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Scalable Microencapsulation of Plant-Beneficial Bacteria for Sustainable Agriculture

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Scalable Microencapsulation of Plant-Beneficial Bacteria for Sustainable Agriculture

By

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DISSERTATION

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DAVIS

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Abstract

Currently in the agricultural industry, the application of pesticides to benefit crop health and performance is unavoidable, yet major reform is needed for safer and more sustainable practices. Plant-beneficial bacteria are of interest because they are able to address many of the issues traditional pesticides target. These biopesticides are isolated from the environment thus completely natural, and are not harmful to humans. Unfortunately, the types of viable bacteria for biopesticide products are mostly limited to spore-formers due to some challenges in commercialization. Microencapsulation, a process where a wall material surrounds and protects a cargo component to form a microcapsule, is perhaps the best method for non-spore-forming plant-beneficial bacteria to be implemented in the agricultural industry. Encapsulation of non-spore-formers appears to be most promising in dry alginate beads. Alginate is a highly desirable naturally-derived wall material for its safety and ability to form a gel in the presence of multivalent cations. However, forming dry alginate beads is a tedious process requiring a gelling step, curing step, collection step, and drying step, making it difficult to efficiently scale up. A recently developed UC Davis spray-drying process forms in situ cross-linked alginate microcapsules (CLAMs) in a single step. In this work, I hypothesized that formulation and process development of the single step encapsulation process by spray-drying or fluidized bed spray-coating, will stabilize non-spore-forming bacteria such that high viability is maintained throughout the encapsulation process and in long term storage.

To identify key formulation and process parameters which influenced survival of bacteria during spray-drying, the non-spore-forming plant-protective antifungal bacterium *Collimonas arenae* Cal35 was encapsulated in CLAMs. Response surface methodology was used in this study with the optimization objectives of maximizing survival of Cal35, and maximizing yield of spray-

dried powder. For the control parameters, only inlet temperature significantly impacted survival, while inlet temperature and spray rate influenced yield. Alginate concentration of the CLAMs formulation only impacted yield. By lowering inlet temperature to 95°C, the greatest survival was achieved, whereas greater inlet temperatures, and low spray rates and alginate concentrations produced the highest yield. When Cal35 was spray-dried with common excipients, i.e. maltodextrin or modified starch, there was no survival. Combining the CLAMs formulation with modified starch yielded the lowest loss of 3 log units while extending the shelf survival to over three weeks in a low oxygen and low humidity storage environment. Most importantly, Cal35 was able to retain its antifungal activity after spray-drying and shelf storage supporting its potential as a biofungicide.

Since the physical encapsulation of bacteria in spray-dried CLAMs is driven by atomization, fluidized bed spray-coating was investigated as another potential encapsulation method for seed coating applications. Here, enzyme was used as a substitute for bacteria to facilitate the process development of forming in situ cross-linked alginate matrix shell (CLAMshell) particles by fluidized bed spray-coating. The CLAMshell process maximized process efficiency while simultaneously improving the coating quality. Incorporation of the CLAMshell formulation allowed a 40% faster spray rate to be achieved over coatings with cargo-only, with coating efficiencies above 90%, and aggregates and fines below 2% of the product. Process interruptions were also eliminated. Increasing alginate concentration and using polyvinyl alcohol as an excipient in the CLAMshell formulation produced coatings with the greatest mechanical strength with minor particle damage and low attrition. CLAMshell particles also delayed dissolution of the salt core in solution, indicating potential for controlled release.

Finally, the non-spore-forming plant-beneficial bacteria *Collimonas arenae* Cal35 was encapsulated using the same formulation in CLAMs by spray-drying, and in CLAMshells by fluidized bed spray-coating, as both encapsulation processes are feasible in practice depending on how a bacterial biopesticide might be applied. Cal35 encapsulation in CLAMs had greater survival with a 3 log reduction in viability. Encapsulation in CLAMshells resulted in a 4 log reduction. Testing the sensitivity of Cal35 to heat and desiccation individually revealed the bacteria are somewhat tolerant to high temperatures, but not tolerant to desiccation. High temperatures and fast drying time are the conditions seen during spray-drying, which had better survival. Overall, CLAMs were between 5-20 μm in diameter allowing them to be applied to crops by spraying, while CLAMshells ranging between 250-300 μm would be more suitable for applying biopesticides as seed coatings.

Overall, this research demonstrates that through formulation and process development of the highly scalable CLAMs and CLAMshell encapsulation methods, there is potential for industrially application of non-spore-forming bacterial biopesticides. Low in-process drying temperatures but fast drying times were found to be most beneficial to the survival of Cal35. Addition of excipients in the formulation such as modified starch which is known for its oxygen barrier properties improved the shelf stability of Cal35; however, controlling the direct storage environment greatly affected its stability. Future work is highly recommended for scale up of both processes.

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His openness to helping other students and his obvious talent as a scientist gave me a great example to follow and look up to ever since I joined Tina's Lab.

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Chapter 1 Introduction

1.1 Overview

The agricultural industry has been increasing its awareness and employment of plant-beneficial microorganisms with the goal of improving crop performance. These improvements include availing nutrients for plant absorption, promoting plant growth, and providing protection against pests and pathogens. And while these beneficial microorganisms contribute towards greater overall crop yield, they also have high potential to address the need for more sustainable agricultural practices.

Pesticides are of particular interest because their application to crops have had significant, but unintended, consequences to human health and the environment. Traditional chemical pesticides destroy insects, weeds, or any other harmful organisms; however, with many of these chemicals now understood to be dangerous and toxic agents, the option for safer alternatives is highly sought after. Biopesticides address this issue. Defined as naturally occurring substances or microorganisms that control pests, biopesticides are becoming more recognized and applied in practice. Yet, the number of marketable products containing biopesticides, especially in the form of bacteria, remains low because of challenges in finding low-cost and scalable production processes and maintaining high bacterial viability from formulation to application.

Microencapsulation has been increasingly studied as a solution to these issues. While there are many different encapsulation methods for preserving and protecting plant-beneficial bacteria, a more recent in situ cross-linking process by spray-drying is perhaps the most notable. These spray-dried cross-linked alginate microcapsules (CLAMs) are formed using a more easily scalable process and have shown success in encapsulating a variety of bioactive ingredients. However, there is still significant consideration and work required to improve and optimize this

encapsulation process for plant-beneficial bacteria before it can be industrially relevant in agriculture.

1.2 Research Goal

This research aimed to develop an industrially scalable process to stabilize the non-spore-forming plant-beneficial bacteria, *Collimonas arenae* Cal35, in CLAMs. With the majority of commercial biopesticide products made up of robust spore-forming bacteria, there is great need to diversify and expand the possibilities to reduce and replace the more dangerous chemical pesticides. *C. arenae* Cal35 was suitable as a model non-spore-forming and Gram-negative bacterium for this work because of its ability to form antifungal compounds and chitinases and to weather and solubilize inorganics from the soil matrix. I hypothesized that through formulation and process development, more sensitive non-spore-forming bacteria like *C. arenae* Cal35 could overcome commercialization challenges using CLAMs technology to become more widespread in the agricultural industry. And because this method of in situ cross-linking of alginate is achieved through atomization, two encapsulation processes – spray-drying and fluidized bed spray-coating – were assessed to address application by foliar or field spraying and by seed coatings. The specific objectives of this research were:

1. To optimize spray-drying control parameters and the CLAMs formulation to maximize survival of *C. arenae* Cal35 during encapsulation and storage (Chapter 3),
2. To understand and investigate the fluidized bed spray-coating process through encapsulation of enzyme as a bioactive cargo (Chapter 4),
3. To compare spray-drying and fluidized bed spray-coating as encapsulation methods for *C. arenae* Cal35 (Chapter 5).

Chapter 2 Background and Literature Review

2.1 Pesticides and Biopesticides in Agriculture

There are approximately 2.3 billion acres of land surface area in the United States, 15% of which is devoted to crop production (Bigelow et al., 2017). The entire world's pesticide usage totals 6 billion pounds per year, while the U.S. is responsible for approximately one-sixth of that (Atwood et al., 2017). As defined by the EPA, a pesticide is considered any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest such as weeds, insects, and plant pathogens (Atwood et al., 2017). Early pesticide usage vastly improved crop yield while reducing labor and machine costs for pest control; however, apparent risks to human health and the environment quickly emerged (Fernandez-Cornejo et al., 2014). Farm workers are easily exposed when applying pesticides infield while consumers are indirectly exposed to residue on produce. Some of the most serious health risks include cancer and reproductive toxicity (Alavanja, 2009; Kim et al., 2017). There is very strong evidence linking pesticides to bladder and colon cancer (Koutros et al., 2009), lung cancer (Lerro et al., 2015b), and breast cancer (Arrebola et al., 2015; Lerro et al., 2015a), to name a few. Furthermore, potential environmental effects include surface and ground water contamination, and toxicity to wildlife (Covert et al., 2020; Hladik et al., 2016). While some of the most toxic chemical pesticides have been banned or regulated, there are still many other controversial pesticides in use. There is an urgent need to reduce and eliminate the use of harmful chemical pesticides in the agricultural industry.

Biopesticides are becoming rather popular in industry as a safer alternative. They are defined as microbial or naturally occurring compounds that control pests and pathogens by non-toxic mechanisms (California Department of Pesticide Regulation, 2017). While the integration of biopesticides at commercial levels has been increasing, it is still very limited and there is

significant room for improvement. For example, in California, 205 million pounds of total pesticide were applied, 25% of which were known to cause reproductive toxicity or cancer, and less than 4% were biopesticides (**Figure 2-1**) (California Department of Pesticide Regulation, 2017). The mechanisms that underlie the use of biopesticides as protectors of plant health represent traits and properties that have evolved among members of plant-associated microbiota as a way to antagonize, compete, or interfere with co-habitating pathogens (Berg, 2009; Berg et al., 2014; Finkel et al., 2017). In addition, microorganisms can protect plants by supporting plant health through induction of systemic plant defense, fixation or weathering of nutrients for the plant to absorb, and the release of hormones to stimulate root and shoot growth (Backer et al., 2018; Hayat et al., 2010; Lobo et al., 2019; Vacheron et al., 2013).

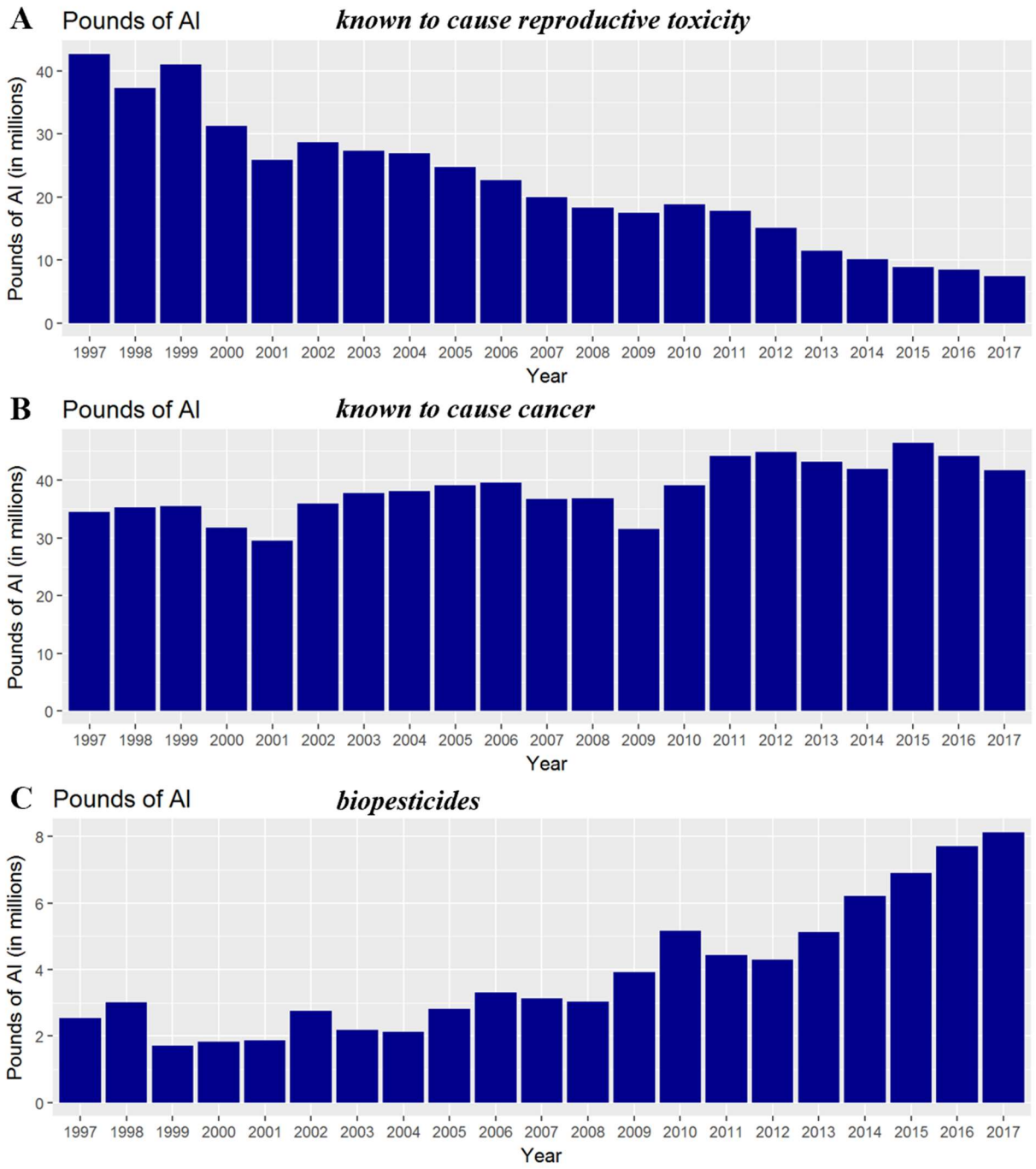

Figure 2-1 Pounds of active ingredient (AI) applied to crops in California between 1997 and 2017 for: (A) pesticides know not cause reproductive toxicity, (B) pesticides known to cause cancer, and (C) biopesticides. From California Department of Pesticide Regulation (2017).

Spore-forming bacteria belonging to the genus *Bacillus* are the most abundant and successful example of biopesticides (**Table 2-1**) (California Department of Pesticide Regulation,

2017; O'Callaghan, 2016; Pérez-García et al., 2011). Having the ability to form spores gives *Bacillus* great advantages which allows it to be easily commercialized and widely applied to crops. Spores are very durable and provide protection against external stresses, for example, those that are experienced by microorganisms during formulation, storage, and upon release in the field (Murphy et al., 2018). Non-spore-formers face considerably more challenges and thus have less commercial appeal. With lower tolerance to stresses like heat or desiccation, non-spore-forming bacteria are more often formulated as wet products (**Table 2-2**) compared to *Bacillus* which are predominantly dried into powders (**Table 2-3**) (Berninger et al., 2018; O'Callaghan, 2016; Preininger et al., 2018). Moreover, *Bacillus* products tend to have a longer shelf life on the scale of years at room temperature or even higher temperatures, whereas products containing non-spore-forming bacteria must be kept in freezing or refrigerated conditions and have a much shorter shelf life, on the scale of weeks.

Table 2-1 List of microbial biopesticides used in California from 2013 to 2017. From California Department of Pesticide Regulation (2017).

BIOPESTICIDE	**LBS 2013**	**LBS 2014**	**LBS 2015**	**LBS 2016**	**LBS 2017**
BACILLUS AMYLOLIQUEFACIENS STRAIN D747	84,957	177,589	131,295	209,773	395,702
BACILLUS AMYLOLIQUEFACIENS STRAIN MBI 600	<1	None	None	15	79
BACILLUS FIRMUS (STRAIN I-1582)	None	42	190	170	212
BACILLUS MYCOIDES ISOLATE J	None	None	None	None	1,085
BACILLUS POPILLIAE	<1	<1	<1	<1	None
BACILLUS PUMILUS, STRAIN QST 2808	6,245	7,957	8,123	7,889	9,239
BACILLUS SPHAERICUS 2362, SEROTYPE H5A5B, STRAIN ABTS 1743 FERMENTATION SOLIDS, SPORES AND INSECTICIDAL TOXINS	10,500	10,499	12,357	13,122	16,362
BACILLUS SUBTILIS GB03	1	2	3	3	4
BACILLUS SUBTILIS STRAIN IAB/BS03	None	None	None	None	None
BACILLUS SUBTILIS VAR. AMYLOLIQUEFACIENS STRAIN FZB24	94	119	178	6	<1
BACILLUS THURINGIENSIS (BERLINER)	11	4	29	21	14
BACILLUS THURINGIENSIS (BERLINER), SUBSP. AIZAWAI, GC-91 PROTEIN	13,265	18,776	16,771	18,882	34,097
BACILLUS THURINGIENSIS (BERLINER), SUBSP. AIZAWAI, SEROTYPE H-7	359	333	184	73	118

BACILLUS THURINGIENSIS (BERLINER), SUBSP. ISRAELENSIS, SEROTYPE H-14	9,269	11,779	15,761	15,839	17,733
BACILLUS THURINGIENSIS (BERLINER), SUBSP. KURSTAKI STRAIN SA-12	8,246	7,971	8,579	9,804	2,218
BACILLUS THURINGIENSIS (BERLINER), SUBSP. KURSTAKI, SEROTYPE 3A,3B	53	41	18	34	76
BACILLUS THURINGIENSIS (BERLINER), SUBSP. KURSTAKI, STRAIN EG 2348	500	514	344	645	396
BACILLUS THURINGIENSIS (BERLINER), SUBSP. KURSTAKI, STRAIN EG2371	None	None	None	None	None
BACILLUS THURINGIENSIS (BERLINER), SUBSP. KURSTAKI, STRAIN SA-11	77,932	80,401	80,953	74,963	96,271
BACILLUS THURINGIENSIS (BERLINER), SUBSP. SAN DIEGO	None	None	None	None	None
BACILLUS THURINGIENSIS SUBSPECIES KURSTAKI STRAIN BMP 123	None	None	None	None	None
BACILLUS THURINGIENSIS SUBSPECIES KURSTAKI, GENETICALLY ENGINEERED STRAIN EG7841 LEPIDOPTERAN ACTIVE TOXIN	116	65	3	43	3
BACILLUS THURINGIENSIS VAR. KURSTAKI STRAIN M-200	None	None	<1	None	<1
BACILLUS THURINGIENSIS VAR. KURSTAKI, GENETICALLY ENGINEERED STRAIN EG7826	None	None	None	7	None
BACILLUS THURINGIENSIS, SUBSP. AIZAWAI, STRAIN ABTS-1857	29,863	49,186	55,914	72,261	92,917
BACILLUS THURINGIENSIS, SUBSP. AIZAWAI, STRAIN SD-1372, LEPIDOPTERAN ACTIVE TOXIN(S)	18	6	43	13	6
BACILLUS THURINGIENSIS, SUBSP. ISRAELENSIS, STRAIN AM 65-52	49,682	42,763	46,599	70,128	61,729
BACILLUS THURINGIENSIS, SUBSP. KURSTAKI, STRAIN ABTS-351, FERMENTATION SOLIDS AND SOLUBLES	83,409	111,388	95,431	117,645	134,263
BACILLUS THURINGIENSIS, SUBSP. KURSTAKI, STRAIN HD-1	2,323	1,928	1,916	441	646
BACILLUS THURINGIENSIS, VAR. KURSTAKI DELTA ENDOTOXINS CRY 1A(C) AND CRY 1C (GENETICALLY ENGINEERED) ENCAPSULATED IN PSEUDOMONAS FLUORESCENS (KILLED)	None	<1	None	<1	None
PSEUDOMONAS FLUORESCENS, STRAIN A506	92	270	87	123	111
PSEUDOMONAS SYRINGAE, STRAIN ESC-10	3	None	None	None	None

Table 2-2 Examples of commercially available non-spore-forming microbial biopesticides. From Berninger et al. (2018); O'Callaghan (2016); Preininger et al. (2018).

Product Name	**Bacteria**	**Type**	**Storage/Shelf life**
BlightBan A506 (NuFarm)	*Pseudomonas fluorescens*	Liquid suspension	Freezer
Bio Gold (Bio Power Lanka)	*Azotobacter chroococcum, Pseudomonas fluorescens*	Liquid suspension	Not available

Bio-Save 10 LP (JET Harvest Solutions)	*Pseudomonas syringae*	Freeze-dried powder	Not available
Cedomon (BioAgri AB)	*Pseudomonas chlororaphis*	Liquid suspension	4-8°C/8 weeks Room Temperature/3 weeks
Cerall (BioAgri AB)	*Pseudomonas chlororaphis*	Liquid suspension	4-8°C/8 weeks
Galtrol-A (AgBioChem)	*Agrobacterium radiobacter*	Culture on solid media	2-4°C/4 weeks
Sheathguard (Agri Life)	*Pseudomonas fluorescens*	Dry powder	Unspecified/1 year

Table 2-3 Examples of commercially available *Bacillus* biopesticide products. From Preininger et al. (2018).

Product Name	**Bacteria**	**Type**	**Storage/Shelf life**
Biotilis (AgriLife)	*Bacillus subtilis*	Dry powder	<40°C/1 year
XenTari (Valent Biosciences Corp)	*Bacillus thuringiensis* subsp. *aizawai*	Dry granule	Not available
Serenade Opti (Bayer CropScience)	*Bacillus subtilis*	Dry powder	<25°C/3 years
Companion (growth products)	*Bacillus subtilis*	Dry powder	Not available
Vectobar (AgriLife)	*Bacillus thuringiensis* subsp. *israelensis*	Liquid suspensions	45°C/2 years

2.2 *Collimonas* species

The genus *Collimonas*, typically found in vegetated soil environments, contains four recognized species: *C. arenae*, *C. fungivorans*, *C. pratensis*, and *C. antrihumi* (De Boer et al., 2004; S. D. Lee, 2018; Leveau et al., 2010). These bacteria are Gram-negative rods and are not known to form spores (De Boer et al., 2004). Soil environments from which *Collimonas* has been isolated have been characterized by slight acidity, low nutrient availability, limited human disturbance, and fungal presence (Leveau et al., 2010). Collimonads share several traits which

give them potential as biofungicides. They have the ability to hydrolyze chitin and grow at the expense of fungal hyphae (De Boer et al., 2001; De Boer et al., 2004). They have also demonstrated antifungal activity by inhibiting fungal growth in vitro (Akum et al., 2020; Doan et al., 2020; Fritsche et al., 2014; Kamilova et al., 2007; Mela et al., 2011) and suppressing disease in vivo (Doan et al., 2020; Kamilova et al., 2007), in addition to interrupting fungal spore germination (Mosquera et al., 2020). Lastly, the ability to weather nutrients, i.e. to dissolve rocks and minerals, make *Collimonas* an even more versatile plant-beneficial bacteria (Leveau et al., 2010; Uroz et al., 2009).

2.3 Microencapsulation of Bacteria

Microencapsulation is a method of preserving and protecting a cargo component by surrounding it with a wall or carrier component (Jyothi et al., 2010). Used generally across many industries, microencapsulation of bacteria is mostly seen in the food industry for controlled release of probiotic bacteria in the gut (Cook et al., 2012), however it is becoming more valuable in agriculture for bacterial biopesticides sprayed in-field or in-furrow (Alonso et al., 2014; Bashan et al., 2014). Microencapsulation can be especially helpful to non-spore-forming bacteria because they are less tolerant to induced and environmental stresses experienced during formulation, storage, or application to crops, all of which have potential to lower bacterial viability (Bashan et al., 2014; Schoebitz et al., 2013). There are many ways of producing microcapsules, however, the exact process is mainly determined by the properties of the cargo and the final application.

For sensitive cargo such as non-spore-forming bacteria used in crop applications, microencapsulation in cross-linked alginate beads is a suitable method because alginate has been shown to be a protective carrier to bacteria and cross-linking of alginate allows for controlled release into soil (Bashan, 1986; Bashan et al., 2002; Rohman et al., 2021; Schoebitz et al., 2012;

Wu et al., 2012; Wu et al., 2014). Alginate is widely accepted as a carrier for encapsulation due to its relatively low cost, safe handling, and gel forming ability (Draget et al., 2011; K. Y. Lee et al., 2012). It is composed of alternating or repeating units of β-D-mannuronic acid and α-L-guluronic acid, where carboxylate functional groups lined along the polyuronic backbone allow multivalent cations (e.g. calcium) to cross-link the linear polymer chain to form a gel structure (**Figure 2-2**) (Bruchet et al., 2015; Grant et al., 1973; Hecht et al., 2016). Alginate gels are insoluble in water, however, the gelation process is reversible. Calcium cross-links can be dissociated in the presence of chelating agents such as citrate, EDTA, or phosphate, which scavenge calcium ions away from cross-linked alginates and allows release of cargo (Gombotz et al., 1998; Murata et al., 1993). The basic method of forming alginate beads containing bacteria involves suspending bacteria in an alginate solution, where it is dripped into a calcium chloride bath to achieve cross-linking at the surface. This is then followed by a curing step, a washing step, and a drying step to achieve dry beads. More developed methods using this technique exist (e.g. vibrating nozzle (Whelehan et al., 2011), electrostatic droplet extrusion (Al-Hajry et al., 1999)), however, the need for a separate calcium water bath to form alginate beads and the additional steps (curing, washing, drying) to attain usable dry beads make this challenging to scale up (Ching et al., 2017).

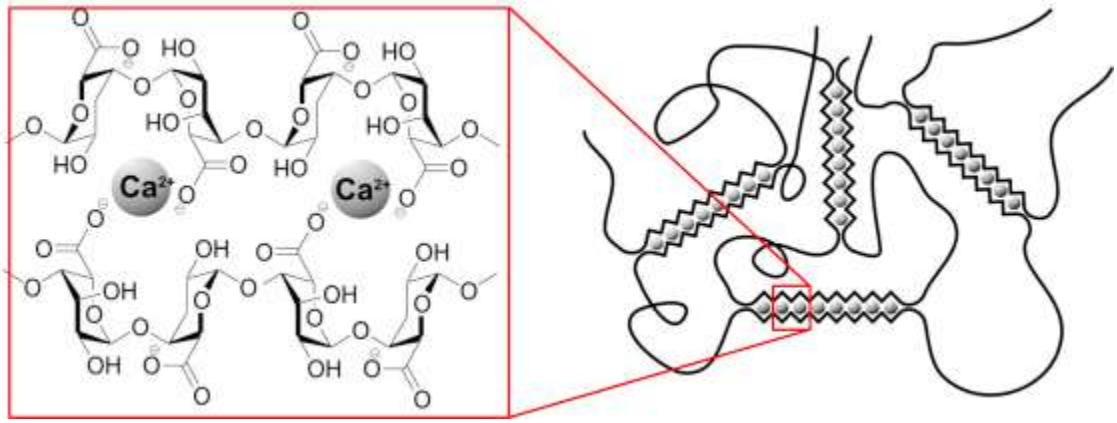

Figure 2-2 Structure of cross-linked alginate with calcium ions. From Bruchet et al. (2015); Grant et al. (1973).

2.4 Industrially Scalable Microencapsulation of Bacteria

Spray-drying is perhaps the most widely used industrial process for producing microcapsules, mainly for its scalability and low cost relative to other encapsulation processes (Poozesh et al., 2019; Rezvankhah et al., 2020; Schoebitz et al., 2013; Strobel et al., 2020b). The thermal drying process sprays a liquid feed containing the suspended cargo and carrier components into a hot air current where dry particles of the cargo embedded in the carrier matrix are formed and collected at the outlet. Spray-drying of probiotics is a widely studied and successful area of research, mainly due to the Gram-positive cell wall of probiotic bacteria, such as those belonging to the genus *Bifidobacterium* or *Lactobacillus*, allowing these bacteria to tolerate the high temperature drying process (Broeckx et al., 2017; Huang et al., 2017; Liu et al., 2019). Spray-drying of biopesticides such as *Bacillus* which have the ability to form spores are also widely successful and result in easy formulation and long shelf stability (Eski et al., 2019; Ma et al., 2015), also illustrated by the many available products listed in **Table 2-1** and **Table 2-3**. Spray-drying of biopesticides like *Pseudomonas* which are Gram-negative and cannot form spores is very difficult because of their high sensitivity to heat and desiccation, leading to low viability after encapsulation and a shorter shelf life (Amiet-Charpentier et al., 1999; Amiet-Charpentier et al., 1998).

Recently, a novel formulation process of forming in situ cross-linked alginate microcapsules (CLAMs) by spray-drying was introduced (Jeoh-Zicari et al., 2020a). CLAMs have previously demonstrated success in encapsulating a variety of cargo including enzymes (Santa-Maria et al., 2012), polymers (Wong et al., 2020), lipids (Strobel et al., 2020a; Strobel et al., 2016) and bacteria (Strobel et al., 2018). The production of bacteria-filled CLAMs, for example, begins with a feed suspension of alginate, an organic acid titrated to a higher pH with a volatile base, a calcium salt insoluble at the higher pH, and bacteria cells. Once pumped to the nozzle, atomization

of the feed suspension in the heated concurrent airflow volatilizes the base and lowers the pH of the sprayed droplet to the pK_a of the acid, solubilizing the calcium and enabling calcium mediated cross-linking of alginate while drying in the evaporation chamber and cyclone (**Figure 2-3**). Bacteria-filled CLAMs are collected in the collection chamber.

