis and interpretation. Nucleic Acids Research 37:W652-W660.
[321] Xia JG, Sinelnikov IV, Han B, Wishart DS. 2015. MetaboAnalyst 3.0-making metabolomics more meaningful. Nucleic Acids Research 43:W251-W257.
[322] Yadav S, Yadav PK, Yadav D, Yadav KDS. 2008. Purification and characterization of an alkaline pectin lyase from Aspergillus flavus. Process Biochemistry 43:547-552.

[323] Yaish MW, Al-Lawati A, Jana GA, Patankar HV, Glick BR. 2016. Impact of Soil Salinity on the Structure of the Bacterial Endophytic Community Identified from the Roots of Caliph Medic (Medicago truncatula). PLoS One 11.
[324] Yang H, Puri A, Padda KP, Chanway CP. 2017. Substrate utilization by endophytic bacteria Paenibacillus polymyxa P2b-2R that may facilitate bacterial entrance and survival inside diverse plant hosts. Facets 2.
[325] Yi YJ, Li YS, Xia B, Li WP, Pang L, Tong ZD. 2015. Optimization of medium composition and culture conditions for antifungal activity of a tomato endophytic bacterium. Biological Control 82:69-75.
[326] Yin H, Zhang R, Xia M, Bai X, Mou J, Zheng Y, Wang M. (Yin2017 co-authors). 2017. Effect of aspartic acid and glutamate on metabolism and acid stress resistance of Acetobacter pasteurianus. Microbial Cell Factories 16:109.
[327] Young CC, Rekha P, Lai WA, Arun AB. 2006. Encapsulation of plant growth-promoting bacteria in alginate beads enriched with humic acid. Biotechnology and Bioengineering 95:76-83.
[328] Youssef HH, Hamza MA, Fayez M, Mourad EF, Saleh MY, Sarhan MS, Suker RM, Eltahlawy AA, Nemr RA, El-Tahan M, et al. 2016. Plant-based culture media: Efficiently support culturing rhizobacteria and correctly mirror their in-situ diversity. Journal of Advanced Research 7:305-316.
[329] Zhao S, Zhang Y, Liu Y, Yang F, Yu WG, Zhang SX, Ma XJ, Sun GW. 2018. Optimization of preparation conditions for calcium pectinate with response surface methodology and its application for cell encapsulation. International Journal of Biological Macromolecules 115:29-34.