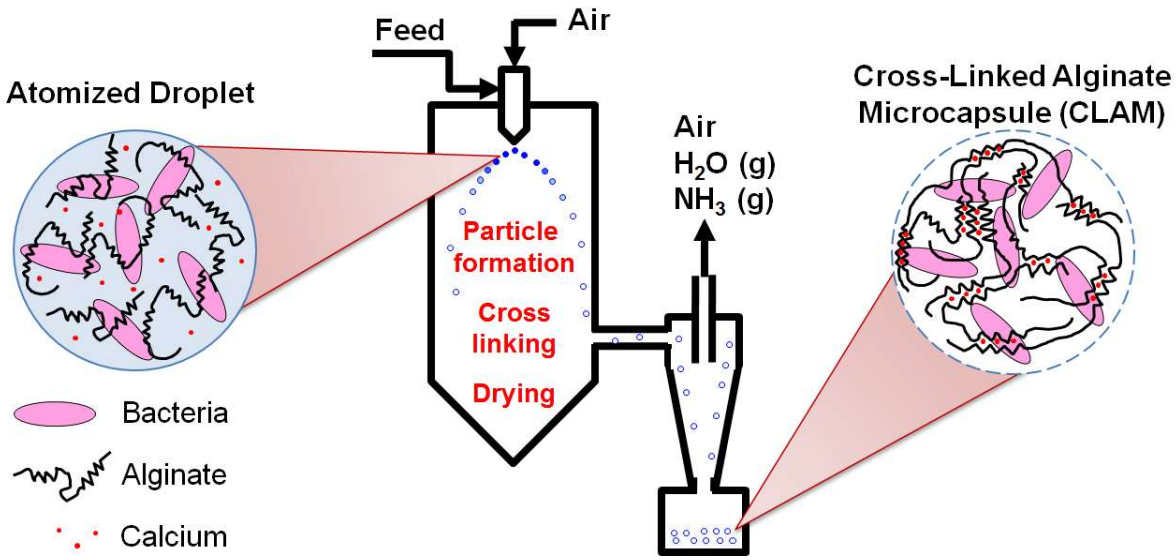

Figure 2-3 Novel spray-drying process for forming in situ cross-linked alginate microcapsules (CLAMs).

Because this formulation process relies on atomization to produce in situ cross-links, other physical microencapsulation methods that have similar mechanisms have been adapted to fit this process. More recently, a method of producing in situ cross-linked alginate matrix shell (CLAMshell) particles in a fluidized bed spray-coated was introduced (Jeoh-Zicari et al., 2020b). To produce CLAMshell coatings containing bacteria, the feed suspension is similar to that of the spray-drying process. Although the chemistry is the same upon atomization in the fluidized bed spray-coater, in the coating process, droplets collide with the fluidizing core particles, where they slowly but continually form a coating layer of in situ cross-linked alginate embedding the bacteria. The product is a cross-linked alginate matrix shell (CLAMshell) coating around a core particle.

Enzymes have been shown to be successfully encapsulated in CLAMshell particles (Kawakita et al., 2021); however, encapsulation of bacteria in CLAMshell particles produced by fluidized bed spray-coating has not been investigated. While the application of spray-dried particles containing biopesticides is more suited for spray applications, using fluidized bed spray-coating as a method for coating biopesticides onto seed is a potential opportunity. Nonetheless, both spray-drying and fluidized bed spray-coating are already established in industry and are highly scalable microencapsulation processes that can meet commercial needs required for practical utilization in the agricultural industry.

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Chapter 3 Optimizing viability and yield and improving stability of Gram-negative, non-spore-forming plant-beneficial bacteria encapsulated by spray-drying

Under Review: Kawakita, R; Leveau, J.H.J.; Jeoh, T. (2021). Optimizing viability and yield and improving stability of Gram-negative, non-spore-forming plant-beneficial bacteria encapsulated by spray-drying. *Bioprocess and Biosystems Engineering*.

3.1 Abstract

This study investigates methods to commercialize safer alternatives to chemical pesticides that pose risks to human safety and the environment. Spray-drying encapsulation of the plant-protective, antifungal bacterium *Collimonas arenae* Cal35 in *in situ* cross-linked alginate microcapsules (CLAMs) was optimized to minimize losses during spray-drying and maximize yield of spray-dried powder. At the benchtop scale, only inlet temperature significantly affected survival during spray-drying, while inlet temperature, spray rate, and alginate concentration significantly affected yield of spray-dried powder. Lowering inlet temperature to 95°C provided the greatest survival during spray-drying, while increasing inlet temperature and lowering spray rate and alginate concentration produced the highest yield. Without the CLAMs formulation, Cal35 did not survive spray-drying. When Cal35 was encapsulated in CLAMs in the presence of HICAP-100 modified starch, shelf survival was extended to three weeks in a low oxygen, low humidity storage environment. Cal35 retained its antifungal activity through spray-drying and shelf storage, supporting its potential use as a formulated biofungicide product.

3.2 Abbreviations

Amb	ambient storage condition with no oxygen or humidity control
Cal35	*Collimonas arenae* Cal35
CFU	colony forming unit
CLAM	cross-linked alginate microcapsule
KB	King's medium B
lowH	storage condition with humidity control
lowH, -O_2	storage condition with humidity and oxygen control
M	maltodextrin
M-CLAM	CLAM containing maltodextrin
PBS	phosphate buffered saline
PDA	potato dextrose agar
psia	pounds per square inch absolute
RSM	response surface methodology
S	modified starch
S-CLAM	CLAM containing modified starch
SEM	scanning electron microscopy

3.3 Introduction

U.S. pesticide usage totaled over 1.1 billion pounds, nearly one-sixth of the entire world's usage (Atwood et al., 2017). California in particular applied 205 million pounds of active ingredient, 50 million pounds of which were made up of pesticides known to cause reproductive toxicity or cancer (California Department of Pesticide Regulation, 2017). Biopesticides, which are microbial or biochemical pesticides that control pests and pathogens by non-toxic mechanisms, have recently become more relevant and desirable due to their low risk to human health and the environment; unfortunately, they make up less than 4% of total pesticide use in California (California Department of Pesticide Regulation, 2017). Some of the most abundant and successful bacterial biopesticides are spore-formers, such as those belonging to the genus *Bacillus* (California Department of Pesticide Regulation, 2017; Pérez-García et al., 2011). Spores are robust and can tolerate different kinds of stresses, including those that bacteria are typically subjected to during the commercial process of formulation, storage, and release into the field (Murphy et al., 2018). In contrast, non-spore-forming bacteria face considerable challenges during formulation and commercialization that prevent widespread application in the agricultural industry.

Collimonas arenae (*C. arenae*) Cal35 is a Gram-negative, non-spore-forming bacterial strain that was originally isolated from forest soils in California (Uroz et al., 2014). Its sequenced genome (J.-J. Wu et al., 2015) harbors a number of gene clusters whose predicted functions are consistent with the reported antifungal properties of Cal35 and other collimonads (Doan et al., 2020; Fritsche et al., 2014; Leveau et al., 2010; Mela et al., 2011; Mosquera et al., 2020). One of these is the *cpl* gene cluster, which in Cal35 codes for the biosynthesis of the fungistatic secondary metabolite carenaemin and contributes to the ability of root-applied Cal35 to protect tomato plants, in synergy with *Bacillus* bacteria, from a soilborne fungal pathogen (Akum et al., 2020). Collimonads can be found in the rhizosphere, the compartment of soil influencing the plant root system (Leveau et al.,

2010), where their ability to produce antifungal compounds (Akum et al., 2020; Fritsche et al., 2014) and chitinases (Fritsche et al., 2008) and to weather and solubilize inorganics from the soil matrix (Uroz et al., 2009) can be beneficial to plants. A key stumbling block in the commercialization of these plant-beneficial properties of collimonads is the issue of formulation. Unlike Gram-positive spore-formers such as *Bacillus* species, Gram-negative non-spore-formers, including *Collimonas*, are notoriously sensitive to the stresses (e.g. heat, moisture, oxygen, light) that are typically involved in the process of product formulation and subsequent product storage (Bashan et al., 2014; Schoebitz et al., 2013). Products containing these types of bacteria mainly exist in liquid form, and must be applied relatively quickly due to poor shelf stability (O'Callaghan, 2016).

These challenges can be addressed using microencapsulation, a method of creating protective environments for sensitive cargo including bacteria. Microcapsules consist of a cargo protected by a carrier matrix. Natural polymer carriers are favored in the food and agricultural industries, for example alginate, which is safe, biodegradable, and has the ability to gel (Draget et al., 2011). Common microencapsulation processes include spray-drying, fluidized bed spray-coating, extrusion and oven drying, and freeze-drying; each of these has its unique advantages and disadvantages (Jyothi et al., 2010; Schoebitz et al., 2013). Spray-drying is generally the most desirable because it is industrially scalable, relatively economical, and can simultaneously encapsulate and dry the intended cargo (Rezvankhah et al., 2020; Strobel et al., 2020b). Because it is a convective drying process with a very short residence time, high inlet temperatures are required to form dry microcapsules, introducing a potential concern when encapsulating non-spore-forming bacteria (O'Callaghan, 2016; Schoebitz et al., 2016). Moreover, careful selection

of process parameters depending on the formulation and species of bacteria requires optimization and makes the ideal spray-drying conditions cargo-specific and difficult to generalize.

We aimed to optimize survival of the Gram-negative non-spore-former *C. arenae* Cal35 during spray-drying and shelf storage. More specifically, Cal35 was encapsulated by spray-drying using a UC Davis patented process to form in situ cross-linked alginate microcapsules (CLAMs) (Jeoh-Zicari et al., 2020). This formulation process has previously demonstrated effective microencapsulation of bacteria (Strobel et al., 2018), enzymes (Santa-Maria et al., 2012), polymers (Wong et al., 2020), and lipids (Strobel et al., 2020a; Strobel et al., 2016). Two spray-drying parameters (inlet temperature and spray rate) and one formulation parameter (alginate concentration) were optimized using response surface methodology. Loss of bacterial viability during spray-drying and shelf storage under humidity- and oxygen-controlled environments were measured. Characterization of spray-dried powders included scanning electron microscopy (SEM), particle size analysis, mercury porosimetry, and moisture analysis. The antifungal activity of Cal35 after spray-drying and shelf storage was tested against *Aspergillus niger* (*A. niger*) N402.

3.4 Materials and Methods

3.4.1 Materials

Peptone, potassium phosphate dibasic (K_2PO_4), magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), glycerol, potato dextrose broth, succinic acid, 28-30% ammonium hydroxide (NH_4OH), dicalcium phosphate (Ca_2HPO_4), potassium hydroxide (KOH), Tween 80, and cheesecloth were purchased from Fisher (Waltham, MA). Maltodextrin (dextrose equivalent 4.0-7.0) and low-viscosity (4-12 cP), MQ 200, alginate derived from brown algae (A1112) were from Millipore-Sigma (Burlington, MA). Agar was from BTS (Houston, TX) and concentrated 20x phosphate-buffered saline (PBS) from VWR (Radnor, PA). HI-CAP 100 modified starch was from Ingredion (Westchester, IL). Milli-Q water from a Millipore Ultrapure Water Purification System

was used to prepare all solutions. RP-1AN packs were supplied by Mitsubishi Gas Chemical America, Inc. (New York, NY). *C. arenae* Cal35 was originally isolated from forest soil in the Jug Handle State Natural Reserve in Mendocino County, CA (Uroz et al., 2014). *A. niger* N402 (ATCC 64974) is a derivative of CBS120.49 (Bos et al., 1988) and has been used previously in assays to demonstrate the antifungal activity of *C. arenae* Cal35 (Akum et al., 2020; Doan et al., 2020; Fritsche et al., 2014; Mela et al., 2011; Mosquera et al., 2020).

3.4.2 Methods

3.4.2.1 Culture conditions for C. arenae Cal35

A stock of *C. arenae* Cal35 was stored in 50% glycerol at -80°C. King's medium B (KB) was prepared with the following formula: 20 g/L peptone, 1.15 g/L K_2HPO_4, 0.73 g/L $MgSO_4 \cdot 7H_2O$, and 12.6 g/L glycerol. KB agar contained 15 g agar per L. Cal35 was streaked from glycerol stocks onto KB agar and incubated at 28°C. After 48-72 hours, a single colony was transferred into 3 mL KB and incubated at 28°C and 200 rpm for 24 hr. From this overnight culture, 1% inoculum was transferred into larger volumes of fresh KB to scale up for spray-drying by incubation for 24 hr at 28°C and 200 rpm before harvesting and processing into the feed for spray-drying (*section 3.4.2.3*).

3.4.2.2 Experimental design and response surface methodology

A face-centered, central composite design with four center points (18 treatments) was used to optimize bacterial survival and powder yield after spray-drying. Three control factors including two process parameters (inlet temperature and spray rate) and one formulation parameter (alginate concentration) were varied with equal spacing at three levels. Actual values for each factor in the experimental design are shown in **Table 3-1**.

Table 3-1 Values of alginate concentration, inlet temperature, and spray rate at low and high levels.

Factor	Units	Factor Levels

		Low	High
Alginate Concentration (x_1)	%, w/w	1	3
Inlet Temperature (x_2)	°C	95	125
Spray Rate (x_3)	g/min	10	17

Coded values (X_i), required for each factor as inputs to the response surface model, can be converted from actual values (x_i) following Eq. [3-1]:

$$Coded\ value\ (X_i) = \frac{x_i - \overline{x}}{\frac{1}{2}(x_{high} - x_{low})}, i = 1, 2, or\ 3 \qquad [3\text{-}1]$$

where $\overline{x}$ is the mean of the actual values and x_{high} and x_{low} are the maximum and minimum values, for factor i.

Assuming the sparsity-of-effects principle that higher order (>2) effects are not significant (Neter et al., 1996), the behavior of a response (Y) may be described by Eq. [3-2], derived from a second-order regression model:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i<j}^{n} \sum_{j}^{n} \beta_{ij} X_i X_j \qquad [3\text{-}2]$$

where n is the number of control factors, β_0 is the constant coefficient that represents the mean response of Y when $X = 0$, β_i is the linear effect coefficient, β_{ii} is the quadratic effect coefficient, β_{ij} is the interaction effect coefficient, and X_i and X_j are the coded values of the process and formulation parameters. The second-order polynomial function correlates each response variable with alginate concentration (X_1), inlet temperature (X_2), and spray rate (X_3), in terms of their coded value (X_i). Treatments of the response surface design were constructed and randomized in JMP

(Statistical Discovery from SAS, Cary, NC). The desirability function in JMP calculated the optimal set of formulation and process parameters for maximizing survival and yield.

3.4.2.3 Formulation of CLAMs containing C. arenae Cal35

To prepare the spray-drying feed, 1.5 L of Cal35 culture were grown as specified in section 2.2.1. Cells were prepared for formulation by centrifuging cultures at 4,200 rpm for 15 min at room temperature and removing the supernatant; cell pellets were suspended in succinic acid, as detailed below.

For the response surface experiment, three formulations for producing cross-linked alginate microcapsules (CLAMs) with varying alginate concentrations were prepared. Here, the CLAMs formulation consists of alginate, succinic acid, and dicalcium phosphate (Jeoh-Zicari et al., 2020). The final concentration on a weight basis of the feed (final weight 50 g) included 10% maltodextrin (as the excipient), 1, 2, or 3% sodium alginate, 0.5, 1, or 1.5% succinic acid, and 0.25, 0.5, or 0.75% dicalcium phosphate, to maintain a 4:2:1 ratio of alginate-to-succinic acid-to-dicalcium phosphate. First, 5 g maltodextrin, the appropriate mass of dicalcium phosphate, Milli-Q water, and the corresponding mass of alginate were combined up to 25 g. This suspension was autoclaved at 121°C and 15 psi for three minutes. Second, a filter-sterilized concentrated solution of succinic acid adjusted to pH 7 with 28-30% ammonium hydroxide was diluted to the correct concentration up to 25 g and used to resuspend the pelleted Cal35 from the centrifugation step. Finally, these two 25-g suspensions were combined forming the 50 g feed.

For the shelf stability experiment, four formulations were prepared to 50 g for spray-drying and immediate shelf storage. Two contained the CLAMs formulation with a final concentration on a weight basis of 2% (w/w) alginate, 1% (w/w) succinic acid, 0.5% (w/w) dicalcium phosphate with the addition of either 10% maltodextrin (M-CLAMs), or 10% modified starch (S-CLAMs),

prepared using the same method described for the response surface experiment. Two formulations were also prepared without CLAMs that were comprised of only the excipient: either 10% maltodextrin (M) or 10% modified starch (S).

Spray-drying was performed using a BUCHI B-290 benchtop spray-dryer (BUCHI Corporation, New Castle, DE). Combinations of inlet temperatures and spray rates were chosen based on the randomized experimental design setup described in section 2.2.2 (**Supplementary Table 3-1**). For all treatments, aspirator airflow rate and nozzle pressure remained constant at 35 m^3/hr and 0.5 bar, respectively. By analyzing the data from the response surface experiment, we derived optimized process parameters to set the inlet temperature and spray rate for spray-drying in the shelf stability study.

3.4.2.4 Enumeration and characterization of Cal35 in CLAMs

Loss of bacteria viability during spray-drying was quantified as the log reduction in viable cell counts before and after spray-drying (Eq. [3-3]).

$$Loss\ in\ viability = Viability_{feed} - Viability_{powder} \quad [3\text{-}3]$$

where $Viability_{feed}$ represents the count of viable bacteria in the feed suspension prior to spray-drying, determined as log [CFU/g solids] using the serial dilution method, by assuming the density of the feed was equal to water (1 g/mL) and knowing the total solids of the feed suspension; $Viability_{powder}$ represents the count of viable bacteria after spray-drying, also determined as log [CFU/g solids] using the serial dilution method, by dissolving 0.03 ± 0.001 g of powder in 12.5 mL 1X PBS, mixing by inversion for one hour before plating, and factoring in the moisture content of the powder. The limit of detection for bacteria in the spray-dried powder was 4 log[CFU/g].

Yield was calculated by taking the ratio of solids (i.e. powder) collected in the cyclone and collection chamber of the spray-dryer and the solids in the feed suspension (Eq. [3-4]).

$$Yield\ (\%) = (Total\ Solids_{powder}/Total\ Solids_{feed})*100 \quad [3\text{-}4]$$

Particle size distribution and geometric weighted mean diameter of spray-dried powders were measured by light diffraction using a Malvern Mastersizer 3000 (Malvern PANalytical Inc., Westborough MA).

Particle porosity was investigated using a mercury intrusion method completed by Particle Testing Authority (Norcross, GA). Approximately 0.1 g of spray-dried powder was subjected to mercury using a pressure range from 0.1 to 61,000 psia.

Moisture content was determined gravimetrically by drying in an oven at 60 - 70°C for three days. Water activity was measured using an Aqualab series 3TE water activity meter (METER Group Inc., Pullman, WA).

3.4.2.5 Assessing shelf stability of Cal35 in CLAMs

Spray-dried bacterial cells from four formulations, two containing maltodextrin alone (M) or maltodextrin with CLAMs (M-CLAMs) and two containing modified starch alone (S) or modified starch with CLAMs (S-CLAMs), were subjected to three different storage conditions immediately after spray-drying to measure the viability of encapsulated bacteria over time. Samples of spray-dried powder were weighed to 0.03 ± 0.001 g in triplicate per time point for each storage condition, and sealed in vials. Each storage condition was created by enclosing the vials in an airtight container (BD GasPak^{TM}). Storage condition #1 (Amb) had no environmental controls. Storage condition #2 (lowH) had controlled relative humidity (RH) kept at less than 10% using a saturated

salt solution of KOH; vials in this environment were left partially open to maintain equilibrium with the controlled humidity. Storage condition #3 (lowH, -O2) had controlled relative humidity (<10%) and oxygen levels (< 0.1%) using a moisture/oxygen scavenging RP packet that was placed inside the vial with the powder. All conditions were at room temperature (~21°C). Samples were removed from the containers every other day for the first week, then weekly afterwards. Powders were dissolved in 12.5 mL of 1X PBS, mixed by inversion for one hour, and plated onto KB using the serial dilution method to count CFUs.

3.4.2.6 Testing antifungal activity of spray-dried and stored Cal35

From KB plates used to count CFUs of Cal35 that survived spray-drying and shelf storage, single colonies were inoculated in fresh KB broth and incubated at 28°C and 200 rpm for 24 h, before being stored in a 25% glycerol solution at -80°C. These stock samples were inoculated onto KB agar plates and allowed to grow for 48 hr at 28°C, prior to use in antifungal assays against *A. niger* N402 as the target, as described below.

Sporulating *A. niger* N402 grown on potato dextrose agar (PDA) was cut from the agar, and inserted into a vial containing sterile saline solution supplemented with 0.005% Tween 80. After mixing by vortex for one minute, the suspension containing the spores was filtered using sterile cheesecloth to remove agar chunks and mycelia.

Following a previously published assay (Doan et al., 2020; Fritsche et al., 2014; Mela et al., 2011; Mosquera et al., 2020), biomass from the KB agar plates inoculated with spray-dried and shelf-stored Cal35 samples was streaked in a line at the center of a PDA plate. Then, from the filtered fungal spore suspension, 5 µL were pipetted onto the agar surface 2 cm away from the bacterial streak. These plates were incubated at 28°C for 48 hr and photographed.

3.4.2.7 Statistical analysis

The randomized three factor response surface design (**Supplementary Table 3-1**) was set up and analyzed with the statistical software JMP. Analysis of variance (ANOVA) and linear regression determined significance of individual factors and any interaction or second-order effects while correlating the formulation (alginate concentration) and process (inlet temperature and spray rate) variables to the response variables, which was expressed by a second-order polynomial function. Simple linear regression was used to investigate any correlation between response variables. P values less than 0.05 were considered significant.

3.5 Results

3.5.1 Optimum spray-drying parameters for minimal Cal35 cell death and maximal powder yield

We utilized response surface methodology (RSM) to identify formulation and spray-drying parameters (x_1 = alginate concentration, x_2 = inlet temperature, and x_3 = spray rate) that minimized loss of viable *C. arenae* Cal35 and maximized yield of spray-dried cross-linked alginate microcapsules (CLAMs). The resulting second-order models for loss of viability, and yield of spray-dried powder in terms of the formulation and process variables' coded values (X_i) (Eq. [3-1]) are presented in Eq. [3-5] and Eq. [3-6].

$$Loss = 4.19 + 0.09X_1 + 0.34X_2 - 0.18X_3 + 0.19X_1X_2 + 0.13X_1X_3 - 0.07X_2X_3 + 0.09X_1^2 - 0.38X_2^2 + 0.31X_3^2 \quad [3\text{-}5]$$

$$Yield = 45.64 - 5.42X_1 + 1.92X_2 - 6.17X_3 + 0.49X_1X_2 - 0.59X_1X_3 - 0.39X_2X_3 - 1.35X_1^2 + 2.85X_2^2 - 1.80X_3^2 \quad [3\text{-}6]$$

3.5.1.1 Inlet temperature impacts survival of Cal35 during spray-drying

The regression model representing loss of viable Cal35 during spray-drying in CLAMs (Eq. [3-5]) was not significant ($p = 0.27$), with just 23% of the variability explained by all three factors – alginate concentration (x_1), inlet temperature (x_2), and spray rate (x_3) – combined (**Figure 3-1A**, **Table 3-2**). Only inlet temperature had a significant effect on loss of viability ($p = 0.034$) (**Table 3-2**). The lowest losses of viable Cal35 ranged from 3.4 to 3.9 log units at an inlet temperature of 95°C (low), while the greatest losses ranged from 4.5 to 4.9 log units at 125°C (high) (**Figure 3-2A**, **Supplementary Table 3-1**). Cooling of the inlet temperature by 30°C reduced loss of viable Cal35 by nearly 1.5 log units, with no significant contribution from alginate concentration or spray rate.

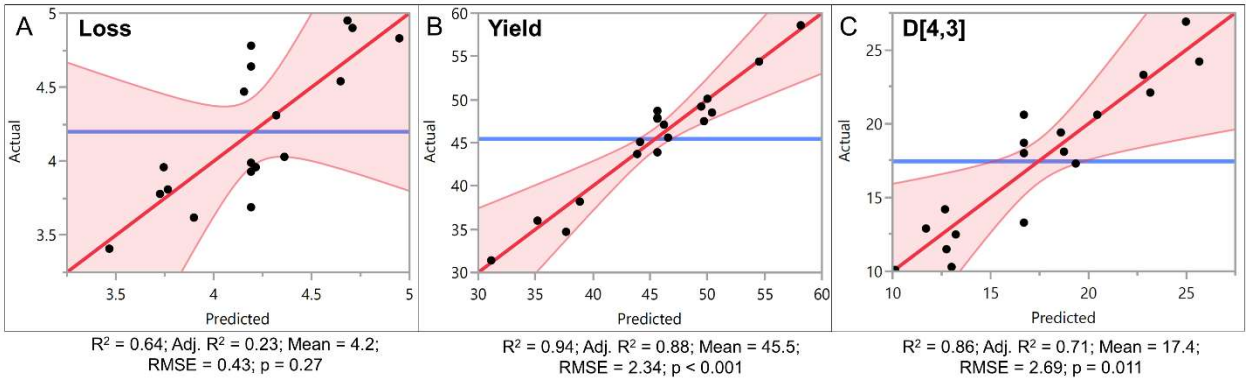


Figure 3-1 Predicted vs actual response plots for: (A) loss of viability (log reduction), (B) powder yield (%), and (C) volume weighted mean particle size (μm). Coefficient of determination (R^2), adjusted R^2, mean response value, root mean squared error (RMSE), and p-value of the prediction model are reported below the plot. The diagonal red line represents the linear fit of the data points. The horizontal blue line is the mean.

Table 3-2 P-values of first-order, second-order, and interaction effects of alginate concentration (x_1), inlet temperature (x_2), and spray rate (x_3) for each response. Significant effects ($p < 0.05$) are in bold.

	p-values					
Source	Loss	Yield	D[4,3]	Outlet Temp.	Moisture Content	Water Activity

Alginate concentration (x_1)	0.545	**<0.001**	**0.011**	**0.001**	0.060	0.525
Inlet temperature (x_2)	**0.034**	**0.032**	0.537	**<0.001**	0.135	0.305
Spray rate (x_3)	0.213	**<0.001**	**0.008**	**<0.001**	0.069	0.050
$x_1 \cdot x_2$	0.236	0.571	**0.017**	0.560	1.000	0.818
$x_1 \cdot x_3$	0.421	0.497	0.398	0.844	0.553	0.949
$x_2 \cdot x_3$	0.677	0.652	**0.006**	0.106	0.087	0.097
x_1^2	0.750	0.368	0.596	0.794	0.258	0.396
x_2^2	0.180	0.080	0.087	0.519	0.758	0.859
x_3^2	0.264	0.240	0.576	0.063	0.778	0.830

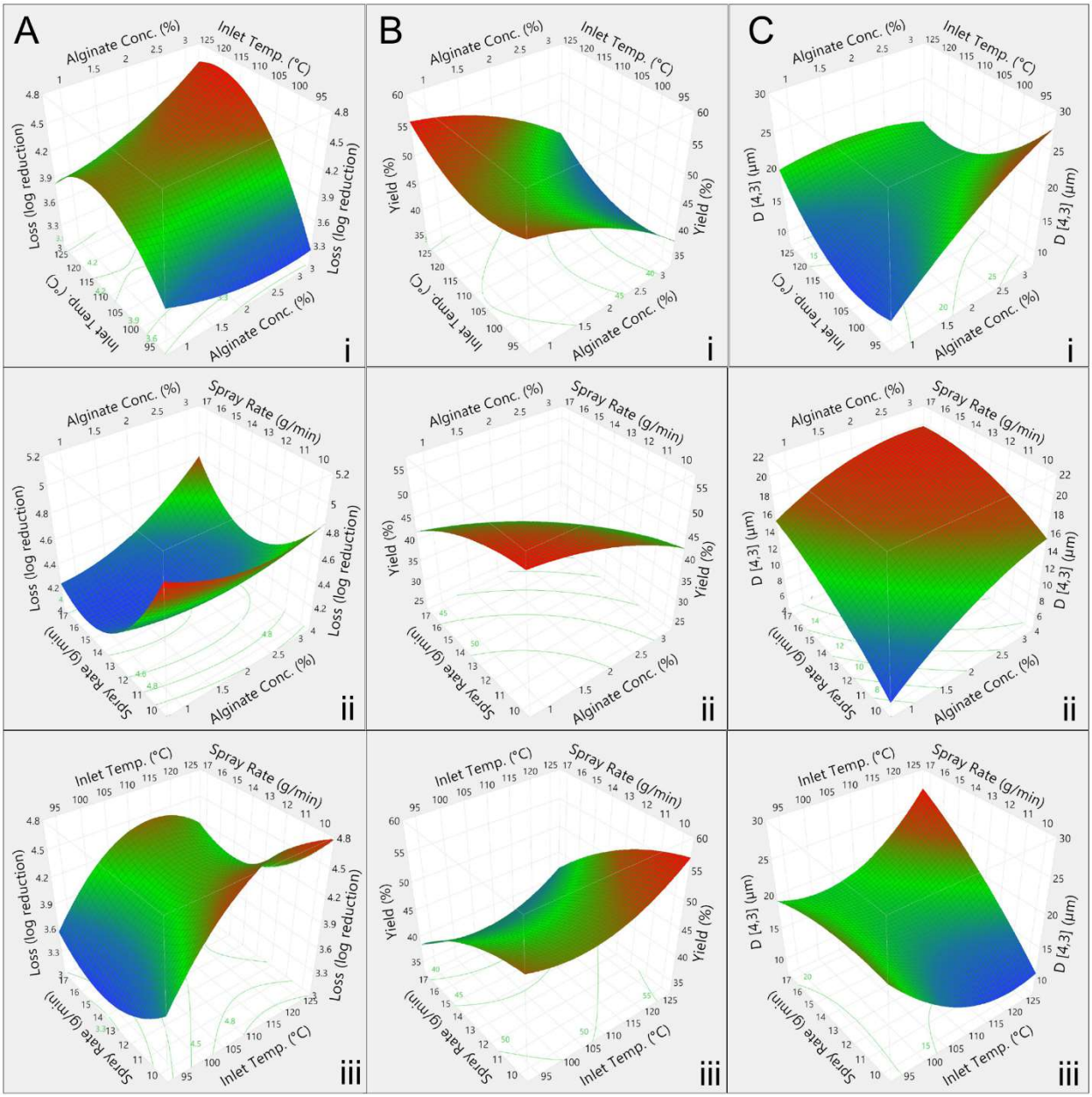


Figure 3-2 Surface plots with underlying contours illustrating the response of (A) loss in viability during spray-drying (log reduction), (B) spray-dried powder yield (%), and (C) D[4,3] or volume weighted mean particle diameter (µm), to alginate concentration (%), inlet temperature (°C), and spray rate (g/min). Each row varies two factors while keeping the third constant at the middle level: (i) alginate concentration and inlet temperature, (ii) alginate concentration and spray rate, and (iii) inlet temperature and spray rate.

3.5.1.2 Alginate concentration, inlet temperature, and spray rate impact yield of spray-dried powder containing Cal35

The regression model describing powder yield during spray-drying of CLAMs (Eq. [3-6]) was significant ($p < 0.001$), with high R^2 (0.94) and adjusted R^2 (0.88) values (**Figure 3-1B**). Alginate concentration ($p < 0.001$), inlet temperature ($p = 0.032$), and spray rate ($p < 0.001$) all significantly affected yield, supporting high accuracy of the model (**Table 3-2**). A maximum yield of 59% was achieved when the 1% alginate formulation (low) was sprayed with an inlet temperature of 125°C (high) at 10 g/min (low). Conversely, spraying the 3% alginate formulation (high) at 95°C (low) and 17 g/min (high) resulted in the lowest yield of 31% (**Supplementary Table 3-1, Figure 3-2B**). Raising inlet temperature improved yields by about 5% with a constant spray rate (**Figure 3-2B**, **i**) or alginate concentration (**Figure 3-2B**, **iii**). Yield increased over 20% when lowering both alginate concentration and spray rate (**Figure 3-2B**, **ii**).

3.5.1.3 Alginate concentration and spray rate impact particle size of spray-dried powder containing Cal35

The regression model describing particle size was significant ($p = 0.011$) (Error! Reference source not found.**C**). Higher alginate concentrations ($p = 0.011$) and faster spray rates ($p = 0.008$) resulted in a greater volumetric weighted mean particle diameter of CLAMs (**Table 3-2**, **Figure 3-2C**). Mean particle size was at a minimum of 10 μm when alginate concentration and spray rate were low, and at a maximum of 27 μm at their respective high levels (**Figure 3-2C**, **ii**, **Supplementary Table 3-1**). Although inlet temperature had no significant effect ($p = 0.537$) on particle size, significant interaction effects were observed between inlet temperature and alginate concentration ($x_1 \cdot x_2$) and inlet temperature and spray rate ($x_1 \cdot x_2$) (**Table 3-2)**. Only at low inlet temperatures did greater alginate concentrations generate larger particle sizes (**Figure 3-2C**, **i**).

Similarly, only at high inlet temperatures did faster spray rates produce larger particle sizes (**Figure 3-2C**, **iii**).

3.5.1.4 Parameter optimization to maximize survival and yield during spray-drying

We determined that the optimal conditions for spray-drying Cal35 and minimizing loss of bacterial viability and maximizing powder yield involved using a concentration of 1.3% alginate in the CLAMs formulation at 95°C inlet temperature and 12.9 g/min spray rate (**Supplementary Figure 3-4**). Inlet temperature significantly affected loss in viability of Cal35 during spray-drying for this design space, while all three factors did for powder yield (**Table 3-2**).

Additionally, lower losses in viability correlated with cooler outlet temperatures ($p < 0.001$), larger particle size ($p = 0.036$), and higher water activity ($p = 0.046$) (**Figure 3-3A**, **3B**, and **3C**). Greater powder yield correlated with decreasing particle size ($p = 0.025$) and lower moisture content ($p = 0.019$) (**Figure 3-3D**, **Supplementary Figure 3-3A**). Both moisture content and water activity, however, were not significantly influenced by alginate concentration, inlet temperature, or spray rate (**Table 3-2**).

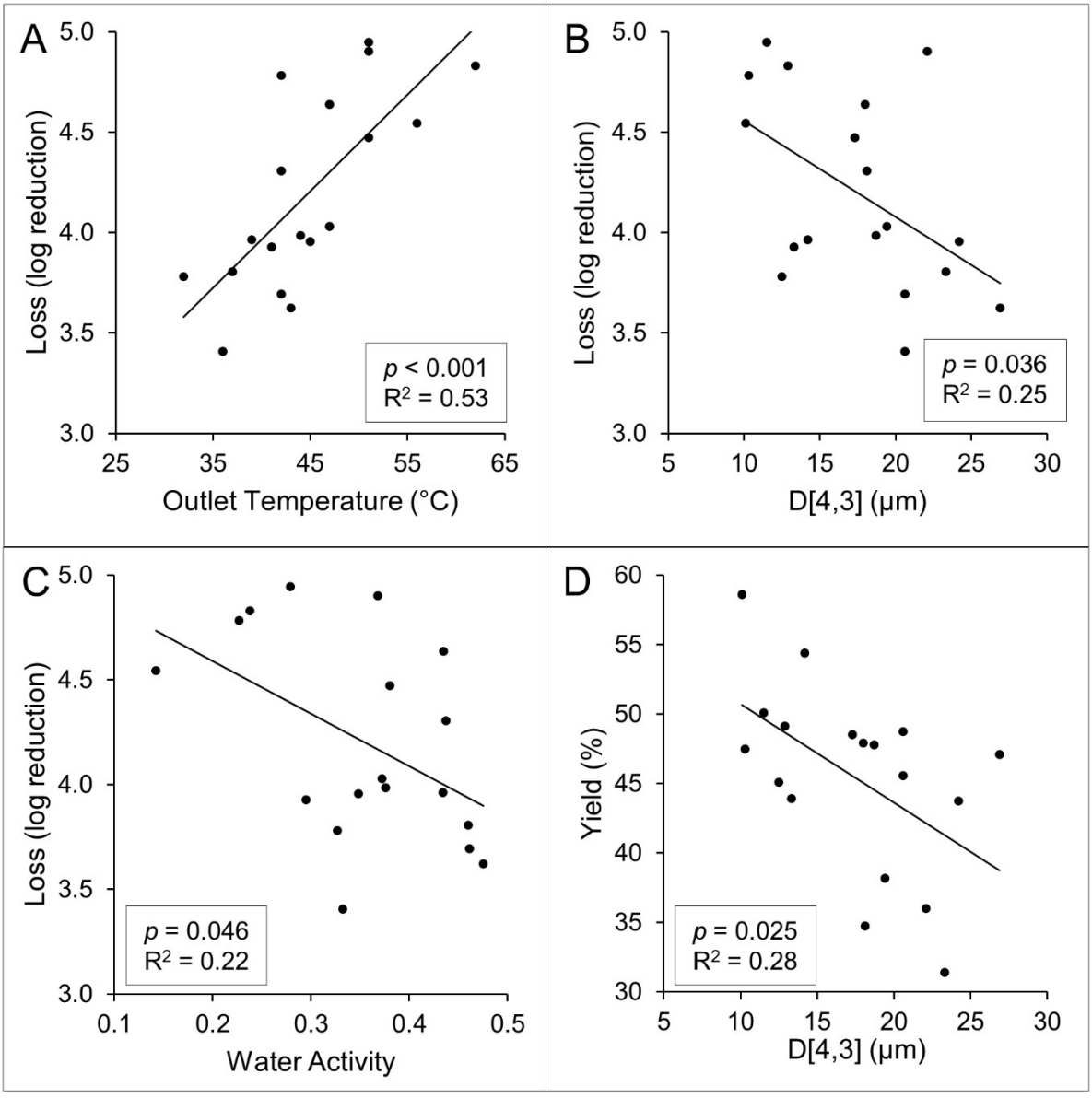


Figure 3-3 Linear regression of significant correlations between (A) loss and outlet temperature, (B) loss and particle size, (C) loss and water activity, and (D) yield and particle size. The p-value and coefficient of determination (R^2) are displayed in each graph.

3.5.2 Shelf-stability of Cal35 in CLAMs

Starches and starch-derivatives are used extensively in encapsulation because they are highly soluble and can greatly increase solids loading at low viscosity (Schoebitz et al., 2013). Using optimal spray-drying parameters (section 3.1.4), we examined how loss of viability during spray-drying and subsequent shelf storage was impacted by replacing maltodextrin with modified starch

as the excipient, or by excluding the CLAMs from the formulation altogether (i.e. spray-drying only in the presence of maltodextrin or modified starch). We found that size and morphology of powder particles were more dictated by the type of excipient than by the addition of CLAMs. Spray-dried particles containing only maltodextrin (M) or maltodextrin with CLAMs (M-CLAMs) resembled collapsed spheres (**Figure 3-4A** and **4B**), whereas particles containing only modified starch (S) or modified starch with CLAMs (S-CLAMs) appeared more spherical (**Figure 3-4C** and **4D**). S and S-CLAMs exhibited a bimodal distribution with a second peak near 250 μm, due to visible particle aggregates (**Figure 3-5**, **Figure 3-4C** and **4D**). Consequently, the volume weighted mean particle diameter for S and S-CLAMs was overestimated (**Supplementary Table 3-2**). Based off the higher first peak, most particles were sized between 15 and 20 μm.

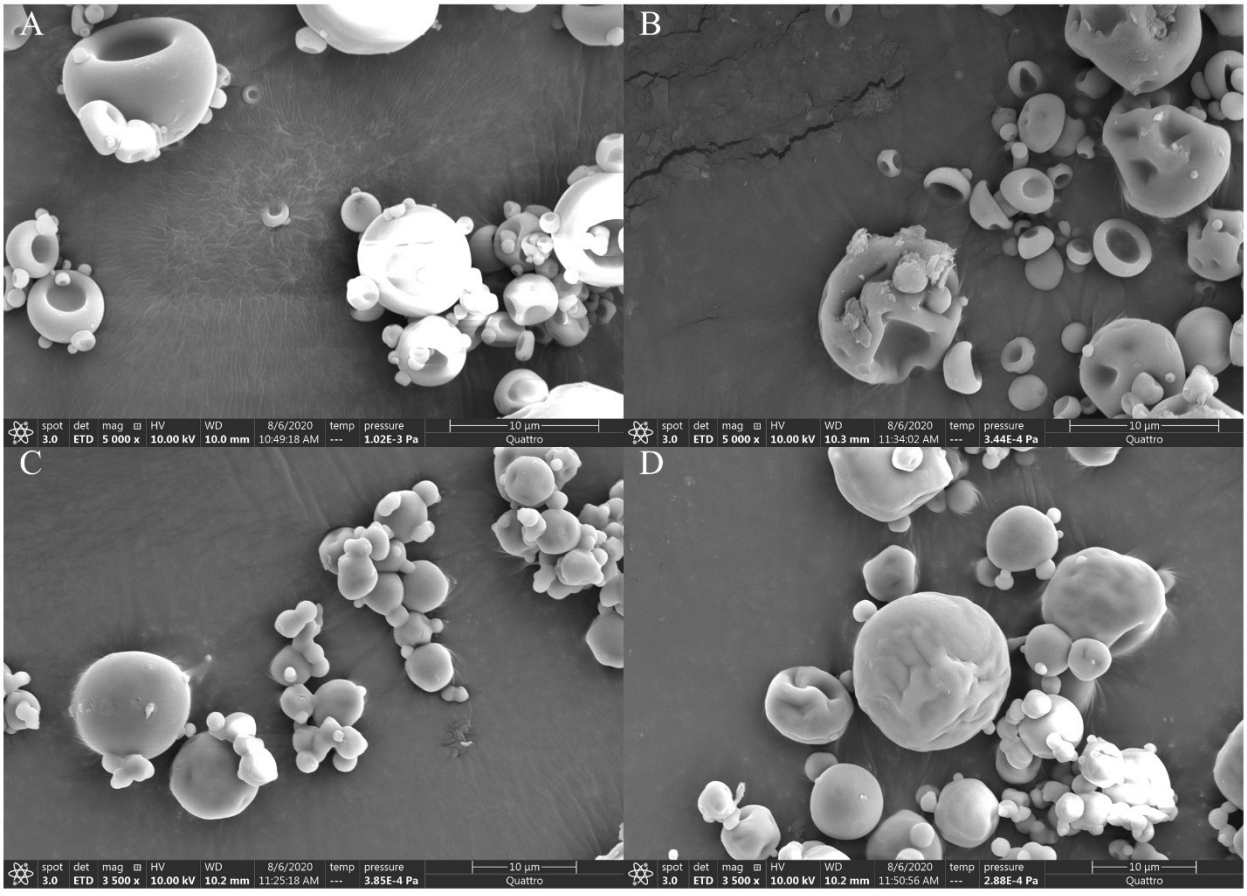


Figure 3-4 Surface SEMs of (A) maltodextrin-only particles (M), (B) maltodextrin plus CLAMs (M-CLAMs), (C) modified starch-only particles (S), and (D) modified starch CLAMs (S-CLAMs). All error bars read 10 µm.

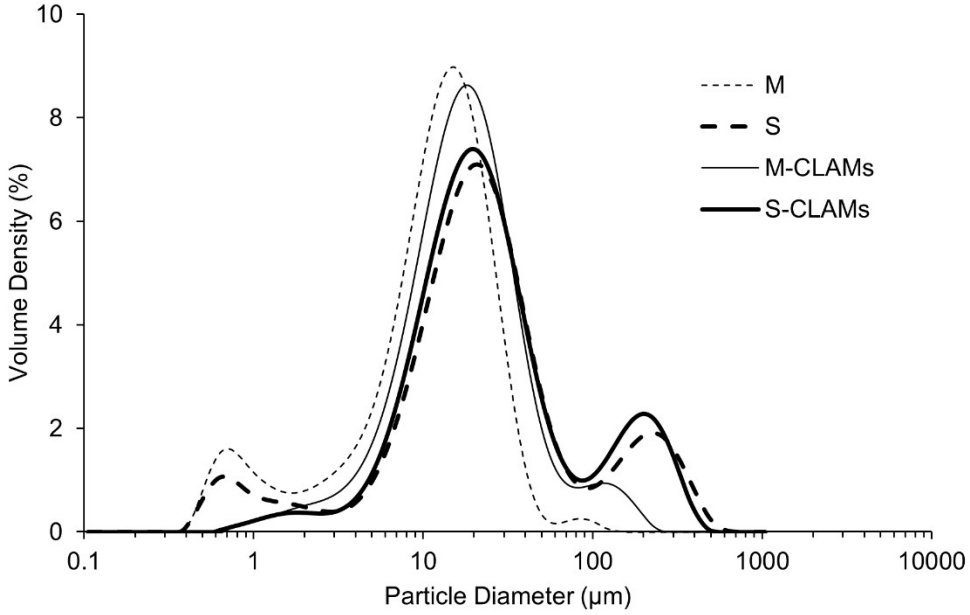


Figure 3-5 Particle size distribution of maltodextrin-only particles (M), maltodextrin plus CLAMs (M-CLAMs), modified starch-only particles (S), and modified starch plus CLAMs (S-CLAMs).

3.5.2.1 Survival of Cal35 spray-dried in CLAMs with maltodextrin or modified starch

In all formulations we tested (i.e. M, M-CLAMs, S, and S-CLAMs), the viability of Cal35 in the feed consistently measured ~10.5 log CFU/g. Those formulations lacking CLAMs (i.e. M and S) showed no survival above the detection limit after spray-drying (**Supplementary Table 3-2**). By contrast, incorporating CLAMs (i.e. M-CLAMs and S-CLAMs) significantly helped Cal35 survive, and reduced losses of Cal35 during spray-drying to 4 and 3 log units, respectively (**Supplementary Table 3-2**). Spray-drying of CLAMs without excipients (3% solids) proved impractical due to unusable levels of collectable powder and significantly higher temperature requirements to remove the extra moisture as a result of a very low solids loading.

3.5.2.2 Modified starch CLAMs in oxygen and humidity scavenged conditions prolonged viability of Cal35

Encapsulated Cal35 (M- or S-CLAMs) was stored at ambient temperature in an uncontrolled environment (Amb), in a low humidity environment (lowH), and in an oxygen and humidity scavenged (lowH, -O_2) environment. Overall, Cal35 survived longer in S-CLAMs than in M-CLAMs (**Figure 3-6**). Cal35 encapsulated in S-CLAMs experienced a 2.5 log reduction by Week 1 in lowH and a 2.1 log reduction by Week 3 in lowH, -O_2, with no detectable CFUs thereafter. When encapsulated in M-CLAMs, Cal35 experienced a 1.3 log reduction by Day 3 in lowH, -O_2, with no CFUs recoverable on Day 1 in lowH. No viable Cal35 were detected after 1 day in M-CLAMs or S-CLAMs without humidity or oxygen control (Amb). One thing all treatments had in common (**Figure 3-6**) was a 1-1.5 log unit reduction in CFUs after the first day in storage, followed by a more steady decline in viability until there were no longer any CFUs above the limit of detection.

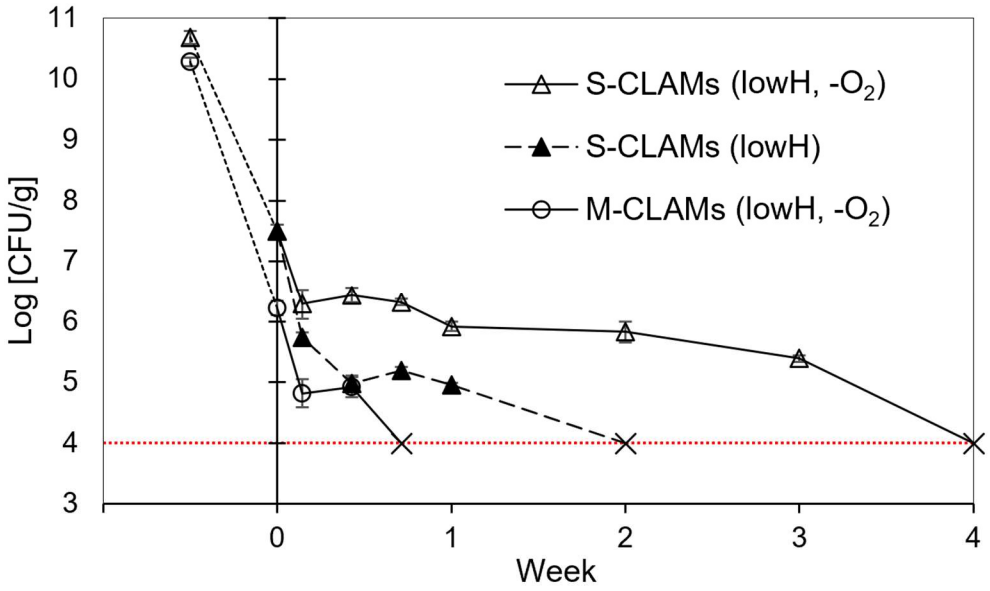


Figure 3-6 Stability of Cal35 encapsulated in S-CLAMs and M-CLAMs over a four week period in a humidity controlled environment with a saturated salt solution (lowH) or an oxygen and humidity controlled environment with RP packs (lowH, -O_2). Points before Week 0 represent CFUs of the feed. Points marked with an 'X' indicate instances where CFU counts dropped below

the limit of detection of 4 log[CFU/g] (indicated by the dotted red line). M-CLAMs in lowH and Amb, and S-CLAMs in Amb were excluded from this graph because no CFUs were detected on Day 1 for any of these treatments.

3.5.2.3 Confirming antifungal activity of Cal35 after spray-drying and storage

The antifungal activity of encapsulated *C. arenae* Cal35 was tested in standardized confrontation assays with *Aspergillus niger* N402 (**Figure 3-7**). Figures 7a and b show control experiments: without any Cal35 present, the fungi grew radially from the point of inoculation (**Figure 3-7A**), whereas with Cal35 on the same plate, fungal growth close to the bacteria was suppressed (**Figure 3-7B**). This is the expected antifungal behavior of Cal35, as shown previously (Doan et al., 2020). To verify that spray-drying and shelf storage had no effect on the antifungal capability of Cal35, bacteria recovered from M-CLAMs or S-CLAMs were used in the confrontations assays to reveal that activity was indeed retained (**Figure 3-7C** and **7D**, respectively) even after 3 weeks of storage as S-CLAMs under lowH, -O_2 conditions (**Figure 3-7D**, **ii**).

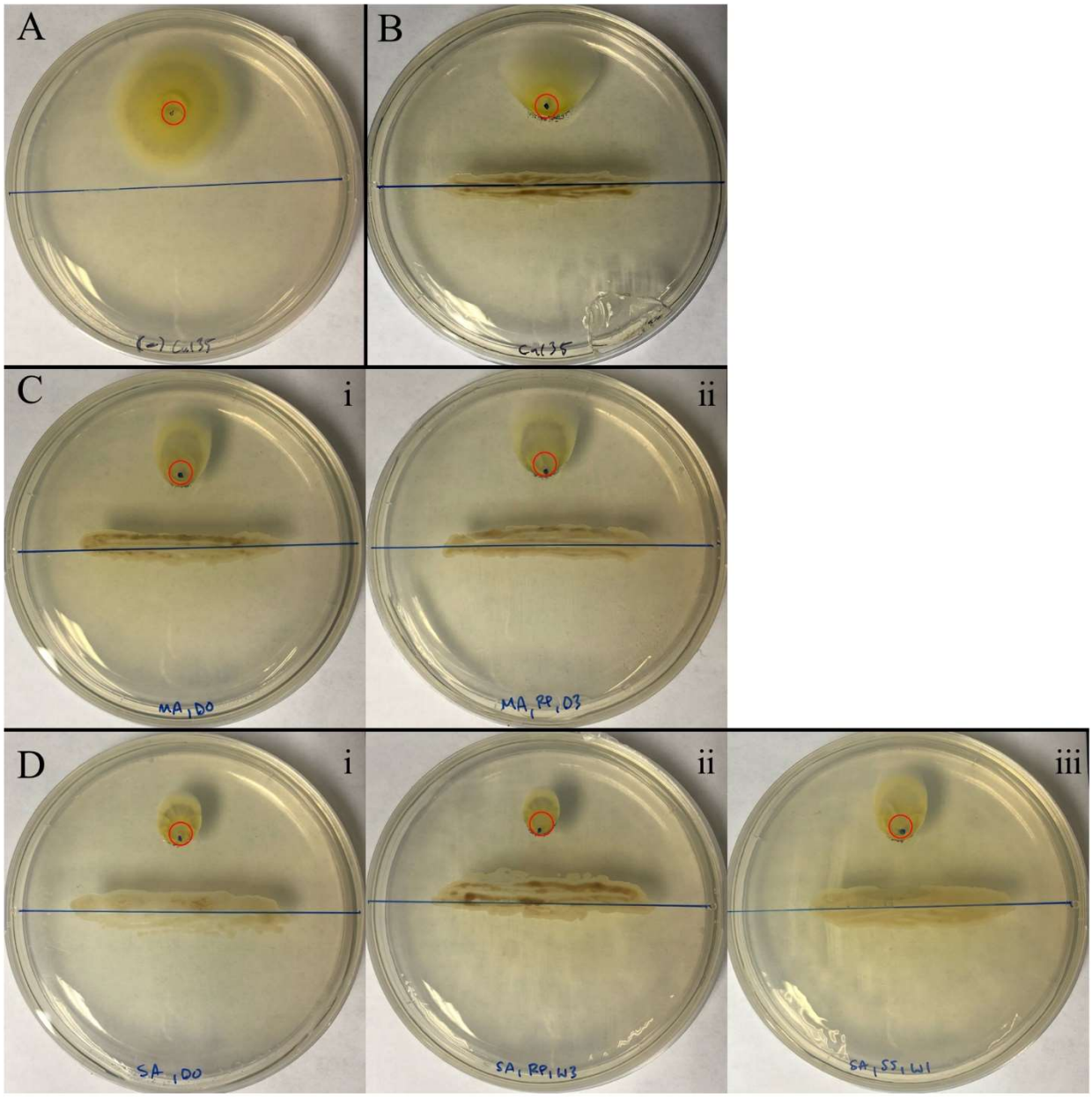


Figure 3-7 Confrontation assay of *C. arenae* Cal35 and *A. niger* N402 on PDA agar plates. (A) Negative control containing only *A. niger* N402. (B) Freshly cultured Cal35 from KB, not spray-dried. (c) Cal35 encapsulated in M-CLAMs on: (i) Day 0, and (ii) Day 3 in lowH, -O_2. (D) Cal35 encapsulated in S-CLAMs on: (i) Day 0, (ii) Week 3 in lowH, -O2, and (iii) Week 1 in lowH.

3.6 Discussion

Many plant-beneficial bacterial species qualify, based on performance in lab, greenhouse or even field experiments, as potential candidates for use as biopesticides in agriculture. However,

whether applied on seed, in-furrow, or on foliage, these bacteria must survive and remain active sufficiently long to provide their intended benefit. To ensure such survival, encapsulation is an often proposed solution (Bashan et al., 2014; Schoebitz et al., 2013). Most non-spore-forming Gram-negative bacteria do not tolerate the encapsulation process and therefore do not maintain high viability and efficacy in storage and until application, except in cases where the bacteria is accustomed to extreme environments, such as leaf surfaces (Strobel et al., 2018). This is one of the reasons why *Bacillus*-based products dominate the biofungicide market (O'Callaghan, 2016). Some of the less stressful encapsulation methods, such as extrusion into a curing bath, have demonstrated excellent survival rates with non-spore-formers (Humbert et al., 2017; Schoebitz et al., 2012; Z. S. Wu et al., 2014); unfortunately, these methods are not easily or readily scaled up to be feasible in industry. Spray-drying is one of the most attractive encapsulation processes that meets this need.

3.6.1 Spray-drying

In this study, we demonstrated the potential of our patented spray-dried cross-linked alginate microencapsulation (CLAM) technology for encapsulating Gram-negative non-spore-forming plant-beneficial bacteria as a dry product. Encapsulation of *M. radiotolerans*, a leaf dwelling bacteria, in CLAMs constituted a 0.5 log reduction, however, influence of formulation and encapsulation parameters were not explored (Strobel et al., 2018). By utilizing response surface methodology (RSM), we identified meaningful process and formulation parameters that provided optimal survival of Cal35 and yield of spray-dried powder. The overwhelming majority of studies that use RSM and optimization experiments to maximize survival and yield for spray-drying involves Gram-positive probiotics in the food industry (Behboudi-Jobbehdar et al., 2013; Broeckx et al., 2017; Hernández-López et al., 2018) or spore-formers in the agricultural industry (Eski et

al., 2019; Ma et al., 2015). Here, we applied this methodology towards the non-spore-forming, Gram-negative bacterium *Collimonas arenae* Cal35, which has demonstrated potential as a biofungicide with use on economically important crops (Akum et al., 2020).

Inlet temperature was the only parameter that had a statistically significant effect on both bacterial survival and powder yield; but whereas the lowest inlet temperature benefitted survival rates, it also diminished yields. Lower spray rate or alginate improved powder yield without compromising high survival rates, since both parameters impacted yield. But perhaps only moderate changes in these two parameters would be possible since greater losses strongly correlated with high outlet temperature, especially since outlet temperature itself is easily controlled by both formulation and process parameters (**Table 3-2**, **Supplementary Figure 3-1**). Although the sensitivity of bacteria to spray-drying is largely species-dependent, there generally is a strong correlation between low outlet temperature and high survival rates (Berninger et al., 2018; Fu et al., 2011; Greffe et al., 2020). Tolerance of Cal35 or other collimonads to high temperatures has not previously been looked at.

Typically in RSM, if the optimal conditions lie on an edge of the design space, parameters are adjusted such that a more optimal response may be found. While this was the case for inlet temperature, spraying any lower than 95°C would lead to inadequate drying, which results in droplets that stick to the walls of the drying chamber and are unable to be collected. Furthermore, physical constraints of particle formation at the benchtop scale limit the space that can be explored. The interaction of faster spray rates producing larger CLAMs only at a high inlet temperature is one example (**Figure 3-2C**, **iii**). Spraying at low inlet temperatures might only dry droplets up to a certain size, with anything larger sticking to the walls of the drying chamber due to insufficient heat. Indeed, we saw a negative correlation between particle size and yield (**Figure 3-2D**). By

scaling up, however, larger chamber dimensions would increase the residence time and evaporation rate to allow those larger droplets to form dry particles at equivalent outlet temperatures (Poozesh et al., 2019). Compared to the Buchi B-290 benchtop spray-dryer which is specified to produce particles between 2-25 μm, pilot and production scale spray-dryers may extend that range up to 80 μm and achieve yields nearing 100% (Poozesh et al., 2019; Sosnik et al., 2015; Thybo et al., 2008). Most noteworthy was the significant correlation between larger sized CLAMs and lower losses in bacteria viability (**Figure 3-3B**), expanding the implications of scale-up to not only maximize yield, but to vastly improve bacteria survival rates during spray-drying as well.

3.6.2 Shelf stability

Spray-drying two types of formulations – with or without CLAMs – and subjecting them to oxygen- and humidity-controlled environments allowed us to investigate the effect of encapsulating Cal35 in CLAMs, and to better understand the role of oxygen and moisture during storage. Cal35 benefitted greatly when encapsulated in either modified starch CLAMs (S-CLAMs) or maltodextrin CLAMs (M-CLAMs), compared to being sprayed with maltodextrin alone (M) or modified starch alone (S). And although alginate formulations containing starch or its derivates have improved encapsulation of plant-beneficial bacteria in alginate beads formed by extrusion into a calcium bath and subsequent oven drying (Rohman et al., 2021; Schoebitz et al., 2012; Z. S. Wu et al., 2012), we have incorporated *in situ* cross-linked alginate with maltodextrin and modified starch and demonstrated similar improvements but with a much more industrially relevant method.

Starting with the highest levels of viable bacteria is a key factor when considering how to extend shelf stability. Because Cal35 encapsulated in M or S particles did not survive spray-drying, we could only assess stability of Cal35 in M-CLAMs and S-CLAMs. Cal35 encapsulated in S-

CLAMs had the best survival during spray-drying thus highest initial cell count going into storage. It also exhibited the slowest decline in viability, particularly in the oxygen and humidity limited environment (lowH, -O_2). The presence of oxygen appeared to be the main limiting factor, as no treatment survived one day in the uncontrolled environment (Amb), with the exception of Cal35 encapsulated in S-CLAMs stored with controlled humidity (lowH). HI-CAP 100 modified starch is known for its oxygen barrier properties and has been used as an excipient in food formulations to prevent oxidation of oils (Shaaruddin et al., 2019; Strobel et al., 2020a). Since mercury porosimetry and SEMs revealed no pores larger than 3 nm in M-CLAMs or S-CLAMs (**Supplementary Figure 3-5**) and we assume the excipients formed a solid barrier in the CLAMs matrix, the oxygen barrier properties of modified starch may have slowed diffusion of oxygen into the microcapsule resulting in the overall prolonged survival of Cal35 in S-CLAMs. Furthermore, the spherical morphology of S-CLAMs provides a lower surface area to volume ratio than less spherical M-CLAMs. Seeing that refrigeration was avoided in these experiments, limiting the presence of oxygen or slowing the diffusion of oxygen may be worth more thoroughly investigating as a means to improve shelf stability of Cal35 and similar bacteria.

Increased stability of Cal35 in S-CLAMs in the humidity controlled environment (lowH) compared to ambient conditions (Amb) suggests that low water activity may play a role in prolonging shelf stability. This is consistent with reports that low water activity improves shelf life of microorganisms by slowing biological activity (Mugnier et al., 1985; Teixidó et al., 2006).

3.7 Conclusion

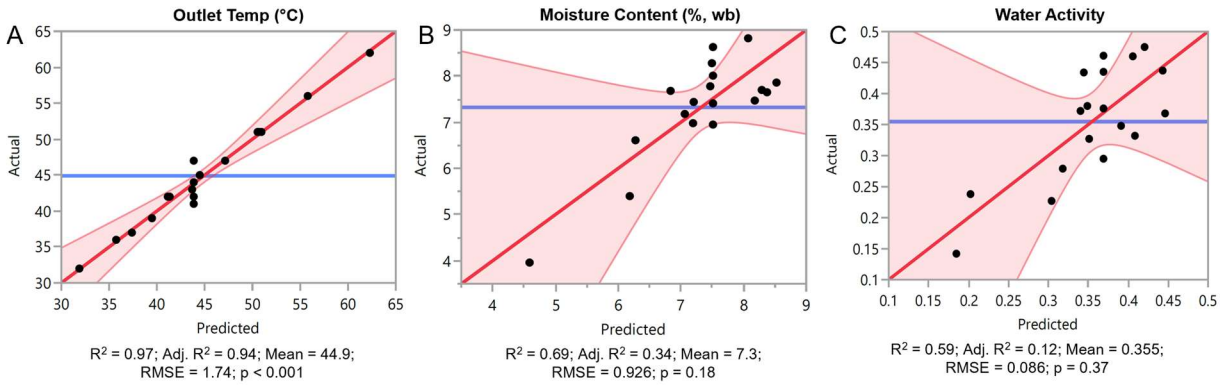
The plant-protective non-spore-forming soil bacterium *Collimonas arenae* Cal35 was encapsulated in in situ cross-linked alginate microcapsules (CLAMs) by spray-drying. Optimization of the formulation and spray-drying process using RSM to minimize losses in

viability during spray-drying and maximize powder yield resulted in the most desirable CLAMs formulated with 1.3% alginate and sprayed at 95°C and a rate of 12.9 g/min. Inlet temperature significantly affected loss in viability, while alginate concentration, inlet temperature, and spray rate all significantly affected yield. Low outlet temperatures and larger particle size benefitted bacteria survival during spray-drying, while smaller particle size led to higher yields. The trade-off between yield and survival was exacerbated by small-scale benchtop spray-drying; scale-up may potentially eliminate this contradiction to maximize both survival and yield. Shelf stability of Cal35 was prolonged when encapsulated in CLAMs, supplemented with modified starch and stored in a humidity- and oxygen- controlled environment. Importantly, the antifungal activity of Cal35 was retained following spray-drying and storage, thus strengthening its potential as a formulated biofungicide, by itself or in combination with *Bacillus*-based products(Akum et al., 2020).

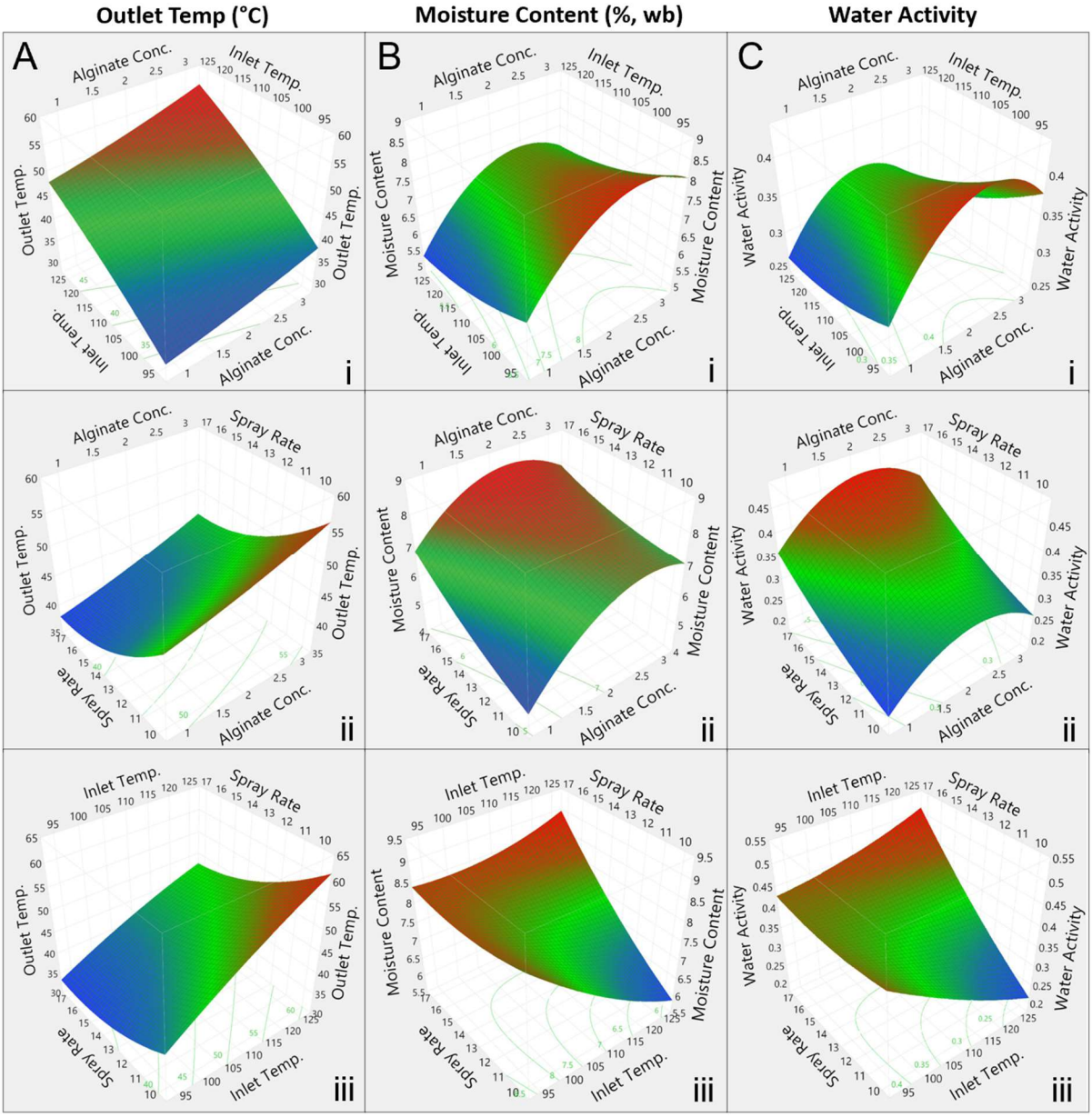
3.8 Supplementary Information

Supplementary Table 3-1 Randomized experimental setup of response surface design displaying coded variables and their responses.

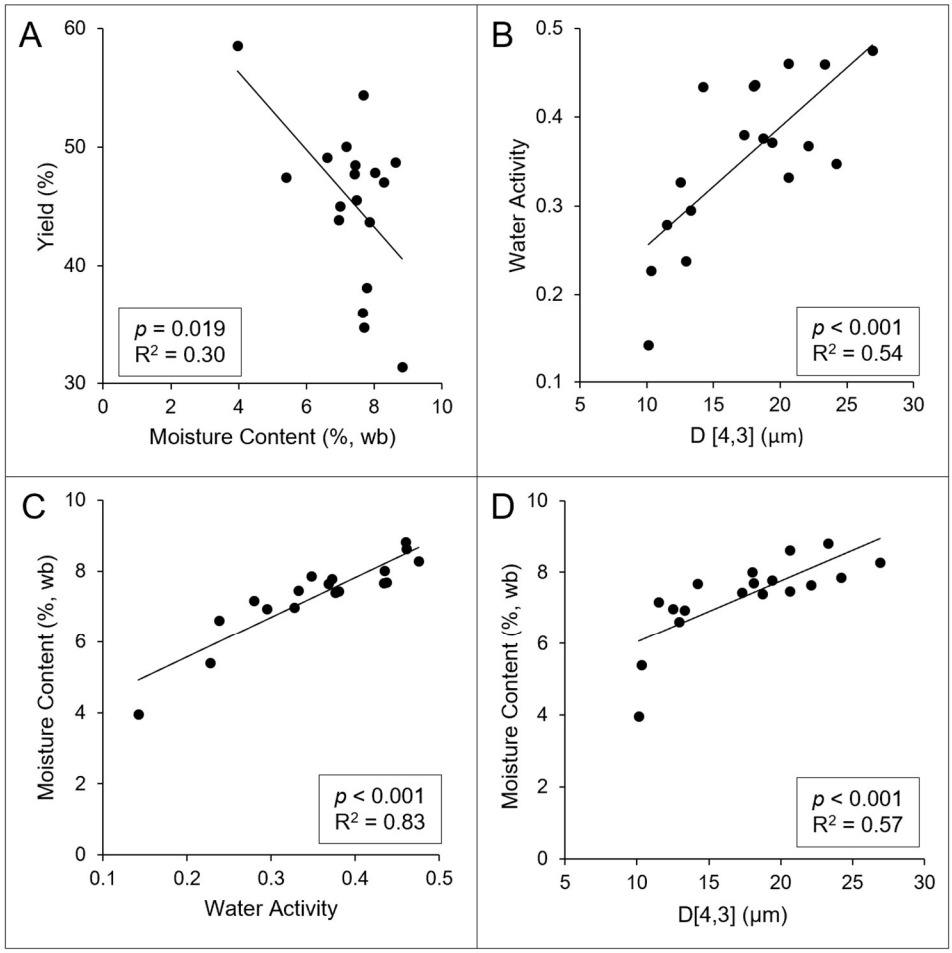
Run	Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3	Response 4	Response 5	Response 6
	Alginate Conc.	Inlet Temp.	Spray Rate	Loss (log reduction)	Yield (%)	D[4,3] (µm)	Outlet Temp. (°C)	MC (%, wb)	Water Activity
1	-1	-1	-1	3.96	54.4	39	14.2	7.68	0.434
2	-1	-1	1	3.78	45.1	32	12.5	6.98	0.327
3	-1	0	0	4.78	47.5	42	10.3	5.4	0.227
4	-1	1	-1	4.54	58.6	56	10.1	3.96	0.142
5	-1	1	1	3.62	47.1	43	26.9	8.28	0.475
6	0	-1	0	3.41	45.6	36	20.6	7.47	0.332
7	0	0	-1	4.95	50.1	51	11.5	7.18	0.279
8	0	0	0	3.93	43.9	41	13.3	6.95	0.295
9	0	0	0	4.64	47.9	47	18	8.01	0.435
10	0	0	0	3.69	48.7	42	20.6	8.63	0.461
11	0	0	0	3.99	47.8	44	18.7	7.41	0.376
12	0	0	1	4.31	34.7	42	18.1	7.7	0.437
13	0	0	0	4.47	48.5	51	17.3	7.44	0.38
14	1	-1	-1	3.96	43.7	45	24.2	7.86	0.348
15	1	-1	1	3.81	31.4	37	23.3	8.82	0.46
16	1	0	0	4.03	38.2	47	19.4	7.78	0.372
17	1	1	-1	4.83	49.2	62	12.9	6.61	0.238
18	1	1	1	4.9	36	51	22.1	7.65	0.368



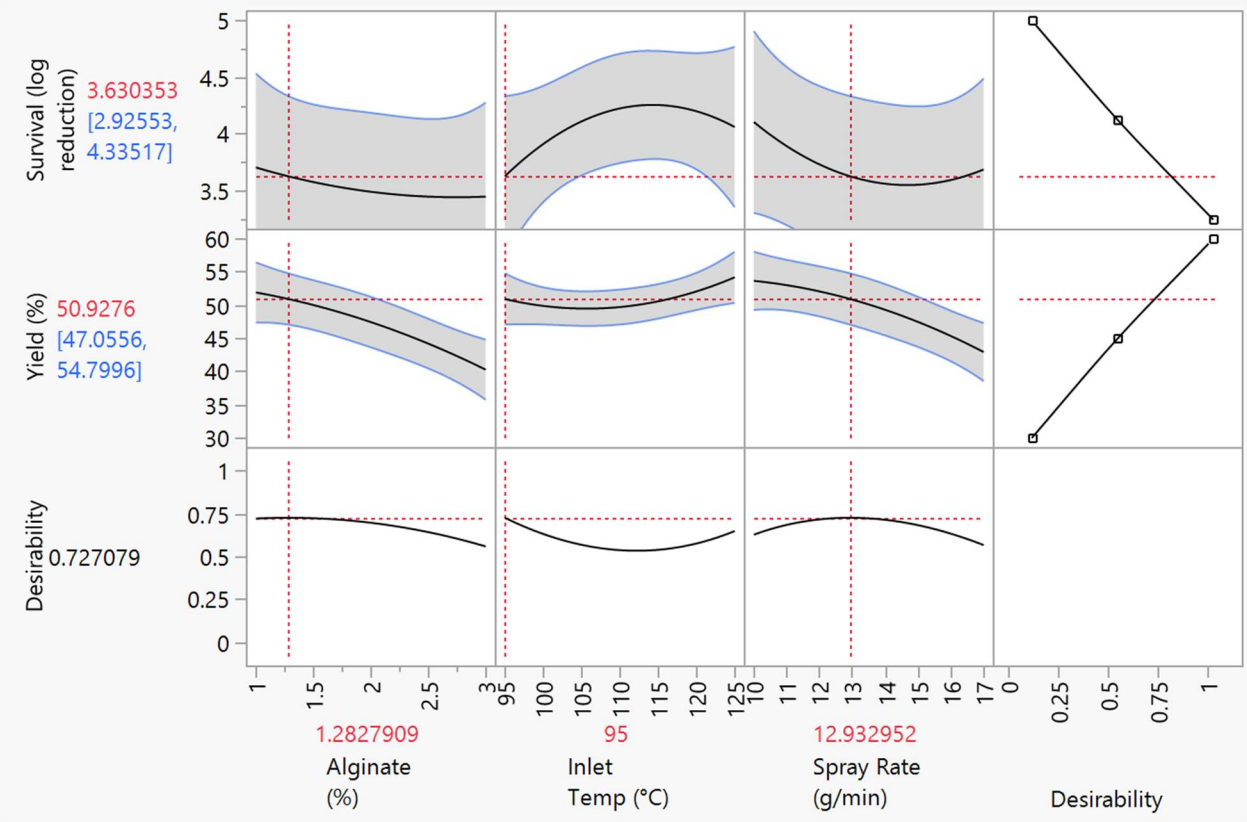
Supplementary Figure 3-1 Predicted vs actual response plots for (A) outlet temperature, (B) wet basis moisture content, and (C) water activity. Coefficient of determination (R^2), adjusted R^2, mean response value, root mean squared error (RMSE), and p-value of the prediction model are reported. The diagonal line (red) represents the linear fit of the data points. The horizontal line (blue) is the mean.



Supplementary Figure 3-2 Surface plots with underlying contours illustrating the response of (A) outlet temperature, (B) wet basis moisture content, and (C) water activity. Each row varies two of the three independent variables while keeping the third constant at the middle level. (i) alginate and inlet temperature, (ii) alginate and spray rate, (iii) inlet temperature and spray rate.



Supplementary Figure 3-3 Linear regression of other significant correlations: (A) yield and moisture content, (B) water activity and volume weighted mean particle size, (C) moisture content and water activity, and (D) moisture content and volume weighted mean particle size.

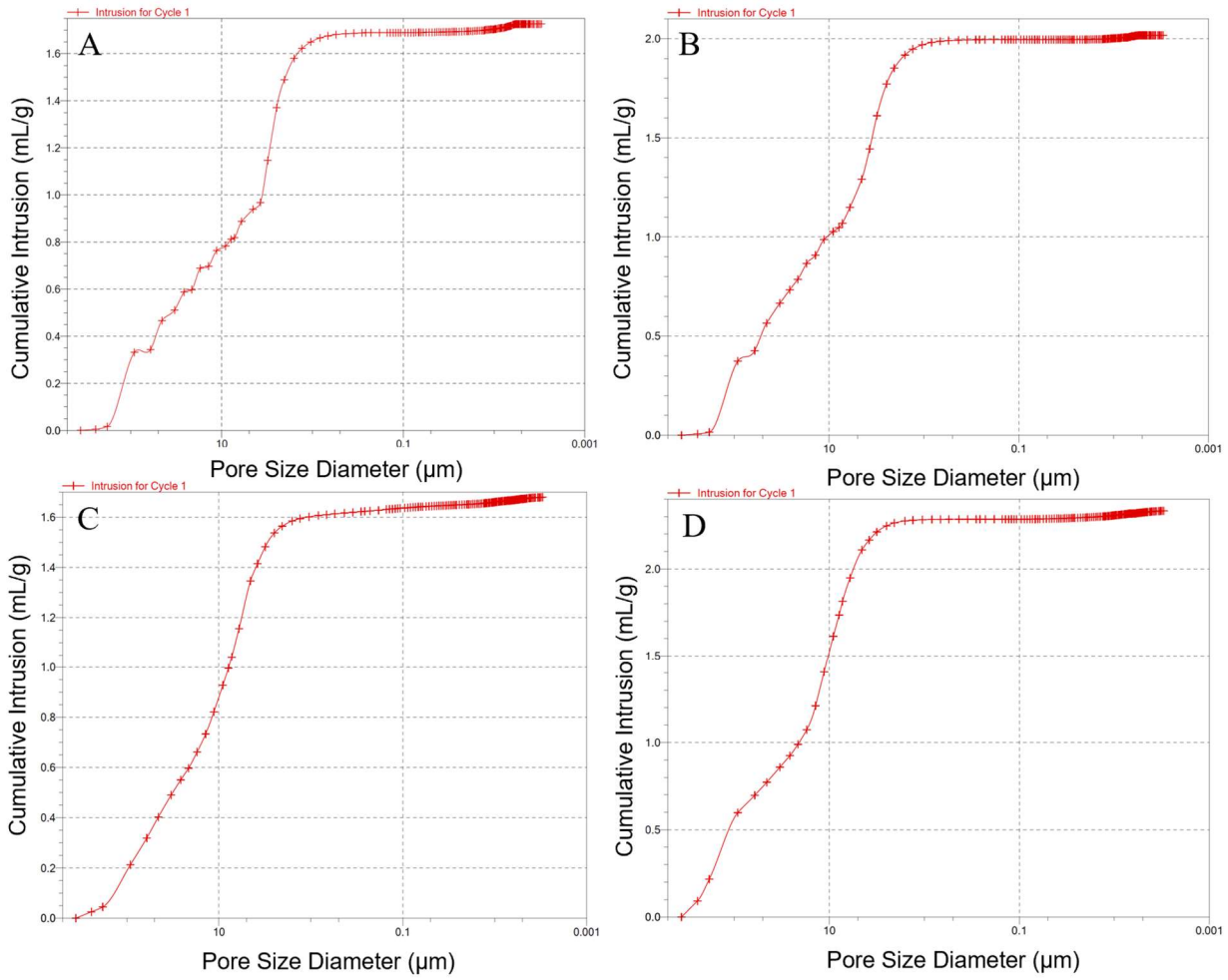


Supplementary Figure 3-4 Desirability function from JMP used to determine formulation and process parameter values to maximize yield and survival.

Supplementary Table 3-2 Characterization of 10% maltodextrin-only particles (M), 10% maltodextrin with CLAMs (M-CLAMs), 10% modified starch- only particles (S), and 10% modified starch with CLAMs (S-CLAMs).

Formulation	Feed (log [CFU/g])	Spray-dried Powder (log [CFU/g])	Particle Size (µm)	Moisture Content, WB (%)	Water Activity
M	10.5 ± 0.3	< 4*[a]	14.3 ± 0.3[a]	8.9 ± 0.5[a]	0.552 ± 0.001[a]
S	10.5 ± 0.1	< 4*[a]	51.7 ± 7.0[c]	8.3 ± 0.4[a]	0.520 ± 0.002[b]
M-CLAMs	10.3 ± 0.1	6.2 ± 0.1[b]	25.0 ± 0.6[b]	9.4 ± 0.7[a]	0.477 ± 0.002[c]
S-CLAMs	10.7 ± 0.1	7.5 ± 0.1[c]	51.2 ± 7.1[c]	11.9 ± 0.2[b]	0.541 ± 0.001[d]

Means with the same letter were not significantly different
*4 log [CFU/g] is the limit of detection



Supplementary Figure 3-5 Mercury intrusion in (A) 10% maltodextrin-only particles (M), (B) 10% maltodextrin with CLAMs (M-CLAMs), (C) 10% modified starch-only particles (S), and (D) 10% modified starch with CLAMs (S-CLAMs).

This analysis method exerts a controlled pressure on mercury to penetrate any void spaces of the spray-dried powder. The cumulative volume of mercury filling any spaces or pores in the sample was displayed over a range of pore size diameters, showing at which pore size mercury intrusion occurred (**Supplementary Figure 3-5**). Because the average particle diameter for each formulation ranged between 15 – 20 µm, the interstitial spacing between particles is estimated to be 1/3 to 1/4 of the particle diameter. In the most extreme case, this means that any measured volume of mercury above a pore size diameter (x-axis) of 3.25 µm is due to intrusion through interstitial space. Every formulation (**Supplementary Figure 3-5A**, **5B**, **5C**, and **5D**) displayed a

rapid increase in cumulative intrusion to just above 1 μm pore diameter, where the graph then remains flat until roughly 0.01 μm. At this point, pressure exerted on the sample exceeds 20,000 psia, likely compressing the powder and causing the minor step increase in intrusion at the tail end of the graph. Mercury porosimetry is limited to detecting pore size down to 3 nm; therefore, we concluded that there are no pores larger than 3 nm in any of these spray-dried powders.

3.9 References

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Chapter 4 Fluidized bed spray-coating of enzyme in a cross-linked alginate matrix shell (CLAMshell)

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This chapter aims to identify the key operational parameters of fluidized bed spray-coating that allow formation of a proper coating. Because the volume of bacteria culture required per batch is unrealistic without large scale equipment, the coating process was optimized using enzymes as a bioactive substitute for bacteria.

4.1 Abstract

One-step encapsulation of subtilisin by fluidized bed spray-coating in a cross-linked alginate matrix shell (CLAMshell) particle is introduced. The CLAMshell process maximizes process productivity, while simultaneously improving coating quality of spray-coated enzyme. Overall, higher spray rates were achieved with improved mechanical strength of the coating, significantly limiting attrition and suppressing surface damage. Incorporating the CLAMshell formulation improved the overall coating process compared to coatings of cargo-only by achieving a 40% faster spray rate and maintaining coating efficiencies above 90%, with aggregates and fines below 2% of the final product. Process interruptions for removing excessive fines obstructing the outlet filter were eliminated and filter fouling was minimized. Increased alginate content and combination with polyvinyl alcohol in the coating yielded the greatest mechanical strength with low attrition and minor particle damage. CLAMshell particles exhibited high coating integrity through delayed dissolution of the salt core in solution, indicating potential controlled release applications.

4.2 Abbreviations

A	alginate, % w/w
Å	Angstrom
A_C	cross-linked alginate, % w/w
ASABE	American Society of Agricultural and Biological Engineers
A^X	alginate, added at X % w/w concentration in the feed suspension
CLAM	cross-linked alginate matrix
CLAMshell	cross-linked alginate matrix, or matrix of other cross-linkable polymer, coated onto a core
E	enzyme
I	coating integrity
kPa	kiloPascal
kV	kilovolt
M	non-fat dry milk powder, also designated as “milk powder”
P	PVA
PVA	polyvinyl alcohol
SEM	scanning electron microscopy
% w/w	percent weight/weight, also designated simply by % except where defined otherwise
Y	spray yield, also called coating efficiency

4.3 Introduction

The detergent, food, animal feed, textile/paper, and pharmaceutical industries are amongst the largest users of enzyme technology (Kirk et al., 2002; Li et al., 2012; van Beilen et al., 2002). Microencapsulation of enzymes has become essential for some industrial and commercial applications to confer protection, increase shelf life and stability, and control release for targeted delivery (Nazzaro et al., 2012; Santa-Maria et al., 2012). For example, granulation of phytase, added to animal feed for availing phosphorous, improves stability in high temperature pelleting conditions and in reactive environments (Misbah et al., 2014; Slominski, 2011). Granulation of proteases, amylases, lipases and mannanases, included in detergents to break down food stains and other soils, protects enzymes from bleaching agents and other harmful components while reducing the risk of human exposure to sensitizing protein dusts (Ahmadian et al., 2007; Chan et al., 2005; Chan et al., 2006). To encapsulate enzymes in coated particles or granules, fluidized bed spray-coating technology is preferred for its large-scale manufacturing capability; however, optimization of this process can be challenging (Seyedina et al., 2017).

During the fluidized bed spray-coating process, a heated air current fluidizes a bed of core particles while a coating liquid containing binding agents and active ingredients is sprayed onto the surface of the particles; layering of droplets on the particles and a continuous cycle of wetting-drying eventually forms a film, or coating, on the particles (Guignon et al., 2002; Teunou et al., 2002). Many industrial and commercial products containing enzyme coated particles already exist (Arnold et al., 1994; Becker et al., 1998; Dale et al., 1999; Ishikawa et al., 1998; Kiuchi et al., 1998; Markussen, 2003; Markussen et al., 1987). Common to all of these is the use of multiple coating layers, each with a designated purpose. For example, applying a coating of polymer such as polyvinyl alcohol or methyl cellulose on top of the enzyme layer may limit exposure to oxygen and moisture, while applying a coating of lubricant or plasticizer helps prevent agglomeration

between particles (Dale et al., 1999). In applying each layer, the spray-coating process can encounter challenges such as particle agglomeration, attrition, and poor coating efficiencies (Dewettinck et al., 1998a; Dewettinck et al., 1998b; Hede et al., 2007; Hede et al., 2009a, 2009b, 2009c). More recently, modeling and simulation of this process has helped advance the improvement and optimization of fluidized bed spray-coating (Bück et al., 2016; Jiang et al., 2018; Jiang et al., 2020). It is of particular importance to develop spray-coating processes and formulations that enable high spray rates and process yields, producing particles with mechanically strong, integral coatings.

Coating formulations are typically composed of an active ingredient or "cargo" stabilized in a carrier matrix. While synthetic polymers appear to be favored as binders or coatings for enzyme cargo, biologically derived polymers such as alginate, carrageenan, pectin, and cellulosics offer a natural alternative (Dewettinck et al., 1998a). Of the naturally derived carriers, alginate is favored for microencapsulation due to its relatively low cost, biocompatibility, safe handling, and gel forming ability (Draget et al., 2011; Lee et al., 2012). In the presence of multivalent cations, the carboxylate groups positioned along alternating and/or repeating units of β-D-mannuronic acid and α-L-guluronic acid serve to cross-link the linear alginate polymer chains forming a gel structure (Draget et al., 2011; Lee et al., 2012). Although forming cross-linked alginate beads or microcapsules is a well-established laboratory technique, forming uniform cross-linked alginate coatings on granular particles is non-trivial.

A recently patented in situ internal gelation process enables physical cross-linking of ionic polymers during atomization processes such as spray-drying (Jeoh-Zicari et al., 2020a). Referred to as the cross-linked alginate matrix (CLAM) process, this spray-drying process has been used to simultaneously form, cross-link, and dehydrate alginate microcapsules in order to stabilize a

variety of cargos, such as enzymes (Santa-Maria et al., 2012), polysaccharides (Strobel et al., 2019; Wong et al., 2020), lipids (Strobel et al., 2020; Strobel et al., 2016), and microbes (Strobel et al., 2018). Here, we describe use of the in situ internal gelation process to form cross-linked alginate matrix shell (CLAMshell) particles in a fluidized bed spray coater (Jeoh-Zicari et al., 2020b). Briefly, a feed suspension of alginate, an organic acid titrated to a higher pH with a volatile base, a calcium salt sparingly soluble at the titrated pH, and cargo material is sprayed onto fluidizing granular core material. Upon atomization and contact with a core particle, volatilization of the base lowers the pH of the sprayed droplet, solubilizing the calcium salt and enabling ion-mediated cross-linking of the alginate while drying and embedding the cargo, forming a coating matrix.

We investigated the effect of integrating the in situ cross-linking alginate formulation with two proteinaceous cargos--non-fat dry milk powder and subtilisin concentrate--on the fluidized bed spray-coating process and coating quality. Specifically, we sought to improve process efficiency by using a single coating step at high spray rates while maintaining low levels of agglomeration, filter fouling and fines creation. Coating strength was assessed using a Heubach dustmeter and coating quality was analyzed using scanning electron microscopy (SEM). Coating integrity was quantified by the relative conductivity of dissolved sodium sulfate cores for CLAMshell particles in a solution.

4.4 Materials and Methods

4.4.1 Materials

Low viscosity alginate (A1112), periodic acid, Schiff's fuchsin sulfite, and sodium metabisulfite were purchased from Millipore-Sigma (Burlington, MA). Ammonium hydroxide, sodium citrate dihydrate, and succinic acid were purchased from Fisher (Waltham, MA). Impact beads (glass cores) were acquired from Grainger (Lake Forest, IL) and sodium sulfate (anhydrous) cores were acquired from Minera de Santa Marta (Belorado, Spain). Calcium hydrogen phosphate

was purchased from Frontier Scientific (Logan, UT). Non-fat dry milk powder was purchased from a local market. Milli-Q water purified in a Millipore Ultrapure Water Purification System was used to create all solutions used in this study. Polyvinyl alcohol (Poval 5-88, Kuraray, Houston, TX) and an ultrafiltration concentrate of a subtilisin protease (102 mg/g of active enzyme and 23.9% total solids) produced by a *Bacillus* fermentation were supplied by DuPont (Wilmington, DE).

4.4.2 Methods

4.4.2.1 Formulation of feed suspensions

Two cargo materials, non-fat dry milk powder (M) and an ultrafiltration concentrate of subtilisin, a protease enzyme (E), were used in this study. Total feed suspension batch sizes of 2000 g were prepared of the formulations listed in **Table 4-1**. First, all carrier and additive components were combined into a 1000 g suspension and mixed overnight to allow full hydration and dissolution of the alginate. Prior to coating, 1000 g of cargo (milk powder or enzyme mixture) was added to complete the formulations of M or E feed suspensions. A feed suspension absent of cargo and additives was also prepared to demonstrate the application of a cross-linked alginate coating layer.

Table 4-1 Compositions of feed suspensions, on a percent weight basis. Cargo is non-fat milk powder (M) or enzyme mixture (E), carrier is cross-linking alginate formulation (A_c), and polyvinyl alcohol (P) additive was included as an additive.

Formulation ID	Cargo	Carrier formulation	Additive
M	15%	-	-
$MA_C^{0.5}$	15%	0.5% alginate, 0.25% succinic acid pH 7 (NH_4OH), 0.125% $CaHPO_4$	-
$MA_C^{1.0}$	15%	1% alginate, 0.5% succinic acid pH 7 (NH_4OH), 0.25% $CaHPO_4$	-
$MA_C^{1.5}$	15%	1.5% alginate, 0.75% succinic acid pH 7 (NH_4OH), 0.375% $CaHPO_4$	-

$MA_C^{2.0}$	15%	2% alginate, 1% succinic acid pH 7 (NH_4OH), 0.5% $CaHPO_4$	-
E	12%	--	-
$EA_C^{2.0}$	12%	2% alginate, 1% succinic acid pH 7 (NH_4OH), 0.5% $CaHPO_4$	-
$EA_C^{2.5}$	12%	2.5% alginate, 1.25% succinic acid pH 7 (NH_4OH), 0.625% $CaHPO_4$	-
$EA_C^{3.0}$	12%	3% alginate, 1.5% succinic acid pH 7 (NH_4OH), 0.75% $CaHPO_4$	-
EP	12%	-	1% polyvinyl alcohol
$EPA_C^{2.0}$	12%	2% alginate, 1% succinic acid pH 7 (NH_4OH), 0.5% $CaHPO_4$	1% polyvinyl alcohol

4.4.2.2 Process optimization of fluidized bed spray coating

Sodium sulfate granules sized at 250-350 μm diameter (glass beads with a similar size range were used for the feed suspension absent of any cargo and additive) were loaded into the product chamber of a 2 L capacity fluidized bed coater (UniGlatt, Glatt Air Industries) in Wurster configuration. To 1500 g of core material, 2000 g of feed suspension was applied. Control parameters include inlet air temperature, airflow flap angle, nozzle pressure, nozzle position, and spray rate. The feed suspension spray rate was controlled by a peristaltic pump, calibrated prior to use. Inlet temperature throughout the study was set at 55-60°C (lower and upper limit). The airflow flap angle was 45 or 25 degrees, adjusted to maintain consistent fluidization levels for all batches. At one point when the outlet filter was replaced, the new filter had notably less resistance to airflow, creating violent particle fluidization that required adjusting the flap angle to 25 degrees.

For each spray coating batch, the following in-process parameters were monitored: outlet temperature, pressure-drop across the fluidized product, pressure-drop across the filter, and filter

hits. Most relevant in this study, pressure-drop across the filter and the number of filter hits were reported. The pressure-drop across the filter compares the pressure above the filter at the outlet and the pressure within the expansion chamber. The pressure-drop is displayed on a gauge ranging from 0-5 kPa. Over the course of a batch, the pressure-drop across the filter tended to increase due to accumulation of material on the product side of the filter. To reduce filter fouling, a manually-engaged filter shaking apparatus delivers a sharp mechanical hit to the filter housing. These filter hits were used only when the pressure-drop across the filter reached 5 kPa. Once initiated, the peristaltic pump and fluidizing air temporarily stop, allowing these hits to remove filter fines. Each interruption consisted of five consecutive hits.

4.4.2.3 Characterization of feed suspensions

Viscosity of feed suspensions without alginate was measured using a Brookfield DV-II+Pro viscometer (AMETEK Inc., Middleboro, MA) with a CPE-40 spindle. Viscosity of feed suspensions containing alginate was measured using a Brookfield RVDV-II+PCP viscometer (AMETEK Inc., Middleboro, MA) with a CPE-41 spindle. Shear stress was measured over a range of shear rates with a volume specified for that spindle. For non-Newtonian feed suspensions (i.e. with alginate), data were fit with a power function to obtain apparent viscosities.

Surface tension was measured using a SensaDyne surface tensiometer (SensaDyne Instrument Division, Mesa, AZ) equipped with 0.5 and 4 mm inverted probes (for more viscous feeds) or non-inverted probes (for less viscous feeds) calibrated with water and ethanol. Probes were inserted into a beaker of the feed suspension until fully submerged. Values were recorded once readings stabilized, after one minute.

4.4.2.4 Characterization of spray-coated particles

Alginate cross-linking was measured by comparing the ratio of soluble to total alginate of CLAMshell coated on glass cores. CLAMshell samples of 1g were mixed either in 10 mL water to dissolve non-cross-linked alginates or 10 mL of 100 mM sodium citrate to fully dissolve all alginates. The suspensions were incubated with end-over-end rotation for two hours at room temperature. The concentrations of soluble alginate dissolved in water and citrate buffer were quantified using a colorimetric assay based on the reaction with periodic acid and Schiff's fuchsin, as described previously (Strobel et al., 2018; Strobel et al., 2019). The ratio of soluble to total alginate was determined as the ratio of alginates dissolved in water to that dissolved in the citrate buffer.

Sieving analyses were completed using a 20 g sample size of coated particles, loaded onto the largest size sieve in a series (500, 354, 300, 250, 212, and 150 μm) and placed into a Sonic Sifter L3P (Allen-Bradley, Milwaukee, WI). Sift amplitude was set at 7 and pulse amplitude at 8. The sieving duration was 5 minutes. After sieving, the mass of particles on each screen was measured using an analytical balance. Size distributions generated from sieving were used to estimate the volume-weighted mean diameter of coated particles (**Supplementary Table 4-1**, **4-2**, and **4-3**) following ASABE (American Society of Agricultural and Biological Engineers) standards (ASAE, 1983). Fines were defined as material passing through the 150 μm sieve, and aggregates were defined as material retained by the 500 μm sieve.

Coating efficiency, or spray yield (Y), was calculated using Eq. [4-1]:

$$Y = \textit{coated solids}/\textit{total sprayed solids} = [P(1 - MC/100) - \mathrm{F} - \mathrm{C}]/\mathrm{TS} \qquad [4\text{-}1]$$

where, P = total product collected (g), MC = wet basis moisture content (%), F = total fines in product (g), C = initial cores amount (g), and TS = total solids sprayed (g).

Coating morphology was visualized by scanning electron microscopy (SEM) using a Thermo Scientific Quattro SEM. Samples were embedded in epoxy resin and sectioned with a microtome using a glass, then diamond blade. Surface and sectioned samples were mounted onto carbon tape and sputter-coated in gold to a target thickness of 120Å. A beam voltage of 5 or 10 kV and spot size of 3.0 were selected. SEM of sectioned samples were used to estimate coating thicknesses, although several other methods of quantifying coating thickness exist (Mezhericher et al., 2020; Sondej et al., 2015; Sondej et al., 2016).

Coating strength was assessed by measuring the generated dust from the product using a Heubach dustmeter Type III (Heubach, Fairless Hills, PA). Samples were sieve cut between 300 and 500 µm and loaded in the sample chamber to a constant volume fill of 16.25 ml, to compensate for differences in bulk density. The program ran for 20 minutes at 45 rpm with an airflow of 20 L/min. Accumulated dust was weighed and compared to the initial mass of product.

Coating integrity (I) was defined as the ability of a coating to retain undissolved core after a given dissolution time, relative to an equivalent uncoated core. It was calculated using Eq. [4-2]:

$$I = 1 - (K_{i,20}/K_{c,20}) \quad [4\text{-}2]$$

where, $K_{i,20}$ = conductivity of a coated particle, i, at 20 seconds, and $K_{c,20}$ = conductivity of an uncoated core sample at 20 seconds. Using a reference control of 5 g uncoated sodium sulfate cores, the sample mass of coated particles that contains 5 g of sodium sulfate was calculated based on the coated solids per mass of cores. Each sample was quickly added to 200g of Milli-Q water

stirring at 400 rpm. Conductivity of the solution was recorded every five seconds for one minute. This dissolution method was similar to one found in previous studies (van Kampen et al., 2015; van Kampen et al., 2016).

Bulk density, moisture content, and water activity of every formulated particle are shown in **Supplementary Table 4-1**, **4-2**, and **4-3**. Bulk density (g/mL) was calculated by weighing approximately 200 g of non-compacted coated particles in a graduated cylinder and recording the corresponding volume. Moisture content (%) on a wet basis was determined gravimetrically. Approximately 3 g of coated particles were dried between 60-70°C in an oven for three days. Water activity was measured using an AquaLab Series 3TE water activity meter (METER, Pullman, WA).

4.4.2.5 Statistical analysis

Statistical analysis was performed using SAS (SAS Institute Inc.). All analyses and measurements were taken in triplicate. One-way and two-way ANOVA and Tukey's post hoc multiple comparison tests determined significance between factors and differences among factor levels. P-values less than 0.05 were considered significant.

4.5 Results

4.5.1 In situ cross-linking of alginate during spray-coating

In situ internal gelation of alginates has previously been accomplished in a spray-drying process (Jeoh-Zicari et al., 2021; Santa-Maria et al., 2012; Strobel et al., 2018; Strobel et al., 2016, 2019); however, this study marks the first time this in situ alginate cross-linking approach has been implemented in a fluidized bed spray-coating process. To validate the process, the in situ cross-linking alginate formulation was first applied to surfaces of glass beads in the fluidized bed coater. The application of the coating was confirmed by SEM and particle size analysis. Surfaces of beads coated with cross-linked alginate appeared textured (**Figure 4-1a, i**) while surfaces of non-coated

glass beads were smooth (**Figure 4-1a, iii**). Furthermore, cross-sections of coated beads revealed a coating layer approximately 2 µm thick (**Figure 4-1a, ii**). A shift to larger sizes in the particle size distribution also confirmed the deposit of a coating (**Figure 4-1c**).

The extent of alginate cross-linking in the coating matrix was quantified as the percentage of insoluble alginate relative to total alginate when the particles were suspended in water. Based on this analysis, 72.2 ± 2.1% of the alginate coating was insoluble, or effectively gelled, while the remainder of the alginate leached out into the surrounding fluid. The alginate gelation achieved in the spray-coating process was similar to that achieved during spray-drying, using the same feed composition (76.3 ± 2.0% (Strobel et al., 2018) and 75.4 ± 0.3% (Strobel et al., 2019)). Thus, this process forms a cross-linked alginate matrix shell (CLAMshell) into which various cargos can be incorporated.

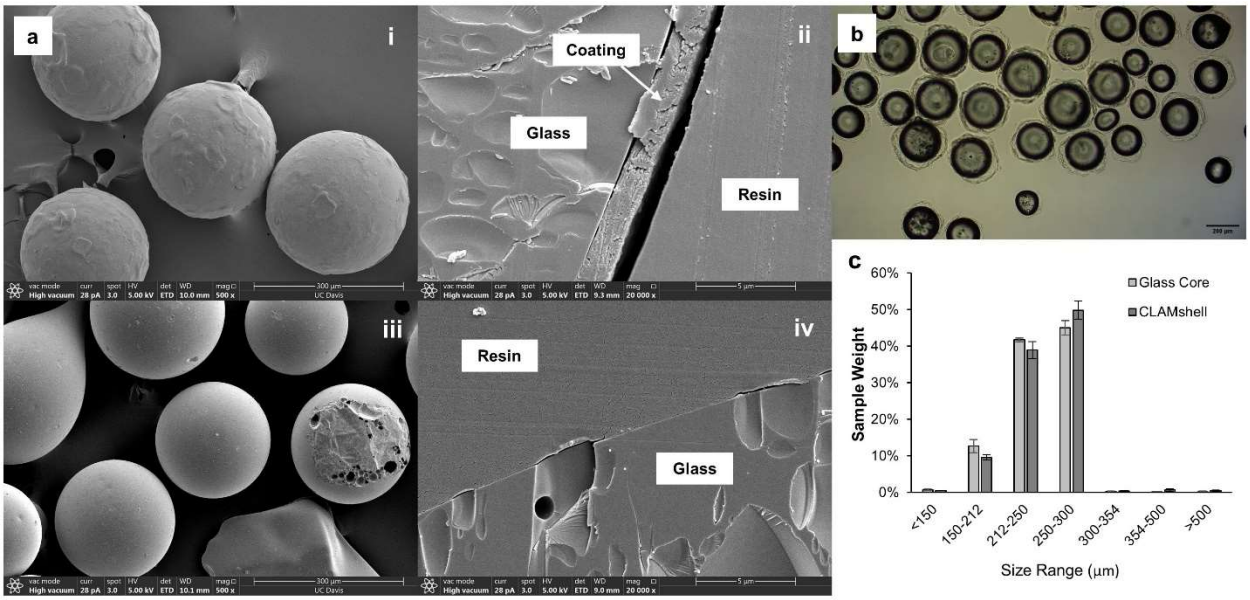


Figure 4-1 Characterization of CLAMshells on glass beads. SEM (a) of surface (i and iii) and cross-sections (ii and iv) of coated glass beads (i and ii) and uncoated glass beads (iii and iv). Optical micrograph of CLAMshells swelling in water (b). Size histogram of coated and uncoated glass beads (c). Scale bars are 300 µm (a, i and iii), 5 µm (a, ii and iv), and 200 µm (b).

4.5.2 In situ cross-linking alginate improves coating process efficiency

4.5.2.1 Influence of CLAMshell formulation on nozzle pressure and spray rate

The advantages of the CLAMshell coating process were demonstrated using milk powder (M) as the cargo coated onto sodium sulfate particles. The criteria for successful spray coating were to generate less than 2% of the total mass in aggregates and less than 2% of the total mass in fines, to achieve at least 85% coating efficiency, and to minimize filter fouling due to fines. The CLAMshell process achieved these success metrics while significantly increasing spray rates for particle coating (**Table 4-2**). In the milk powder formulation (M), a maximum spray rate to coat sodium sulfate core particles was limited to 10 g/min before the fraction of aggregates exceeded the acceptable 2% threshold. Increasing spray rates for the milk powder coating formulation generated larger fractions of aggregates, fines and decreased coating efficiencies. When milk powder was encapsulated in the CLAMshell containing 2% alginate ($MA_C^{2.0}$), however, spray rates of up to 14 g/min were achieved without an increase in aggregates or fines.

Key to reaching higher spray rates with the CLAMshell formulation was to increase the nozzle pressure. The interplay between nozzle pressure and spray rate led to a successful $MA_C^{2.0}$ coating at 14 g/min and 4 bar, yielding 1.68% aggregates, 0% fines and 94.2% coating efficiency (**Table 4-2**). Thus, $MA_C^{2.0}$ was coated at a 40% faster spray rate than M alone, which could only successfully be coated at up to 10 g/min. Spray coating M at 14 g/min generated a significant amount of fines visible through the product chamber window that caused an excessive pressure drop across the outlet filter (**Figure 4-2a**). As a result, the process had to be paused 40 times to clear filter buildup (**Figure 4-2b**) before ultimately failing due to severe agglomeration and full bed quenching. Coating of milk powder in the CLAMshell formulation ($MA_C^{2.0}$) drastically reduced filter clogging and did not require process interruptions to clear the filter (**Figure 4-2a** and **2b**).

Table 4-2 Characterization of M and $MA_C^{2.0}$ milk powder formulations (**Table 4-1**) spray coated with varying spray rates and nozzle pressures.

Formulation	Spray Rate (g/min)	Nozzle Pressure (bar)	Aggregates (%)[1]	Fines (%)	Coating Efficiency (%)
M	6	1.5	0.52 ± 0.13[a]	0.03 ± 0.05	88.1
	8	1.5	0.84 ± 0.30[a]	0.05 ± 0.06	89.0
	10	1.5	1.39 ± 0.55[a]	0.07 ± 0.05	85.6
	12	1.5	**5.42 ± 0.41**[b]	0.22 ± 0.19	85.9
	14	2	--	--	--
	14	4	--	--	--
$MA_C^{2.0}$	6	1.5	1.67 ± 0.34[a]	0.66 ± 0.11	84.4
	8	1.5	**12.9 ± 1.0**[b]	0.08 ± 0.05	91.1
	8	2.5	1.64 ± 0.48	0.01 ± 0.01	92.8
	10	2.5	**7.45 ± 1.04**	0.00	94.4
	10	3	**2.55 ± 0.36**	0.03 ± 0.03	93.4
	12	3.5	0.66 ± 0.21	0.06 ± 0.03	91.8
	14	4	1.68 ± 0.32	0.00	94.2

[1] Significantly affected by spray rate in M and $MA_C^{2.0}$ formulations at 1.5 bar nozzle pressure. Missing values (--) were unable to be determined due to batch failure. Bold values indicate aggregates above the acceptable limit of 2%.

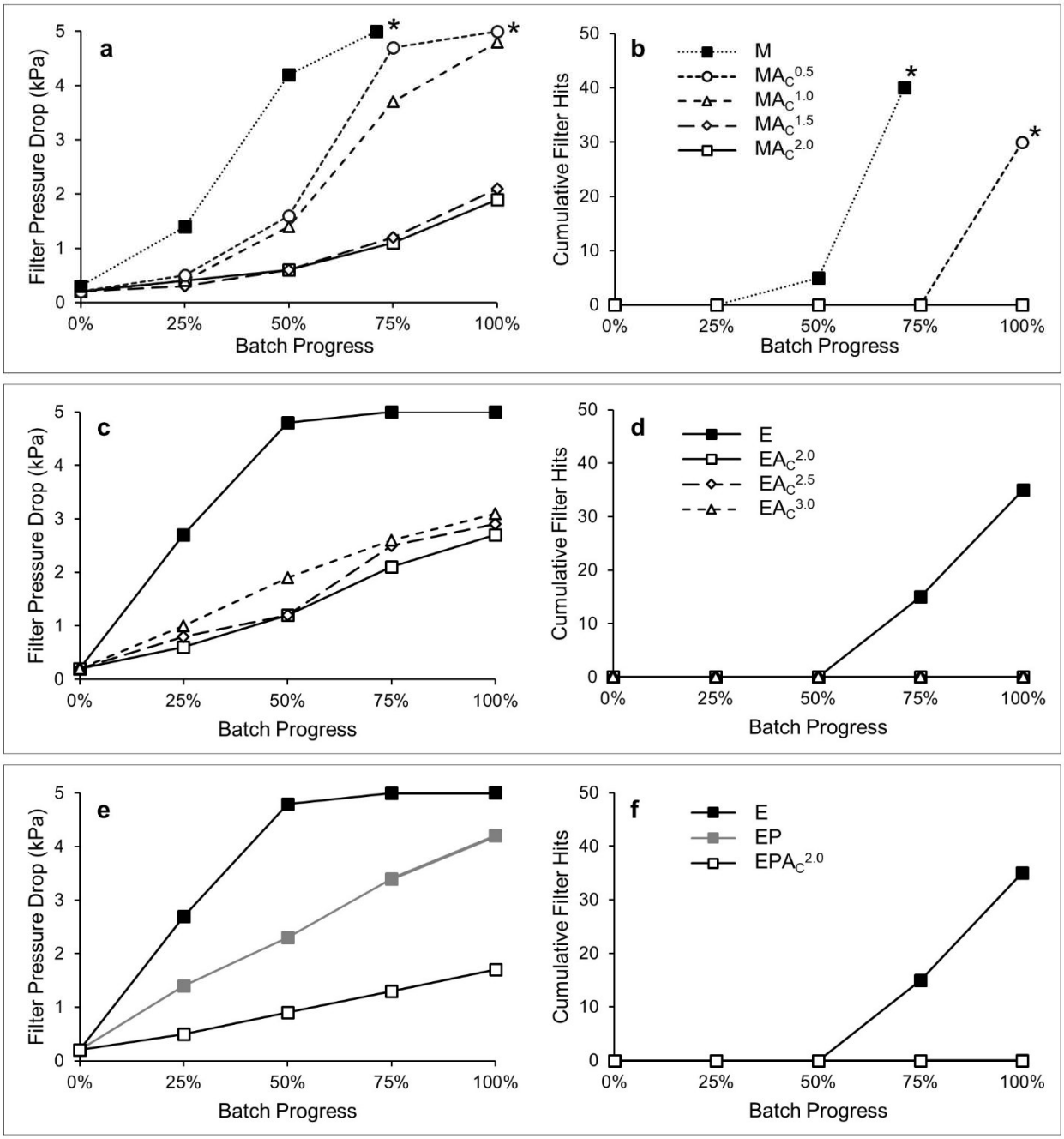


Figure 4-2 In-process parameters for milk powder and enzyme spray-coating formulations, showing pressure drop across the filter (a, c, and e) and cumulative filter hits, i.e. the total number of times the process had to be interrupted to clear filter buildup (b, d, and f). Parameters were monitored during coating of milk powder sprayed with and without the CLAMshell formulation (MA_C and M, respectively) at 14 g/min and 4 bar, and subtilisin enzyme mixture coated with and without the CLAMshell formulation (EA_C and E, respectively) sprayed at 14 g/min and 2 bar. The addition of 1% polyvinyl alcohol to the enzyme feed suspension is indicated by 'P' in e and f. Formulation compositions indicated in the legends are described in **Table 4-1**. Asterisks mark the point during the process when a batch failed.

Encapsulating subtilisin in the CLAMshell with 2% alginate ($EA_C^{2.0}$) similarly allowed for high spray rates (14 g/min) while minimizing aggregates and fines, and achieving high coating efficiencies (**Table 4-3**). For the $EA_C^{2.0}$ formulation, a lower nozzle pressure of 2 bar was found to be the most favorable condition for coating quality and process characteristics (**Supplementary Table 4-3** and **Supplementary Figure 4-1**). When the enzyme mixture was sprayed alone (E) at 14 g/min and 2 bar nozzle pressure, the process required over 30 interruptions to clear the filter, in contrast to EA_C with no interruptions and negligible filter buildup (**Figure 4-2c** and **2d**). With a 10% greater coating efficiency than E, $EA_C^{2.0}$ also had less than 1% aggregates and fines. While the fraction of fines was generally low for all coated products (**Table 4-3**), differences in the impact of the CLAMshell formulation on fines generation were reflected more strongly in the pressure-drop across the filter. The CLAMshell formulation consistently suppressed the formation of fines that normally caused excessive filter fouling (**Figure 4-2**). Polyvinyl alcohol (PVA), a well-established binder used in enzyme coatings (Chan et al., 2006), also helped to decrease filter fouling. Combining both alginate and PVA within the CLAMshell enzyme coating ($EPA_C^{2.0}$) even further reduced filter fouling, while maintaining high coating efficiency (**Figure 4-2e**).

Table 4-3 Characterization of M coated particles sprayed at 14 g/min and 4 bar and E coated particles sprayed at 14 g/min and 2 bar. Details of the formulations are given in **Table 4-1**.

Formulation	Alginate in Feed (%)	Aggregates (%)[1]	Fines (%)[1]	Heubach Dust (mg/g)[1,2]	Coating Efficiency (%)	Coating Integrity @20 sec (%)[1]
M*	0	--	--	--	--	--
$MA_C^{0.5}$*	0.5	--	--	--	--	--
$MA_C^{1.0}$	1	0.76 ± 0.17^{a}	0.01 ± 0.01	--	85.1	37.3 ± 1.4^{a}
$MA_C^{1.5}$	1.5	1.02 ± 0.19^{a}	0.00	--	87.9	$45.8 \pm 6.4^{a,b}$

$MA_C^{2.0}$	2	1.68 ± 0.32^{b}	0.00	--	94.2	55.6 ± 4.7^{b}
E	0	$0.18 \pm 0.05^{a,x}$	1.08 ± 0.20^{a}	$27.1 \pm 1.5^{a,x}$	80.6	$9.5 \pm 3.1^{a,x}$
$EA_C^{2.0}$	2	0.37 ± 0.03^{a}	0.81 ± 0.16^{a}	6.44 ± 0.57^{b}	90.5	56.6 ± 10.8^{c}
$EA_C^{2.5}$	2.5	1.31 ± 0.05^{b}	1.00 ± 0.21^{a}	2.17 ± 0.35^{c}	90.2	31.5 ± 2.4^{b}
$EA_C^{3.0}$	3	2.10 ± 0.31^{c}	1.52 ± 0.31^{b}	2.14 ± 0.17^{c}	86.4	26.4 ± 4.4^{b}
EP	0	0.26 ± 0.16^{x}	1.34 ± 0.47	4.79 ± 0.74^{y}	88.1	8.3 ± 0.9^{x}
$EPA_C^{2.0}$	2	1.25 ± 0.14^{y}	1.66 ± 0.20	1.94 ± 0.08^{z}	87.1	30.8 ± 3.3^{y}

[1] Significantly influenced by alginate concentration (a,b,c,d) or formulation type (x,y,z). Means with the same letter were not significantly different.
[2] Heubach dust analysis of any M formulations could not be completed due to hygroscopicity of milk powder leading to caking.
* Failed runs due to bed quenching. Product from these runs were not characterized.

4.5.2.2 Influence of CLAMshell formulation on feed fluid properties

Increases in either viscosity or surface tension of the feed lead to requiring higher pressures for effective atomization (Hede et al., 2008). Alginate increased the viscosity of the coating formulation nearly linearly with increased concentrations (**Figure 4-3a**). Without alginate, the viscosity of milk powder (M), enzyme (E), and enzyme + PVA (EP) coatings was in the range of 30 – 50 cP. The lowest addition of 0.5% alginate increased the viscosity to 8,500 cP, and up to 38,000 cP at 3% alginate. Thus, the increase in nozzle pressure facilitating higher spray rates with the CLAMshell formulation can be directly attributed to the viscosity increase by the alginate. The cargo, M or E, minimally impacted the feed viscosity, while the addition of 1% PVA had no additional impact (**Figure 4-3a**).

The surface tension of the feed, however, was sensitive to the cargo type but only minimally influenced by the concentration of alginate (**Figure 4-3b**). The surface tension of E was 34.5 ± 0.1 dynes/cm, significantly lower than 56.9 ± 0.1 dynes/cm for M. The addition of PVA had no impact

on the surface tension of the feed. The lower nozzle pressure required for efficient enzyme coating compared to milk powder coating may be due to the reduced surface tension of the feed. Close comparison of **Figure 4-2a** and **2c** shows that filter pressure built up quicker for E than for M; at a batch progress of 25%, the filter pressure drop for the E and M coating processes were ~ 2.5 kPa and ~ 1.5 kPa, respectively. Moreover, the filter pressure drop in the E coating process reached the maximum of 5 kPa by 50% batch progress compared to nearly 75% batch progress in the M coating process. The more rapid accumulation of fines in the outlet filter in the E coating process can be reasonably attributed to smaller droplets due to reduced surface tension of the formulation. However, the M coating process required more process interruptions for filter hits (**Figure 4-2b** and **2d**), suggesting that accumulation of M fines in the outlet filter was more disruptive than that of the E fines. Here, we surmise that the higher surface tension of the milk powder formulation resulted in the accumulation of wetter droplets clogging the outlet filter. Furthermore, the larger, less spreadable droplets on the particle surface may have resulted in wetter and tackier surfaces of the particle facilitating more aggregation (Rieck et al., 2020). The ultimate batch failure for the M coating was because of aggressive agglomeration in the bed leading to bed collapse.

While the viscosity or surface tension of the feed affected the coating process operation, there were no overall trends relating these fluid properties of the CLAMshell feed formulation to the assessed process parameters (coating efficiency, aggregation and fines generation) (**Table 4-3**, **Supplementary Figure 4-1**).

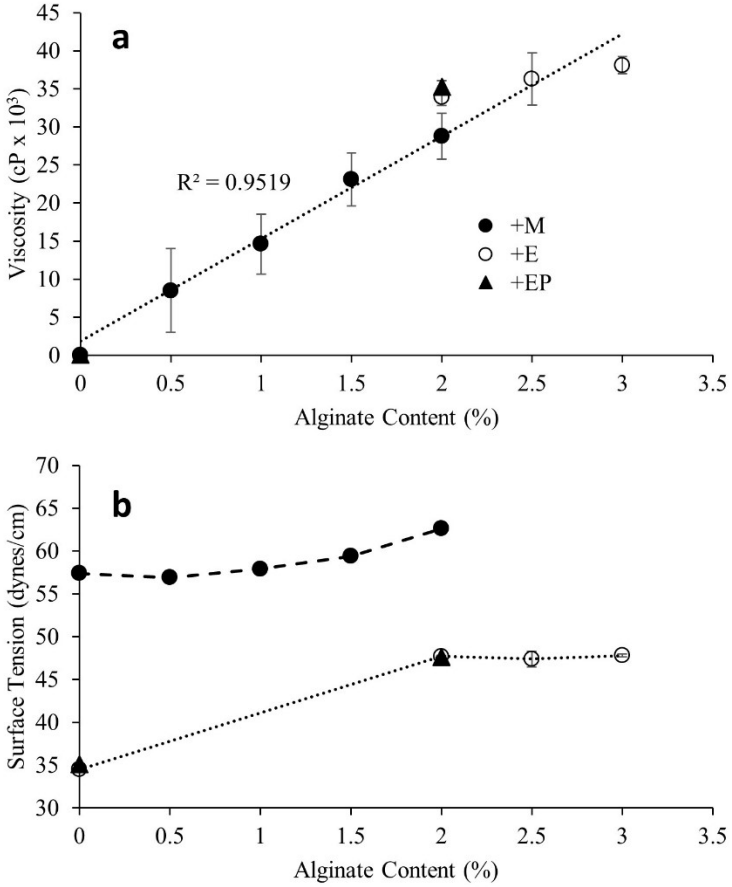


Figure 4-3 The influence of alginate content in CLAMshell formulations with milk powder (+M) or subtilisin enzyme (+E and +EP) on the viscosity (a) and surface tension (b) of the feed. Linear fit to the +E formulations is shown in (a). Lines in (b) are drawn to guide the eye.

4.5.3 In situ cross-linking alginate improves coating quality

4.5.3.1 Improved mechanical strength of enzyme coated in the CLAMshell due to crack suppression

A critical assessment of the quality of the particle coating is its mechanical strength, or the resistance to attrition. Coated particles can be subject to frequent particle-particle collisions while handling, both during and after the coating process. Erosion of the coating generates dust, which is a loss of product and a health risk if the cargo is an allergen. The test of the mechanical strength of the particle coating can be assessed by means of an attrition test using a Heubach dustmeter, whereby particles are subjected to controlled shear forces, and the generated attrition dust is

quantified by passing an airstream through the sample chamber and collecting the elutriated fines on an external filter (**Table 4-3**). The lower the amount of dust generated during the Heubach test, the higher the strength and quality of the coating. Overall, the coated granules produced from spray-coating the subtilisin enzyme feed alone (E) generated nearly 3% of the total original granule mass as dust, but spray-coated granules produced by encapsulating enzyme in the CLAMshell (EA_C) formulation significantly lowered dust generation to less than 0.6% of the original granule mass (**Table 4-3**). The CLAMshell enzyme formulations with alginate (EA_c) and with PVA (EP) were both highly effective at increasing the mechanical strength of the enzyme coating.

Visual assessments by SEM suggest that the increased mechanical strength of the CLAMshell can be attributed to suppression of cracks in the coating structure. All enzyme coated particles exhibited varying coating thicknesses ranging between 15-25 μm (**Figure 4-4**, **Figure 4-5**). Enzyme coated particles containing neither alginate nor PVA (E) displayed severe surface damage due to missing fragments of coating and numerous cracks (**Figure 4-4a**), generating 27.1 mg of dust per gram of particle (**Table 4-3**). Enzyme coated in the CLAMshell with either 2% alginate ($EA_C^{2.0}$) (**Figure 4-4b**) or 1% polyvinyl alcohol (EP) (**Figure 4-5b**) reduced surface cracking, and prevented fragmentation of the coating. $EA_C^{2.0}$ and EP coatings limited Heubach dust generation to 6.4 and 4.8 mg/g, respectively (**Table 4-3**). Supplementing the coating in either of these manners reduced Heubach dust of the resulting granules to 25% of granules coated with enzyme unsupplemented by either of these additives (E). Furthermore, increasing the alginate concentration to 3% in the CLAMshell formulation ($EA_C^{3.0}$) (**Figure 4-4c** and **4d**) or mixing PVA into the CLAMshell formulation ($EPA_C^{2.0}$) (**Figure 4-5c**) made surfaces appear smoother with small scratches, rather than severe cracks, resulting in the least damaged coatings in this study. Most impressively, the combination of cross-linked alginate and PVA in the coating ($EPA_C^{2.0}$)

resulted in the lowest Heubach dust of any coating combination tried: 1.9 mg/g, over 90% lower than that of enzyme alone E (**Table 4-3**).

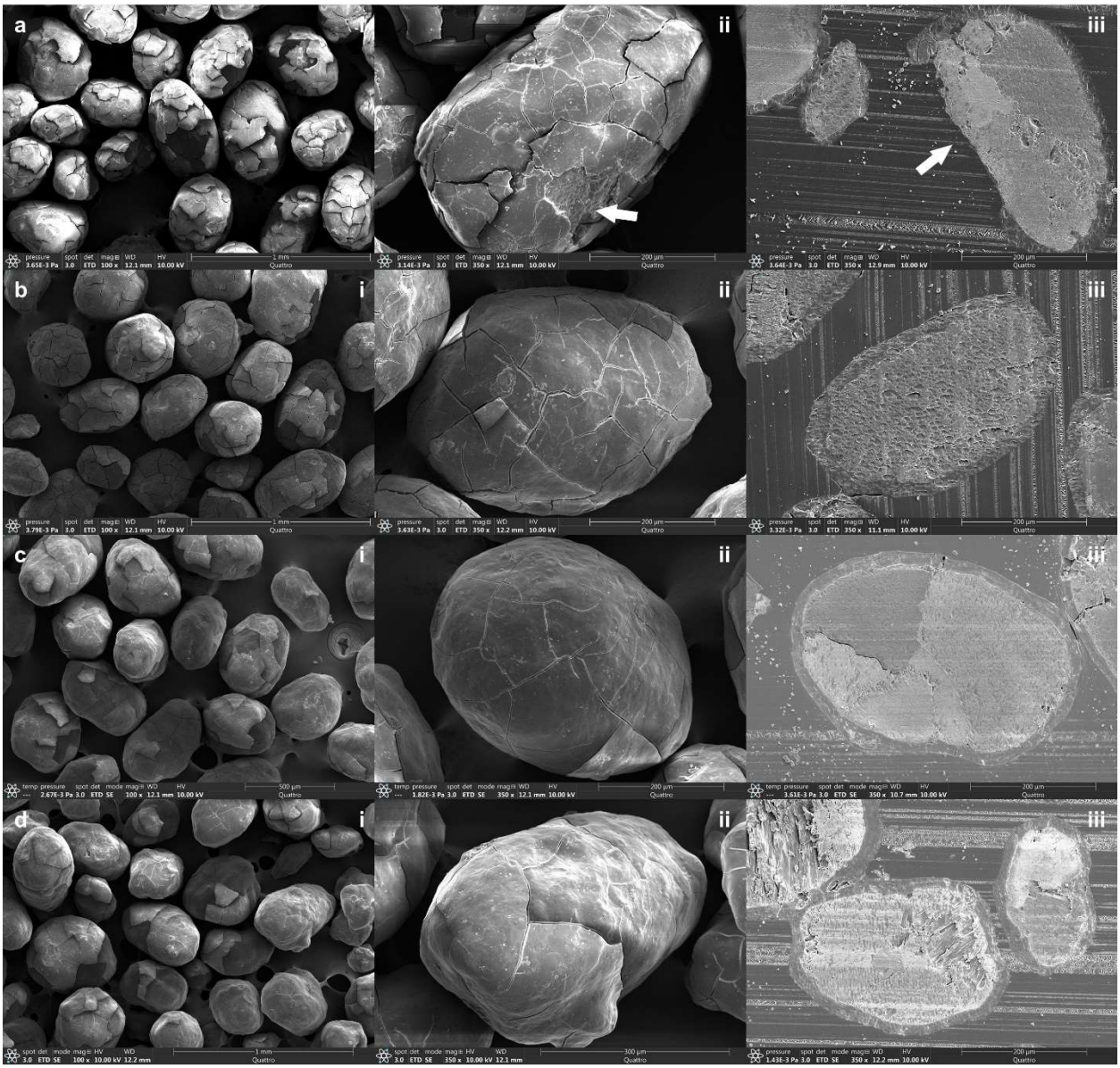


Figure 4-4 SEMs of surfaces (i and ii) and sections (iii) of E (a), $EA_C^{2.0}$ (b), $EA_C^{2.5}$ (c), and $EA_C^{3.0}$ (d) enzyme-coated particles. Arrows indicate missing fragments of coating.

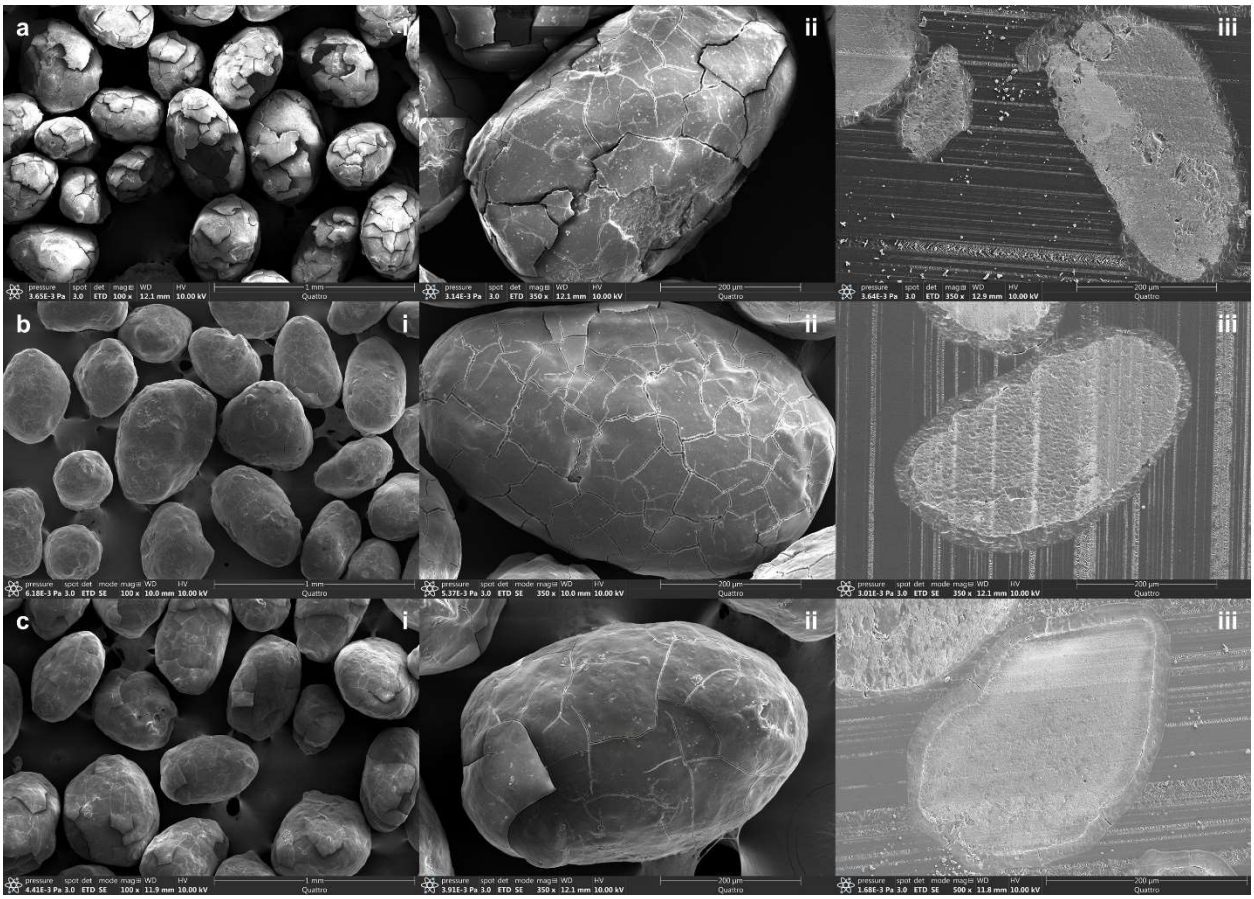


Figure 4-5 SEMs of surfaces (i and ii) and sections (iii) of E (a), EP (b), and $EPA_C^{2.0}$ (c) enzyme-coated particles.

4.5.3.2 Core dissolution rates controlled by cross-linked alginate coating

Unique to the in situ cross-linked alginate coating is its limited solubility in water. In aqueous solutions, the CLAMshell takes up water and swells, but will remain gelled (**Figure 4-1b**). Thus, the cross-linked alginate coat serves as a diffusion barrier at the particle surface. This ability of the CLAMshell coating to delay the dissolution of the sodium sulfate cores was demonstrated by conductivity measurements of the particles over the course of a 60-second dissolution test in a beaker of water (**Figure 4-6**). The coating integrity metric (*I*) (Eq. [4-2]), comparing the conductivity of coated to uncoated particles at 20 s, revealed that particle coating could delay core dissolution by up to 57 % (**Table 4-3**). The CLAMshell, in particular with 2% alginate ($EA_C^{2.0}$)

was the most effective at improving coating integrity (**Table 4-3**). While the addition of PVA in the enzyme coating (EP) was effective at suppressing cracks and decreasing Heubach dust, it did not improve the coating integrity (8.3 ± 0.9 and 9.5 ± 3.1, for E and EP, respectively) (**Figure 4-6c**).

Since CLAMshell coatings can be dissolved readily by chelating agents or pH changes that reverse alginate cross-linking, this provides a means to control the dissolution and release of the cargo from the particles. For example, cross-linked alginate matrices have demonstrated enteric release properties; forming acid gels and remaining intact in the gastric environment while fully dissolving and releasing in the higher pH and chelator-rich environment of the intestine (Agüero et al., 2017; Cook et al., 2012; Jeoh-Zicari et al., 2021).

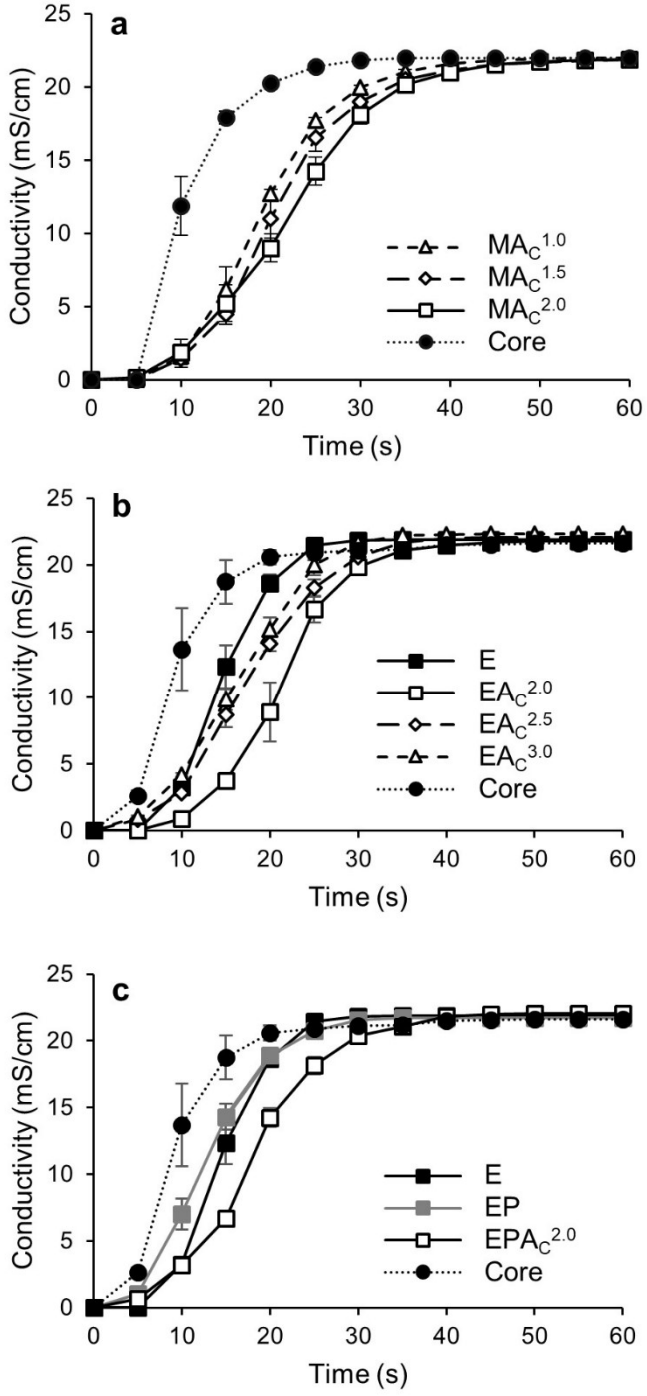


Figure 4-6 Effect of coated M (a) and E (b and c) formulations on the dissolution kinetics of sodium sulfate cores. Conductivity was measured while stirring coating particles in the surrounding Milli-Q water for 60 seconds after particles were added. Core conductivity measurements were repeated for each cargo. The coating integrity (*I*) of each formulation in **Table 4-3** was calculated by Eq. [4-2] using conductivity measurements at 20 seconds.

4.6 Discussion

Fluidized bed spray-coating is an attractive industrial process for encapsulating enzymes and other cargo within particle coatings. Yet, the optimal operation of a spray-coating process is typically an exercise in compromise. While process productivity calls for maximizing spray rates, this can readily lead to overwetting of the core surface due to insufficient drying of the atomized feed, leading to interparticle bridging, agglomeration and reduced product yield (Rieck et al., 2020). To some extent, this can be compensated for by increasing the atomization pressure at the spray nozzle to induce formation of a spray composed of smaller droplets, or by increasing the inlet temperature of the fluidizing air to accelerate drying (Hede et al., 2008). However, this approach easily tips over into premature drying of the smaller droplets before they can contact, spread and adhere to the core surface, thereby producing spray-dried fines. Excessive spray-dried fines collect on and foul the exit filters, reducing fluidization and drying efficiency, reducing yield, and resulting in a dusty product. Thus, spray rates are constrained by the need to strike a careful balance in avoiding agglomeration or spray drying (Vengateson et al., 2016).

This challenge of compromise also plays out in terms of product quality. A high quality coating must exhibit high integrity and mechanical strength, as exemplified by the dissolution rate and Heubach dust metrics used in this study. Production of strong, integral coatings requires minimizing the generation of both agglomerates and spray-dried fines. Coating strength can be improved to some extent by adding a synthetic polymeric binder, such as polyvinyl alcohol. But binders such as PVA typically also increase the risk of agglomeration due to their tackiness, and therefore require reduction of spray rates to allow adequate drying (Dewettinck et al., 1998a; Hede et al., 2009a).

In this study, we have demonstrated a means of maximizing process productivity, while simultaneously improving the coating quality of a spray-coated enzyme. By in situ cross-linking

of alginate incorporated within the enzyme feed suspension sprayed onto core, the resulting cross-linked alginate matrix shell (CLAMshell) particle exhibits low dust and high coating integrity, and can be produced with improved spray rates, and high process yields. While incorporating alginate in the feed suspension increased its viscosity, spray droplet size was kept small by increasing nozzle pressures, thus avoiding overwetting. The in situ cross-linking of the alginate resulted in a strong matrix that adhered strongly to cores, yet showed little tackiness or tendency to agglomerate, even when combined with PVA. Encouragingly, the in situ cross-linked alginate matrix spray also appeared to suppress formation of spray dried fines, perhaps due to its viscosity and drying characteristics. Thus, the CLAMshell process provides a larger operating window for spray-coating at faster rates and reduced risks of agglomeration or fines production.

Enzymes coated in the CLAMshell consistently resulted in uniform, well-adhered particle coats with minimal surface cracking. Additionally, the CLAMshell offers the potential for controlled release of the enzyme in an aqueous suspension, providing the option for a diffusion-limited delay in cargo release or rapid dissolution in the presence of a chelator. While PVA has been proven to be an effective binder and coating for detergent enzyme particles and offer attrition resistance, its tackiness as a binder can lead to agglomeration and places a limit on feasible spray rates (Hede et al., 2009a, 2009b). Of particular note, the combination of in situ cross-linked alginate and PVA within a single CLAMshell particle coating demonstrated a synergistic benefit not only in process throughput and yield improvement, but also in product quality enhancement, resulting in a coated product with lower Heubach dust and higher coating integrity than that of an equivalent CLAMshell composition without the incorporation of PVA in the coating. Reduced attrition, breakage, and coating loss during processing and handling increases enzyme yield and

minimizes the formation of sensitizing dusts that can pose exposure hazards to individuals working within close proximity to such enzyme products (Bonakdar et al., 2015; Jørgensen et al., 2005).

4.7 Conclusion

In situ cross-linking of alginate, previously demonstrated with spray-drying, was successfully applied in a fluidized bed spray-coating process, to encapsulate subtilisin enzyme within a coating applied to sodium sulfate cores, thereby creating novel CLAMshell particles. Inclusion of the CLAMshell formulation in the coating realized a 40% increased spray rate compared to coatings containing cargo only. Coating efficiency increased up to 10% by reducing fines and agglomerates generated in-process, and eliminating the need for process interruptions due to filter fouling. Furthermore, creating a mechanically stronger coat reduced attrition dust generation by 90%, and suppressed the severity and frequency of surface cracking, fragmentation and delamination. The combination of PVA with the CLAMshell formulation was found to synergistically maximize process efficiency and product quality. CLAMshell particles also demonstrate improved coating integrity, as manifested by delayed release of the salt core upon dissolution of the coated particle. The data presented here provide a basis for simultaneously increasing the productivity and coating quality of spray-coated products. This novel CLAMshell particle formulated by fluidized bed spray-coating, demonstrated using an industrial enzyme, has the potential to protect other bioactive or sensitive cargos, and provides a new tool for controlling their release.

4.8 Supplemental Information

Supplementary Table 4-1 Characterization of non-fat dry milk powder (M) and milk powder plus crosslinked alginate (MA_C) particles coated at varying spray rates and nozzle pressures.

Formulation	Spray Rate (g/min)	Nozzle Pressure (bar)	Aggregates (%)**	Fines (%)*	Coating Efficiency (%)	Mean Particle Diameter (µm)*	Bulk Density (g/mL)*	Moisture Content (%)**	Water Activity**
M	6	1.5	0.52 ± 0.13[a]	0.03 ± 0.05	88.1	332.6 ± 6.9[a]	1.40 ± 0.02	0.59 ± 0.02[a]	0.249 ± 0.007[a]
	8	1.5	0.84 ± 0.30[a]	0.05 ± 0.06	89.0	345.7 ± 5.0[a,b]	1.39 ± 0.03	0.84 ± 0.01[b]	0.330 ± 0.005[b]
	10	1.5	1.39 ± 0.55[a]	0.07 ± 0.05	85.6	351.1 ± 9.0[b]	1.40 ± 0.03	0.76 ± 0.02[c]	0.315 ± 0.002[c]
	12	1.5	5.42 ± 0.41[b]	0.22 ± 0.19	85.9	346.4 ± 3.2[a,b]	1.37 ± 0.02	0.84 ± 0.04[b]	0.317 ± 0.002[c]
	14	2	--	--	--	--	--	--	--
	14	4	--	--	--	--	--	--	--
$MA_C^{2.0}$	6	1.5	1.67 ± 0.34	0.66 ± 0.11	84.4	339.8 ± 2.4	1.42 ± 0.03	0.84 ± 0.04	0.247 ± 0.005
	8	1.5	12.9 ± 1.0	0.08 ± 0.05	91.1	374.7 ± 2.2	1.30 ± 0.02	1.10 ± 0.07	0.366 ± 0.006
	8	2.5	1.64 ± 0.48	0.01 ± 0.01	92.8	345.7 ± 5.9	1.35 ± 0.02	0.50 ± 0.05	0.348 ± 0.003
	10	2.5	7.45 ± 1.04	0.00	94.4	359.7 ± 8.1	1.36 ± 0.02	0.42 ± 0.04	0.348 ± 0.002
	10	3	2.55 ± 0.36	0.03 ± 0.03	93.4	346.9 ± 2.0	1.36 ± 0.01	0.33 ± 0.04	0.340 ± 0.004
	12	3.5	0.66 ± 0.21	0.06 ± 0.03	91.8	337.3 ± 3.7	1.36 ± 0.01	0.40 ± 0.11	0.338 ± 0.003
	14	4	1.68 ± 0.32	0.00	94.2	354.0 ± 5.4	1.39 ± 0.02	0.46 ± 0.08	0.354 ± 0.006

*Significantly influenced by spray rate for $MA_C^{2.0}$ formulations sprayed at 1.5 bar.
**Significantly influenced by spray rate for both formulations sprayed at 1.5 bar. Means with the same letter were not significantly different.

Supplementary Table 4-2 Characterization of milk powder (M) and enzyme (E) feed suspensions and particles with varying alginate feed concentrations and PVA amendments.

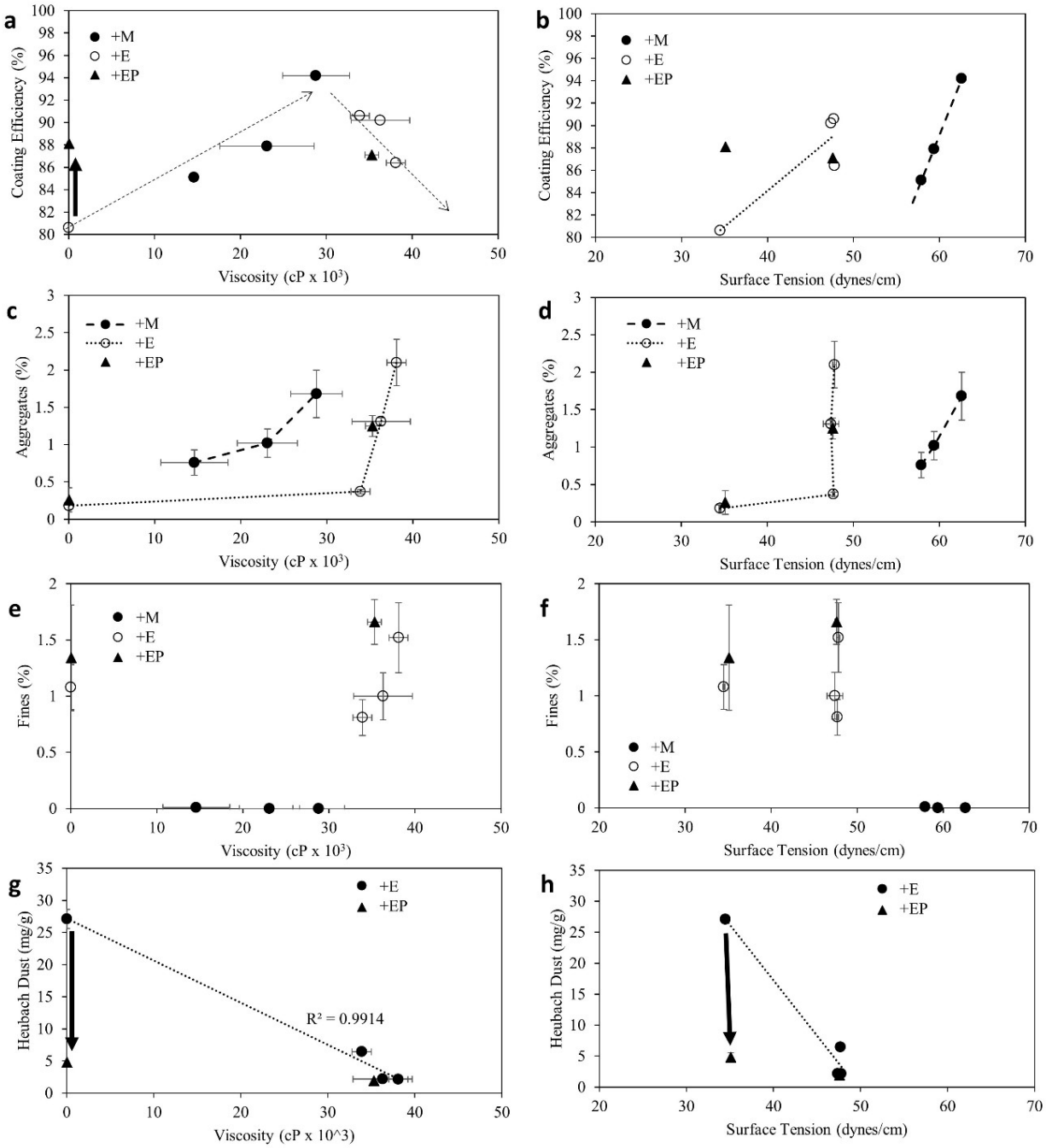
Formulation	Viscosity (cP × 10^3)*	Surface Tension (dynes/cm)*	Aggregates (%)*	Fines (%)*	Heubach Dust (mg/g)*	Coating Efficiency (%)	Particle Dia. (µm)*	Bulk Density (g/mL)*	Moisture Content (%)*	Water Activity*
M*	0.03 ± 0.01^{a}	$57.4 \pm 0.2^{a,b}$	--	--	--	--	--	--	--	--
$MA_C^{0.5}$*	$8.2 \pm 5.5^{a,b}$	56.9 ± 0.1^{a}	--	--	--	--	--	--	--	--
$MA_C^{1.0}$	$14.6 \pm 3.9^{b,c}$	57.9 ± 0.1^{b}	0.76 ± 0.17^{a}	0.01 ± 0.01	--	85.1	345.6 ± 1.9	1.41 ± 0.01	1.44 ± 0.04^{a}	0.355 ± 0.002
$MA_C^{1.5}$	$23.1 \pm 3.5^{c,d}$	59.4 ± 0.5^{c}	1.02 ± 0.19^{a}	0.00	--	87.9	352.4 ± 5.0	1.38 ± 0.03	1.39 ± 0.04^{a}	0.354 ± 0.001
$MA_C^{2.0}$	28.8 ± 3.0^{d}	62.2 ± 0.3^{d}	1.68 ± 0.32^{b}	0.00	--	94.2	354.0 ± 5.4	1.39 ± 0.02	0.46 ± 0.08^{b}	0.354 ± 0.006
E	$0.03 \pm 0.00^{a,x}$	$34.5 \pm 0.1^{a,x}$	$0.18 \pm 0.05^{a,x}$	1.08 ± 0.20^{a}	$27.1 \pm 1.5^{a,x}$	80.6	310.7 ± 2.7^{a}	1.44 ± 0.03^{a}	$0.85 \pm 0.04^{a,x}$	$0.327 \pm 0.010^{a,x}$
$EA_C^{2.0}$	33.9 ± 1.1^{b}	47.7 ± 0.1^{b}	0.37 ± 0.03^{a}	0.81 ± 0.16^{a}	6.44 ± 0.57^{b}	90.5	320.1 ± 1.2^{b}	1.45 ± 0.02^{a}	0.74 ± 0.07^{a}	0.269 ± 0.010^{b}
$EA_C^{2.5}$	36.3 ± 3.4^{b}	47.4 ± 0.9^{b}	1.31 ± 0.05^{b}	1.00 ± 0.21^{a}	2.17 ± 0.35^{c}	90.2	320.0 ± 0.9^{b}	$1.42 \pm 0.02^{a,b}$	1.14 ± 0.01^{b}	0.363 ± 0.014^{c}
$EA_C^{3.0}$	38.1 ± 1.1^{b}	47.8 ± 0.2^{b}	2.10 ± 0.31^{c}	1.52 ± 0.31^{b}	2.14 ± 0.17^{c}	86.4	$316.9 \pm 4.0^{a,b}$	1.38 ± 0.01^{b}	1.20 ± 0.04^{b}	0.365 ± 0.009^{c}
EP	0.05 ± 0.00^{y}	35.1 ± 0.1^{x}	0.26 ± 0.16^{x}	1.34 ± 0.47	4.79 ± 0.74^{y}	88.1	310.6 ± 3.3	1.42 ± 0.01	0.90 ± 0.04^{x}	0.358 ± 0.007^{x}
$EPA_C^{2.0}$	35.3 ± 0.8^{z}	47.6 ± 0.2^{y}	1.25 ± 0.14^{y}	1.66 ± 0.20	1.94 ± 0.08^{z}	87.1	313.2 ± 1.0	1.40 ± 0.01	1.00 ± 0.03^{y}	$0.352 \pm 0.020^{x,y}$

*Significantly influenced by alginate concentration (a,b,c,d) or formulation type (x,y,z). Means with the same letter were not significantly different.
**Failed runs due to bed quenching. Product from these runs were not characterized.

Supplementary Table 4-3 Characterization of enzyme (E) coated particles and enzyme plus crosslinked alginate (EA_C) coated particles, coated at 14 g/min and varying nozzle pressures.

Formulation	Nozzle Pressure (bar)	Aggregates (%)*	Fines (%)*	Heubach Dust (mg/g)**	Coating Efficiency (%)	Mena Particle Diameter (µm)*	Bulk Density (g/mL)**	Moisture Content (%)**	Water Activity**
E	1	0.19 ± 0.02	1.04 ± 0.07	22.8 ± 1.5	87.8	314.9 ± 0.5	1.38 ± 0.01	0.56 ± 0.10	0.199 ± 0.010
	2	0.18 ± 0.05	1.08 ± 0.20	27.1 ± 1.5	80.6	310.7 ± 2.7	1.44 ± 0.03	0.85 ± 0.04	0.327 ± 0.010
$EA_C^{2.0}$	1	9.42 ± 0.37^{a}	0.66 ± 0.07^{a}	3.82 ± 0.34^{a}	92.8	339.1 ± 2.7^{a}	1.38 ± 0.03^{a}	0.96 ± 0.02^{a}	0.324 ± 0.001^{a}
	2	0.37 ± 0.03^{b}	0.81 ± 0.16^{a}	6.44 ± 0.57^{b}	90.5	320.1 ± 1.2^{b}	1.45 ± 0.02^{b}	0.74 ± 0.07^{b}	0.269 ± 0.010^{b}
	3	0.17 ± 0.02^{b}	3.05 ± 0.42^{b}	4.89 ± 0.91^{a,b}	71.2	307.8 ± 2.2^{c}	1.42 ± 0.00^{a,b}	1.04 ± 0.12^{a}	0.325 ± 0.010^{a}
	4	0.13 ± 0.00^{b}	4.66 ± 1.02^{c}	4.78 ± 0.81^{a,b}	57.8	301.9 ± 2.7^{c}	1.43 ± 0.01^{a,b}	1.16 ± 0.07^{a}	0.386 ± 0.010^{c}

*Significantly influenced by nozzle pressure for $EA_C^{2.0}$. Means with the same letter were not significantly different.
**Significantly influenced by nozzle pressure for both formulations.



Supplementary Figure 4-1 Correlation between feed fluid properties (viscosity and surface tension) and process parameters or product characterization – coating efficiency (a and b), sieving aggregates (c and d), sieving fines (e and f), and Heubach Dust (g and h) – for alginate formulations with milk powder (+M) or subtilisin enzyme (+E).

4.9 References

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Chapter 5 Comparing spray-drying and fluidized bed spray-coating to encapsulate non-spore-forming plant-beneficial bacteria

5.1 Abstract

Microencapsulation of plant-beneficial bacteria for use as biopesticides is a growing area of interest in the search for alternatives to harmful chemical pesticides. In this study, we assessed the microencapsulation of the Gram-negative non-spore-forming plant-beneficial bacterial strain *Collimonas arenae* Cal35 in novel cross-linked alginate microcapsules (CLAMs) formed by spray-drying, and in novel cross-linked alginate matrix shell (CLAMshell) particles formed by fluidized bed spray-coating. Spray-drying Cal35 resulted in 10-fold greater survival than fluidized bed spray-coating. During individual stress tests, Cal35 was more tolerant to high temperatures than to desiccation, indicating greater survival would be expected during spray-drying. CLAMs ranged between 5-20µm, indicating potential for spray applications. CLAMshell particles ranged between 250-300 µm, suggesting potential for seed coatings.

5.2 Abbreviations

Cal35	*Collimonas arenae* Cal35
CFU	colony forming unit
CLAM	cross-linked alginate microcapsules
CLAMshell	cross-linked alginate matrix shell
KB	King's medium B
PBS	phosphate buffered saline
SEM	scanning electron microscopy

5.3 Introduction

Microencapsulation is a highly valued process used across many industries. It is a preservation technique where some wall material(s) confers protection to enclosed cargo with a number of additional functions, depending on the application (Jyothi et al., 2010). For example, in the biomedical industry, drugs are encapsulated for targeted delivery and controlled release (Ma, 2014). In the food industry, compounds are encapsulated to mask or retain certain flavors (Nazzaro et al., 2012). And in the agricultural industry, pesticides are often encapsulated for greater stability and controlled release in cropland to combat a variety of pests (Alonso et al., 2014; Tsuji, 2001). It is of particular interest to encapsulate biopesticides, such as plant-beneficial bacteria, as there is a growing need to replace more traditional chemical pesticides that are harmful to humans and the environment.

The two primary means of applying plant beneficial bacteria for crop utilization are by spraying in-furrow or in-field, and by coating onto seed. For spray applications, encapsulation of beneficial bacteria is helpful to confer protection against other ingredients in spray tanks which may damage the bacteria, and against environmental effects in sprayed areas (Preininger et al., 2018). Microcapsules are constrained by the nozzle size of the sprayer, on the scale of microns, as to limit blockages, settlement, and aggregation of particulates (Jambhulkar et al., 2016; Preininger et al., 2018). For coating applications, the seed coating itself serves as the protective encapsulation matrix for the bacteria. Rotary drum dryers are the most widely used method of coating seed; however, bacterial viability is diminished by other ingredients in the seed coating formulation (similar to spray tanks) and by the long drying process (Deaker et al., 2004; Hartley et al., 2012).

Encapsulation of these plant beneficial bacteria has mainly been successfully demonstrated using alginate beads. Alginate is a naturally derived polymer desirable as a microcapsule wall material because it is a relatively low cost ingredient, is safe to handle, and has the ability to form

a gel network (Draget et al., 2011; Lee et al., 2012). Composed of alternating or repeating units of β-D-mannuronic acid and α-L-guluronic acid, the polyurea backbone of alginate contains carboxylate groups allowing ion-mediated cross-linking to form a gel structure (Bruchet et al., 2015; Hecht et al., 2016). Alginate beads containing bacteria are typically formed by dripping a suspension of dissolved alginate and bacterial cells into a calcium chloride bath, where cross-linking occurs upon contact at the surface (Rohman et al., 2021; Schoebitz et al., 2012; Wu et al., 2014). This gelling step is usually followed by a curing step, a washing step, and a drying step, before dry alginate beads are formed. The need for a separate calcium bath and many individual steps make producing alginate beads challenging to scale up (Ching et al., 2017).

Recently, methods for forming in situ cross-linked alginate microcapsules (CLAMs) by spray-drying (Jeoh-Zicari et al., 2020a), and in situ cross-linked alginate matrix shell (CLAMshell) particles by fluidized bed spray-coating (Jeoh-Zicari et al., 2020b) were developed here at UC Davis. Encapsulation of bacteria (Strobel et al., 2018) (Chapter 3), enzymes (Santa-Maria et al., 2012), polymers (Wong et al., 2020), and oils (Strobel et al., 2020a; Strobel et al., 2016) in CLAMs have been demonstrated previously, while encapsulation in CLAMshell particles has been accomplished only for enzymes so far (Kawakita et al., 2021). To produce these microcapsules, atomization at the nozzle evaporates a volatile base lowering the pH of the droplet to allow an insoluble form of calcium at a higher pH to solubilize, inducing ion-mediated cross-linking of alginate as the droplets dry into a particle (CLAM) or form a coating (CLAMshell). This single-step encapsulation process can be industrially scalable while still remaining relatively cheap (Strobel et al., 2020b). In addition, both spray-drying and fluidized bed spray-coating are widespread in industry, making the in situ cross-linking processes easier to implement.

In this study, we compared spray-drying and fluidized bed spray-coating to investigate encapsulation of the potential biofungicide bacterium *Collimonas arenae* (*C. arenae*) Cal35 in CLAM and CLAMshell particles. Cal35 will serve as the model non-spore-forming gram-negative plant beneficial soil bacteria for microencapsulation due to its previously demonstrated success as an antifungal agent in laboratory and greenhouse settings (Akum et al., 2020; Doan et al., 2020; Fritsche et al., 2014; Mela et al., 2011; Mosquera et al., 2020), and the ability to retain its antifungal properties after spray-drying and shelf storage (Chapter 3). Sensitivity of Cal35 to heat and desiccation was tested. Survival of Cal35 during each encapsulation method and the resulting particle yields were assessed. Spray-dried particles and fluidized bed spray-coated particles containing Cal35 were characterized by SEM, moisture content analysis, and water activity analysis.

5.4 Materials and Methods

5.4.1 Materials

Ammonium hydroxide, 28-30% (NH_4OH), dicalcium phosphate (Ca_2HPO_4), glycerol, magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), peptone, potassium phosphate dibasic (K_2PO_4), succinic acid were purchased from Fisher (Waltham, MA). Low-viscosity alginate (A1112) and maltodextrin (dextrose equivalent 4.0-7.0) were acquired from Millipore-Sigma (Burlington, MA). Agar was purchased from BTS (Houston, TX). Concentrated 20x phosphate-buffered saline (PBS) was purchased from VWR (Radnor, PA). HI-CAP 100 modified starch was purchased from Ingredion (Westchester, IL). Impact beads (glass cores) were acquired from Grainger (Lake Forest, IL). Milli-Q water from a Millipore Ultrapure Water Purification System was used to prepare all solutions. *C. arenae* Cal35 was originally isolated from forest soil in the Jug Handle State Natural Reserve in Mendocino County, CA (Uroz et al., 2014).

5.4.2 Methods

5.4.2.1 Culture conditions for Collimonas arenae *Cal35*

Fresh cultures of *C. arenae* Cal35 were prepared and grown in King's medium B (KB) using the conditions described previously (3.4.2.1).

5.4.2.2 Testing sensitivity of Cal35 to temperature and desiccation

Cultures of Cal35 were harvested and collected by centrifugation at 5,000 rpm. Supernatant was removed and the remaining cell pellet was resuspended in 1X phosphate buffered saline (PBS). Initial and final viable cell counts were measured using the serial dilution method by plating each dilution onto KB agar and counting colony forming units (CFUs).

To assess sensitivity towards temperature, Cal35 suspended in 1X PBS was transferred to 15 mL conical tubes. Initial counts before heating were about 9.5 log[CFU/mL]. The conical tubes (n = 3) were placed into a heated water bath from 35 to 60°C and sampled periodically over three hours to measure changes in viable cell counts.

To assess sensitivity towards desiccation, 3 mL of fresh Cal35 culture resuspended in 1X PBS was transferred to empty petri dishes. Initial counts before drying averaged 9.5 log[CFU/mL]. These petri dishes were placed open in a biological safety cabinet at room temperature (~21°C), and dried for 24 hr using the airflow of the unit. Dishes containing the dehydrated Cal35 suspension were rehydrated with 3 mL of 1X PBS and plated to count CFUs.

5.4.2.3 Spray-drying formulation of Cal35 encapsulated in CLAMs

To prepare 50 g of feed for spray-drying, 1.5 L of fresh culture was harvested and centrifuged at 4,200 rpm for 15 min to pellet the cells. The supernatant was removed and the cell pellet was resuspended in 12.5 g of 4% filter-sterilized succinic acid, which was previously adjusted with NH_4OH to pH 7. This was added to an autoclaved suspension containing modified starch, maltodextrin, sodium alginate, and $CaHPO_4$. The final concentration of the feed for spray-drying

was: 13% HI-CAP 100 modified starch, 2% maltodextrin, 2% sodium alginate, 1% succinic acid at pH 7, and 0.5% $CaHPO_4$.

Spray-drying was performed using a BUCHI B-290 benchtop spray-dryer (BUCHI Corporation, New Castle, DE) (**Figure 5-1**). The control parameters used were: 90°C inlet temperature, 40% spray rate (~12 g/min), 40 mm nozzle pressure (0.5 bar), 100% aspirator. The average outlet temperature was 42 ± 1°C. After the 50 g of feed was sprayed, particles were removed and collected from the product chamber.

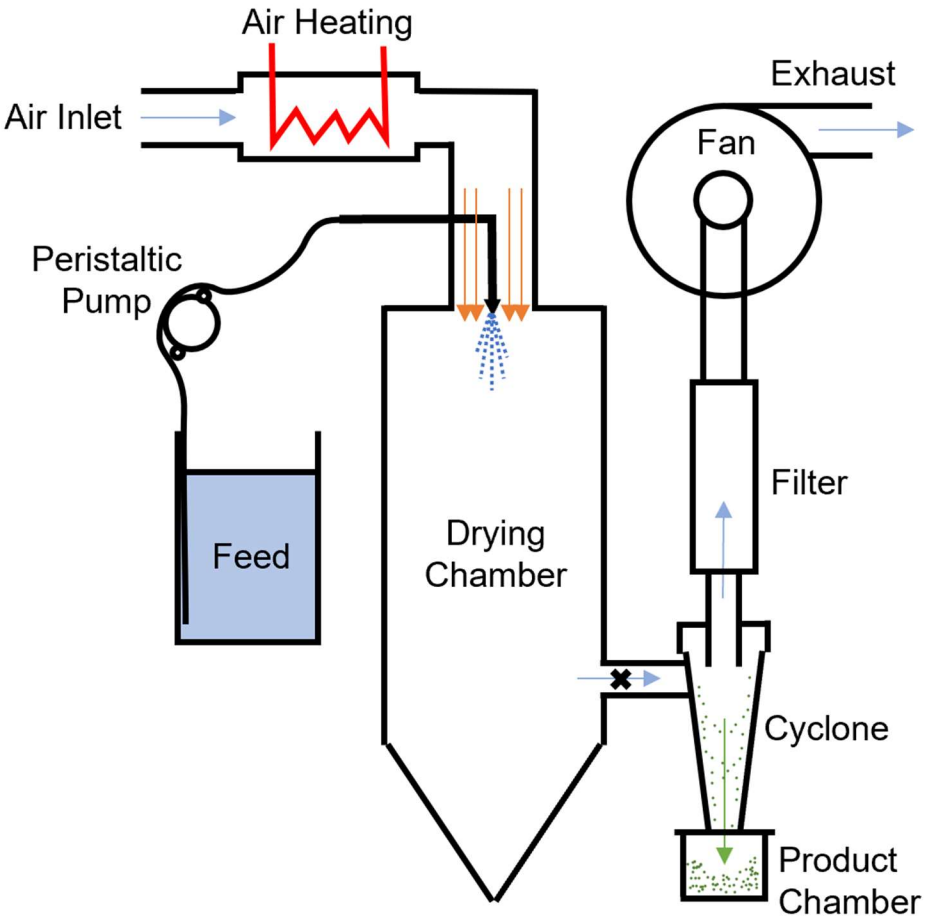

Figure 5-1 Schematic of a Buchi B-290 benchtop spray-dryer. Outlet temperatures is measured in the bridge between the drying chamber and the cyclone, marked by the 'x'.

5.4.2.4 Fluidized bed spray-coating formulation of Cal35 encapsulated in CLAMshells

To prepare 200 g of feed for spray-coating, 6 L of culture was harvested and prepared in the same manner as spray-drying (Section 2.2.3). The final concentration of the feed for spray-coating was the same as the feed for spray-drying: 13% HI-CAP 100 modified starch, 2% maltodextrin, 2% sodium alginate, 1% succinic acid at pH 7, and 0.5% $CaHPO_4$.

Spray-coating was performed using a UniGlatt 2 L capacity fluidized bed coater in Wurster configuration (Glatt Air Techniques, Inc., Ramsey, NJ) (**Figure 5-2**). For the core material, 600 g of glass beads were inserted into the product chamber of the unit. The control parameters used were: 55-60°C inlet temperature (lower and upper limit), 32% spray rate (~10 g/min), 3.5 bar nozzle pressure, 25 deg airflow flap angle. The average outlet temperature was 40 ± 0.6°C. In-process samples were taken using the sample port to measure viability during the coating process. Once all 200 g of the feed was applied, fluidization continued for two minutes to allow more drying of the coatings before collection.

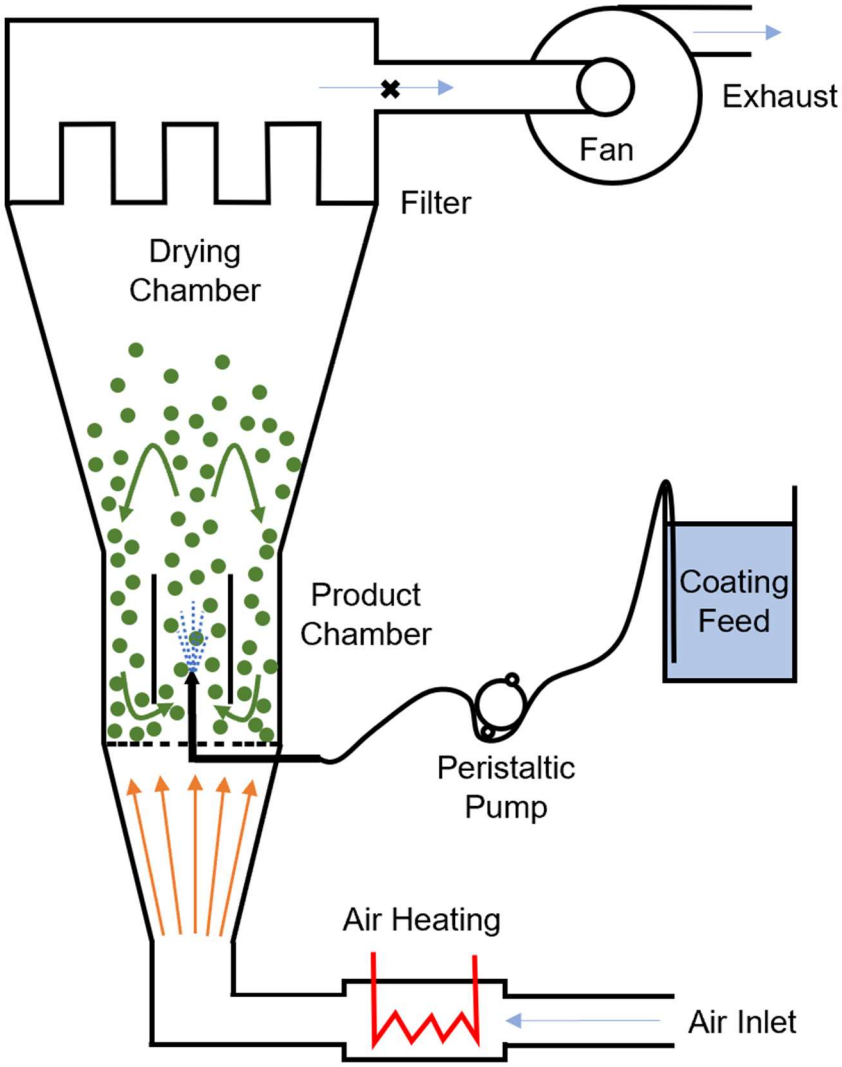

Figure 5-2 Schematic of UniGlatt fluidized bed spray-coater in Wurster configuration. Outlet temperature is measured between the filter and exhaust, marked by the 'x'.

5.4.2.5 Characterization of CLAMs and CLAMshells containing Cal35

Viability of Cal35 before encapsulation was measured by sampling from each feed suspension and using the dilution method to plate onto KB agar and count CFUs. Viability of Cal35 after encapsulation was measured by dissolving 0.03 ± 0.001 g of spray-dried particles and 2.0 ± 0.1 g spray-coated particles in 12.5 mL 1X PBS. Samples were mixed by inversion at room temperature for one hour before being plated onto KB agar to count CFUs.

Yield of product was calculated by taking the percentage of the total feed solids sprayed to the collected solids, either as the total spray-dried solids or total coated solids.

Particle morphology was visualized by scanning electron microscopy (SEM) using a Thermo Scientific Quattro SEM (Thermo Fisher Scientific, Waltham, MA). Samples of spray-dried and spray-coated particles were mounted onto carbon tape and sputter-coated in gold to a thickness of 120 angstrom. A beam voltage of 10 kV and a spot size of 3.0 were used for imaging.

Moisture content was measured gravimetrically by oven drying for three days at 60-70°C. Water activity was measured using an Aqualab Series 3TE water activity meter (METER, Pullman, WA).

5.4.2.6 Statistical analysis

Data was analyzed using the statistical software JMP (Statistical Discovery from SAS, Cary, NC). One way ANOVA and post hoc multiple comparison tests were used to determine significance of factors and differences between means. P values less than 0.05 were considered significant.

5.5 Results and Discussion

Spray-drying and fluidized bed spray-coating are two common industrial drying processes that can be used as methods for microencapsulation. Spray-drying (**Figure 5-1**) relies on significantly hotter air currents to quickly dry atomized droplets into particles, whereas spray-coating (**Figure 5-2**) exploits long drying times of continuously fluidizing particles being coated to require cooler drying temperatures (**Table 5-1**).

Table 5-1 Process parameters for spray-drying and fluidized bed spray-coating used in this study.

Parameter	**Spray-drying**	**Spray-coating**
Inlet Temperature	90°C	55-60°C
Outlet Temperature	42±1°C	40±0.6°C

Residence Time	<1-1.5 sec	20-25 min

5.5.1 Cal35 demonstrated greater survival with spray-drying than with fluidized bed spray-coating

In order to produce a collectable product, a blend of maltodextrin and modified starch was used as excipients to the CLAMs and CLAMshell formulations. Previously, these excipients enhanced the survival of Cal35 during spray-drying and shelf-storage in CLAMs (Chapter 3). However, when spray-coating with modified starch as the only excipient in the CLAMshell formulation, agglomeration of the final product always occurred.

Prior to encapsulation, the viable cell counts of Cal35 in both feed suspensions were above 10 log[CFU/g solids] (**Figure 5-3A**). Cal35 experienced approximately a 3 log reduction during spray-drying in contrast to a 4 log reduction during fluidized bed spray-coating (**Figure 5-3B**). Although outlet temperatures for both processes were around 40°C, it might appear that the inlet temperature of spray-drying being over 50% hotter than that of fluidized bed spray-coating points towards spray-drying being more harmful, however, spray-coating ended up being more harmful to the bacteria. Residence time was the other key difference between these encapsulation processes (**Table 5-1**). For the Buchi B-290 benchtop spray-dryer, the residence time can span in the milliseconds to seconds range, as droplets quickly pass through the evaporation chamber and fall into the collection chamber (**Figure 5-1**). This resulted CLAMs produced by spray-drying containing more viable bacteria (7 log[CFU/g solids]) than CLAMshells produced by spray-coating (6 log[CFU/g coated solids]). In the UniGlatt fluidized bed spray-coater, the residence time ranges from minutes to hours, depending on the amount of coating being applied to the fluidized particles (**Figure 5-2**). In this study, the residence time for spray-coating was about 23 minutes; 20 minutes for the coating suspension to be applied and generally three minutes of extra drying time (**Figure 5-4**). During the coating process, the viability of Cal35 consistently measured

about 7.5 log[CFU/g coated solids], however, the end product of spray-coating was lower in viability compared to spray-drying per gram of sprayed solids due to the final drying step. Although the viability of Cal35 was sustained in-process for the short coating duration, it is possible that thicker coatings may be applied to increase the total CFU in the coating. And while the argument could be made that the total CFU in spray-dried CLAMs can increase by simply spraying a greater volume of feed, the particle size of CLAMs would not change. Perhaps a thicker CLAMshell coating thus larger particle size would confer greater protection and stability to the encapsulated bacteria over time.

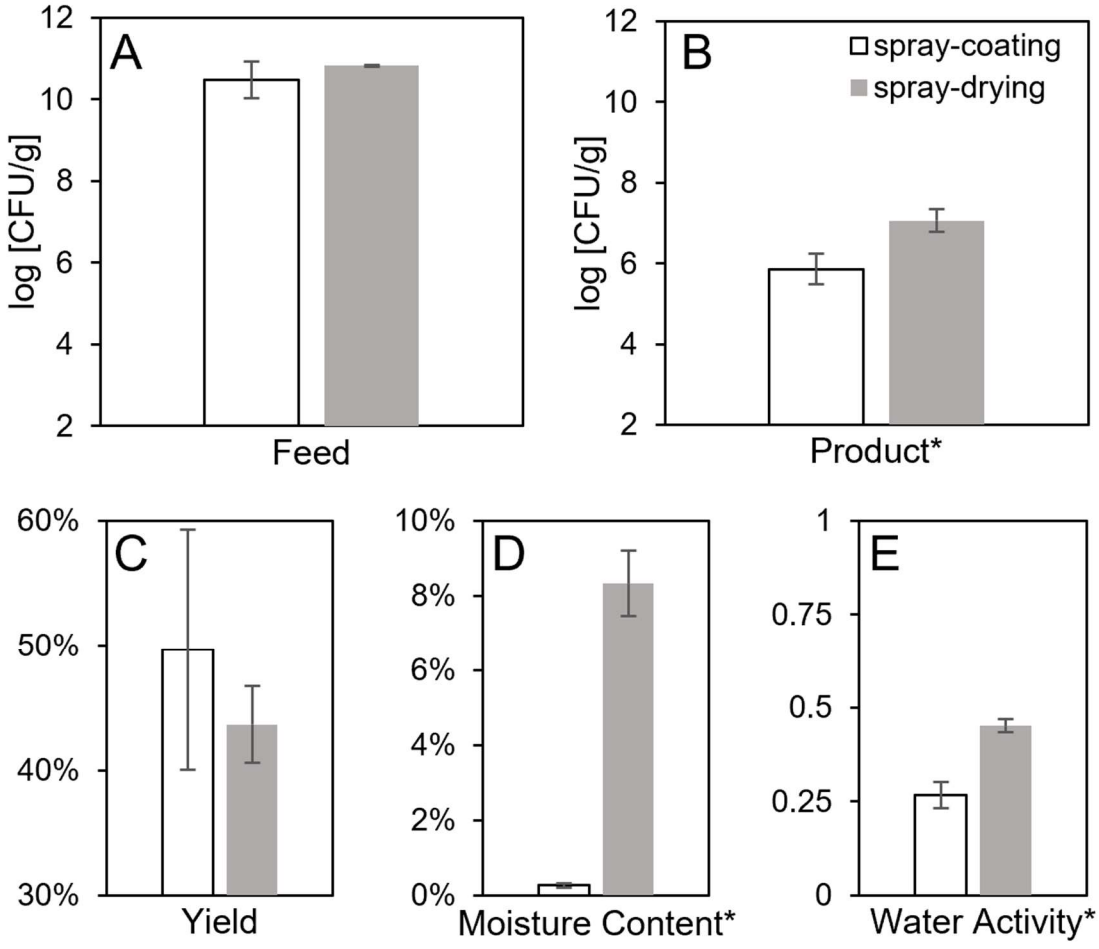

Figure 5-3 Feed and product characterization for each encapsulation method. (A) Viable cell count of Cal35 in the feed as log [CFU/g solids]. (B) Viable cell count of Cal35 in the encapsulated product as log [CFU/g solids] for spray-drying, and log [CFU/g coated solids] for spray-coating.

(C) Yield as a percentage of recoverable solids. (D) Wet basis moisture content of spray-dried powder or spray-coated particles containing Cal35. (E) Water activity of spray-dried powder or spray-coated particles containing Cal35. Asterisks denote statistically significant differences between encapsulation methods.

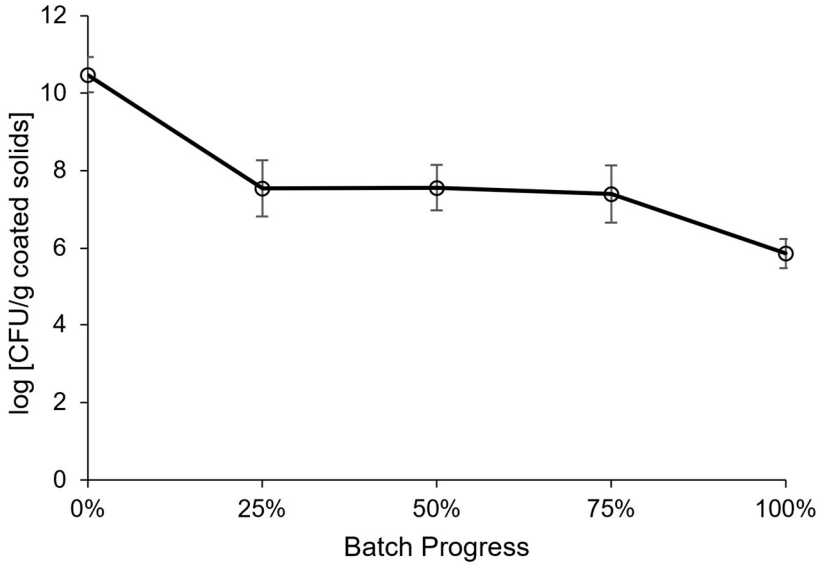

Figure 5-4 Viability of Cal35 during the fluidized bed spray-coating process. Viability of the coating suspension before spraying is represented at 0% Batch Progress. Once the coating suspension was fully sprayed, the coated particles continued to fluidize for two extra minutes to complete drying, represented at 100% Batch Progress.

Here, spray-drying encapsulation, which operates with high temperatures and a short residence time was less damaging to Cal35, yet the 3 log reduction in the viable cell count (**Figure 5-3B**) could still be minimized further. Cellular accumulation of osmoprotectants, mainly in the form of disaccharides, has been attributed to enhancing desiccation stress resistance (Fu et al., 2011). For example, when grown in media supplemented with trehalose, survival of *Raoultella terrigena* dried in alginate beads was improved (Schoebitz et al., 2012). Similarly, cellular accumulation of sucrose by *Pseudomonas chlororaphis* enhanced survival during freeze-drying (Palmfeldt et al., 2003). The benefits of these osmoprotectancts are due to their ability to maintain osmotic balance, prevent unfolding of proteins, and preserve membrane fluidity of the cell (Greffe et al., 2020). This

strategy of introducing osmoprotectants is especially useful because it occurs during the culturing phase of bacteria, prior to formulation, meaning it can be applied in practice prior to any method of encapsulation.

5.5.2 Temperature and desiccation sensitivity of Cal35

Cal35 was most sensitive to temperatures of 60°C or greater, with no viable cells countable after five minutes of exposure (**Figure 5-5**). The number of viable bacteria declined more rapidly with increasing temperature. The viability of Cal35 incubated at 35°C did not over the three hour period of the experiment. After one hour, Cal35 held at 40°C still showed no decrease in viability; however, raising the temperature to 45°C reduced viability by over 5 log units, and up to 50°C resulted in no survival above the limit of detection. In the three hour duration, there was a 2.5 log reduction at 40°C and a 5.5 log reduction at 45°C. At 45 and 50°C, the decline in log CFU begins to flatten, indicating Cal35 is tolerant to temperature (Moats, 1971).

Cal35 could not tolerate desiccation. With an initial cell count of ~9.5 log CFU/mL, no viable bacteria were recovered after the culture was left in a dish for 24 hours of drying at room temperature and rehydrated (21°C).

For bacteria that are more tolerant of heat but highly sensitive to drying, it is sensible that a process like fluidized bed spray-coating where very low moisture environments exist for extended periods of time was more detrimental to Cal35. In fact, the moisture content of a spray-coated particle was 0.28%, over ten times less than a spray-dried particle (**Figure 5-3D)**, yet there was no significant difference in the overall product yield (**Figure 5-3C**). Water activity was also significantly lower in spray-coated particles (**Figure 5-3E**).

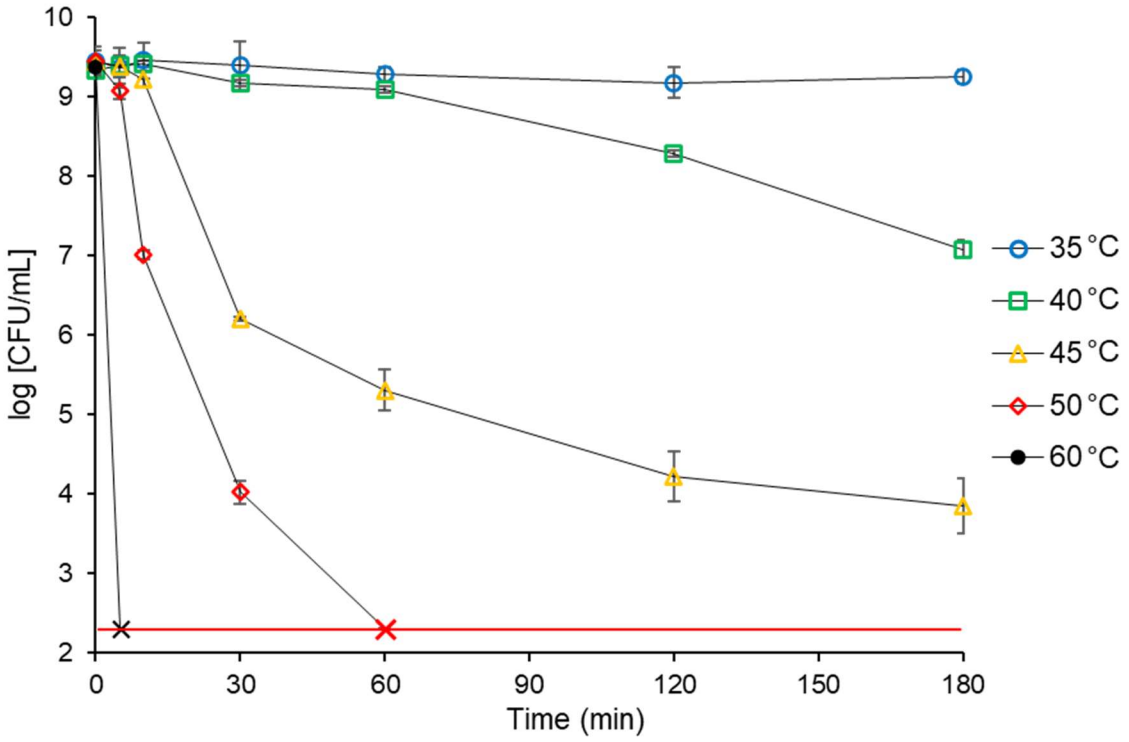

Figure 5-5 Viability of Cal35 heated between 35 to 60°C for three hours. The limit of detection is indicated by the horizontal red line. Data points on the limit of detection marked with an 'x' indicate no viable bacteria were recovered at this timepoint. Lines are drawn to guide the eye.

5.5.3 Outlook and further applications

Whether bacterial biopesticides are applied on seed or in field will largely impact the method of encapsulation. Spray-drying of CLAMs produced particles between 5-20 µm (**Figure 5-6A**). In this size range, it is possible to incorporate these microcapsules into spray-tanks which can be applied in furrow or on foliage. Fluidized bed spray-coating of CLAMshells onto cores produced coated particles between 250-300 µm (**Figure 5-6B**). Despite resulting in slightly greater losses in viability of Cal35 compared to spray-drying (**Figure 5-3B)**, fluidized bed spray-coating could be an enticing method for coating beneficial bacteria directly onto seeds. Seed coatings are conventionally applied by rotary pan or drum drying, where nutrients, pesticides, and other active compounds that benefit the seed and root are sprayed onto seeds as they are mixing or tumbling in

the drum (Rocha et al., 2019). Because drying is completed at ambient conditions, the process is long, resulting in bacteria losing viability due to desiccation (Hartley et al., 2012). However, in addition to the use of osmoprotectants, formulation with polymers such as gum acacia, methylcellulose, polyvinyl alcohol, and polyvinyl pyrrolidone have improved survival of rhizobial legume inoculants in the seed coating process (Deaker et al., 2004, 2007). With bacteria such as Cal35 that are less tolerant to drying, introducing some heat to speed up the coating process, similar to what is happening during fluidized bed spray-coating, may potentially be a more favorable encapsulation method.

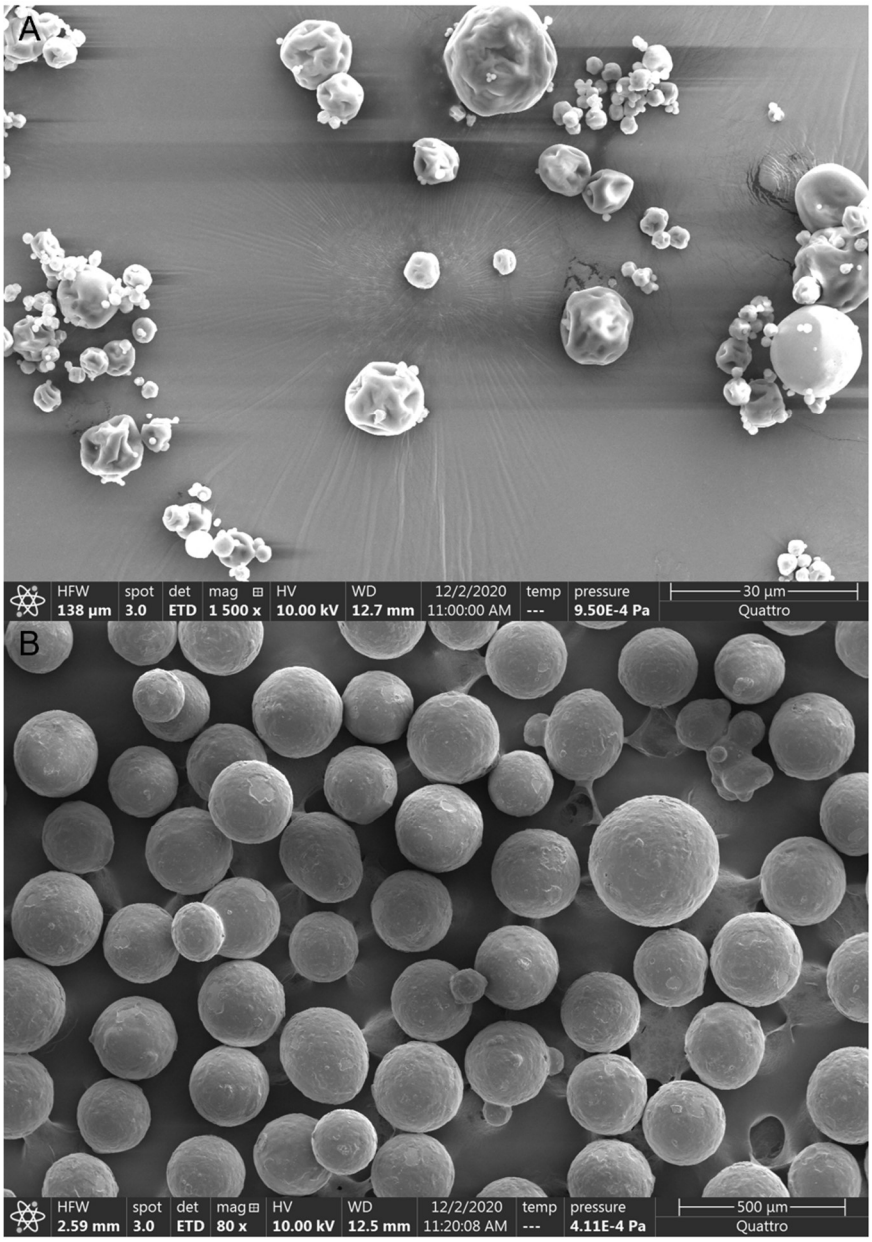

Figure 5-6 SEM images of, (A) CLAMs with 30 µm scale bar, and (B) CLAMshell particles with 500 µm scale bar.

Continuous fluidized bed spray-coating may also offer a unique solution to address issues of long drying times. While the Wurster configuration of spray-coating used this study is a batch process, moving towards a continuous process where seeds are fluidized and directly coated and collected can greatly lower the residence time while at the same time maintaining tolerable drying temperatures. Issues of agglomeration and proper coating formation when spraying with polymers

can be addressed with optimization experiments of process and formulation parameters (Hede et al., 2009; Kawakita et al., 2021; Rieck et al., 2020), however, when coating onto seed specifically, it is important to understand the limits of the drying temperature used where seed germination is not spoiled. As it appears desiccation stress was the more detrimental to Cal35, perhaps the ideal conditions for encapsulating similar bacteria inoculants rely on shorter residence times by elevating drying temperatures, similar to the drying conditions seen in spray-drying where survival of Cal35 was greatest.

Ultimately the survival of bacteria during any encapsulation method may mostly be influenced by their design and mechanisms to tolerate various environmental stresses. Gram-positive probiotic bacteria such as *Bifidobacteria* and *Lactobacillus* lose little viability during spray-drying and can have survival rates greater than 90% due to their more protective membrane structure (Huang et al., 2017). Spore-formers such as *Bacillus* are easily encapsulated and are already used in agriculture as biopesticides because of the robust nature of spores (O'Callaghan, 2016). Even non-spore-forming Gram-negative *Methylobacterium* demonstrated no significant losses in viability during spray-drying, potentially as a consequence of being acclimated to extreme environmental conditions and changes while living on leaf surfaces (Strobel et al., 2018). Future work in encapsulation of plant beneficial bacteria and biopesticides will be to consider how these underlying cellular mechanisms to tolerate or adapt to stresses can improve designing microcapsules for improving the protected bacteria's long-term stability for crop applications.

5.6 Conclusion

Encapsulation of the non-spore-forming plant-beneficial bacterial strain *Collimonas arenae* Cal35 in CLAMs by spray-drying or in CLAMshells by fluidized bed spray-coating was investigated. Spray-drying encapsulation of Cal35 had greater survival compared to fluidized bed

spray-coating, most likely because Cal35 was much more tolerant to heat than desiccation. However, if the coating process would continue for longer periods of time to create thicker coated CLAMshells, it is possible that the total CFUs per sprayed solids would be comparable or better than spray-drying. During spray-drying, high inlet temperatures allow a very short residence time of bacteria being encapsulated, while during fluidized bed spray-coating, low inlet temperatures rely on a long residence time of continuously fluidizing particles. CLAMs produced by spray-drying were smaller, ranging between 5-20 μm, while CLAMshell particles ranged between 250-300 μm. Both methods of encapsulation are relevant for encapsulation of bacterial biopesticides; CLAMs were sized appropriately for spray applications, while CLAMshells may be more useful for coating onto seed.

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Chapter 6 Conclusions and Future Work

6.1 Summary

This research sought to investigate industrially scalable processes for encapsulating non-spore-forming, Gram-negative, plant-beneficial bacteria for use as potential biopesticide products. *Collimonas arenae* Cal35 was chosen as the model organism for this research because it is a Gram-negative bacterium, it cannot form spores, and it has exhibited antifungal properties that make it a realistic candidate as a biopesticide. Spray-drying of cross-linked alginate microcapsules (CLAMs) and fluidized bed spray-coating of cross-linked alginate matrix shell (CLAMshell) particles were explored as microencapsulation systems that could protect such microbial biopesticides, especially those which are not spore-formers. The greatest potential contribution of this research would be full replacement of dangerous chemical pesticides with biopesticides to move towards safe and sustainable agriculture.

I hypothesized that controlling the process parameters (inlet temperature and spray rate) and the formulation parameters (alginate and excipients) will improve the overall microencapsulation of Cal35 by maximizing its survival during encapsulation as well as during storage. Previous work demonstrated that CLAMs were a viable option for bacterial encapsulation; however, the effect of process parameters were not considered and optimization of the encapsulation process to maximize viability was not investigated. With many factors at play in formulation research, this work helped provide a better understanding of some of the underlying variables that might influence how well non-spore-forming bacteria survive during spray-drying and fluidized bed spray-coating encapsulation and during storage, in addition to understanding which parameters could be adjusted to maintain highly viable encapsulated non-spore-forming bacteria.

During spray-drying of Cal35, survival of the bacteria and yield of the spray-dried powder was maximized through optimization of the process inlet temperature and spray-rate and the alginate content of CLAMs formulation. Of the investigated factors, only inlet temperature impacted survival during spray-drying, while all factors (i.e. spray rate and alginate concentration) significantly impacted yield. Minimizing inlet temperature to 95°C resulted in the lowest losses in viability; however, lowering inlet temperature any further would have led to a wet and uncollectable powder in the benchtop scale spray-dryer used in this study. The relationship between low outlet temperature and greater survival rates was already well known; however, identifying that larger CLAMs correlated with low losses of viability was particularly interesting. Unfortunately at the benchtop scale, there was a significant negative correlation between particle size and yield. At pilot and production scale spray-dryers however, larger particles can be created without compromising yield, suggesting greater survival and higher yields may be achieved by scaling up the CLAMs process. When spray-drying Cal35 with excipients, CLAMs were a very necessary component of the formulation as no survival was observed without CLAMs. The addition of modified starch in particular was beneficial to the shelf stability of Cal35 in CLAMs; however, controlling the oxygen and humidity of the storage environment had the greatest effect. Viable bacteria were released from CLAMs with modified starch for at least three weeks after encapsulation when stored in the oxygen (< 0.1%) and humidity (< 10%) controlled environment. At one month, there was no survival. When only humidity was controlled, Cal35 lasted for three days after encapsulation. Storage of Cal35 in CLAMs with maltodextrin as the excipient showed no survival even when controlling for oxygen and humidity. Oxygen appeared to be the most influential factor related to long-term storage. Cal35 that survived spray-drying and storage retained its antifungal properties when tested against *Aspergillus niger* N402.

To compare the encapsulation of Cal35 by spray-drying to fluidized bed spray-coating, a better understanding of the coating process was needed. Non-fat dry milk and enzyme were used as model biological cargo to investigate the effect of coating process parameters and formulation parameters on coating quality and morphology, to prevent the need to grow large volumes of bacteria culture per batch. In situ cross-linked alginate matrix shell (CLAMshell) particles were confirmed to have a similar degree of cross-linked alginate compared to CLAMs formed by spray-drying. When non-fat dry milk was coated onto the sodium sulfate core without the CLAMshell formulation, low spray rates were required to prevent particle aggregates from increasing above the acceptable threshold relative to the total product weight. Including the CLAMshell formulation in addition to increasing the nozzle pressure allowed spray-rates to be increased by 40%. The interplay of nozzle pressure and spray rate only worked with the CLAMshell formulation because it relied on the liquid feed properties to be more viscous. In a low viscous formulation, increasing nozzle pressure led to spray-drying, very low coating efficiencies, and even failed runs where fluidization stopped. For all formulations with the CLAMshell, coating efficiencies were notably greater. Increasing the alginate concentration or adding polyvinyl alcohol (PVA) to the CLAMshell coating produced mechanically stronger coatings. There was visibly less surface cracking and damage to the particle and most importantly, significantly less attrition; dust formation measured by the Heubach dustmeter was reduced over 90% compared to a coating containing only enzymes without the CLAMshell formulation. CLAMshell particles also delayed release of sodium sulfate cores when placed in solution due to the cross-linked alginate acting as a diffusion barrier to water, suggesting there is potential for controlling the release of any cargo encapsulated in the CLAMshell, such as plant beneficial bacteria being released into soil.

Fluidized bed spray-coating encapsulation of Cal35 in CLAMshell particles was compared to spray-drying encapsulation of Cal35 in CLAMs. Drying parameters for each method were controlled such that the spray rates and outlet temperatures were fairly similar. The main differences were the inlet temperatures and the residence times. Despite the inlet temperature of spray-drying being twice as high as spray-coating, spray-drying of Cal35 showed better survival with a reduction of 3 log units down to 7 log[CFU/g solids]. However, the viability of Cal35 during the spray-coating process was sustained at 7.5 log [CFU/g coated solids] until the final drying step, which significantly lowered the survival rate. Individually testing Cal35 against temperature stress and desiccation stress revealed it was able to withstand hotter temperatures for short periods of time, but was very sensitive to drying, even at ambient temperature. CLAMs containing Cal35 were typically around 5-20μm, compared to 250-300μm for CLAMshell particles. Although the spray-coating process was more detrimental to Cal35 due to its low tolerance of desiccation, CLAMshell particles may still be of interest as a seed coating, whereas smaller sized CLAMs would be more practical in spray applications.

6.2 Future Work

Future work is recommended in several areas to help pursue the goal of achieving a scalable encapsulation process for bacterial biopesticides in practice.

A deeper understanding of the cellular mechanisms that affect cell viability, beyond cell membrane type or spore-forming ability, would be useful for improving the survival of bacteria during encapsulation or in storage. With this knowledge, microcapsules may be engineered in ways that could more specifically benefit the encapsulated bacteria by addressing some of their weaknesses. One possible approach would be to assess encapsulation survival of different variants or mutants of a well-performing bacterial strain, which have been genetically sequenced and

mapped, to identify specific genes or gene clusters which can point to certain phenotypes that make some bacteria survive better than others, or make one more suited to withstand certain stresses over others.

More work could also be done on coating bacteria onto seeds. While the fluidized bed spray-coating demonstrated some initial success, it is still possible to lower the drying temperature further, since the residence time of the particle being coated is significantly longer than a particle formed during spray-drying. Paired with this task would be further optimizing the coating formulation, as other protective excipients and/or polymer carriers seem to be key for stabilizing bacteria during drying. In addition, assessing the shelf stability of coated particles is needed.

Formulating for controlled release is also a key area of research. Of course, the mode of application would largely determine the type and speed of release. One example would be the quick release of bacteria into the soil so they can establish themselves and reproduce in high enough quantity to benefit the plant. Another would be the slow release of bacteria if they need to grow and establish themselves inside the microcapsules; here the nutrients could be included in the microcapsules to promote growth of the bacteria after being wetted in the soil. Release may be controlled by varying the extent of cross-linked alginate; however, if excipients are included in the cross-linked alginate microcapsules/matrix, then the effect of these added materials on alginate cross-linking would have to be investigated, as it is very likely they would interfere with the cross-linking network.

Lastly, testing at the pilot scale for both spray-drying and fluidized bed spray-coating is required once an ideal formulation is found and an acceptable level of viable cells remains after encapsulation. Scale-up of spray-drying and fluidized bed spray-coating is non-trivial and requires further process optimization. Significantly larger airflow rates and spray rates, as well as different

nozzle types and drying chamber dimensions are a few of the parameters needing additional assessment. Also at larger scales, drying efficiency of the process would need to be considered.

6.3 Conclusion

This research demonstrated the potential of two scalable microencapsulation processes – spray-drying and fluidized bed spray-coating – for protecting bacteria from the non-spore-forming, plant-beneficial strain *Collimonas arenae* Cal35 in CLAMs and CLAMshell particles. Microencapsulation in CLAMs incorporated with modified starch showed the most promise in extending the shelf stability of Cal35 as long as oxygen was controlled in the storage environment. Although both formulation processes were successful in encapsulating Cal35 with moderately high levels of viability for a non-spore-former, further development of these formulations to achieve greater rates of survival will be necessary before actual implementation as a seed coating or sprayable biopesticide product in the agricultural industry.

Appendix A Microencapsulation of *Pseudomonas chlororaphis* in CLAMs

The work included in this appendix section describes efforts to encapsulate the Gram-negative, non-spore-forming bacteria *Pseudomonas chlororaphis* subsp. c*hlororaphis* (ATCC 9446) in CLAMs. Spray-drying parameters and additives to the CLAMs formulation were investigated to try and identify factors which influenced survival during encapsulation. The moisture properties of CLAMs were also investigated because it is commonly thought that low moisture environments (e.g. low water activity) can maintain longer stability of bacteria in storage.

A.1 Culturing and assessing viability of *P. chlororaphis*

Pseudomonas chlororaphis liquid cultures and solid cultures were incubated at 26°C and 150 rpm. Nutrient agar and broth (Difco) were used as the primary nutrient source unless otherwise noted. F agar was produced with the following recipe:10g pancreatic digest of casein (Fisher Tryptone BP1421), 10g proteose peptone NO3 (Fisher Peptone BP1420), 1.5g K2HPO4 dibasic (Fisher P288), 0.858g MgSO4 (Sigma), 10mL glycerol (Fisher Glycerol G33), 20 g agar (SuperPure Agar A02MG).

Viability of *P. chlororaphis* in the feed suspension was quantified using the following method. Total solids (TS) in the feed suspension was calculated by adding up the solids in the formulation. Density was assumed to be 1 g/ml for calculating CFU/g. Total solids sprayed was calculated by multiplying %TS by total mass of feed suspension. Microbe survivability was measured using the serial dilution method, where a range of diluted samples are plated until colony forming units are counted, giving CFU/ml of suspension. Total CFU was calculated by multiplying CFU/ml by total volume of feed suspension. CFU/g dry solids in the suspension was calculated by

dividing Total CFU by total solids sprayed. Both CFU/g dry solids and total CFU can be used to compare viability before and after spray-drying.

Viability of *P. chlororaphis* in the spray-dried powder was quantified using the following method. Moisture content (wet basis) was measured by drying a known amount of powder to get the TS as it was calculated for the feed suspension, and subtracting the TS from 100%. Bacteria filled CLAMs must be dissolved in 1X PBS (phosphate buffered saline) solution to release any bacteria present in the CLAMs, then viability can be assessed by serial dilution, as described previously. An additional step must be taken in the calculation to account for the volume of 1X PBS per gram of powder, giving CFU/g powder. Total CFU in the powder was calculated by multiplying CFU/g powder by TS (assuming 100% recovery) or mass of powder collected (actual). CFU/g dry solids in the powder was calculated by dividing CFU/g powder by %TS.

A.2 Initial bacteria-CLAMs attempts and factors impacting viability of *P. chlororaphis*

The initial focus for microencapsulation of a gram-negative plant beneficial bacteria was evaluating the compatibility of *Pseudomonas chlororaphis* with the CLAMs process to improve its shelf stability. Initial spray-drying attempts at producing *P.chlororaphis* filled CLAMs were unsuccessful. There was no survival above the detection limit of ~10^4 CFU/g. Sigma alginate was used in this formulation with the standard spray parameters that were used throughout this project unless otherwise noted. Several factors were identified as potential sources of lowering *P. chlororaphis* viability during the spray-drying process. High temperatures and desiccation during the spray-drying process, shear from the nozzle, and pH drop from the CLAMs formation were individually analyzed to assess their impacts on viability (**Figure A-1**).

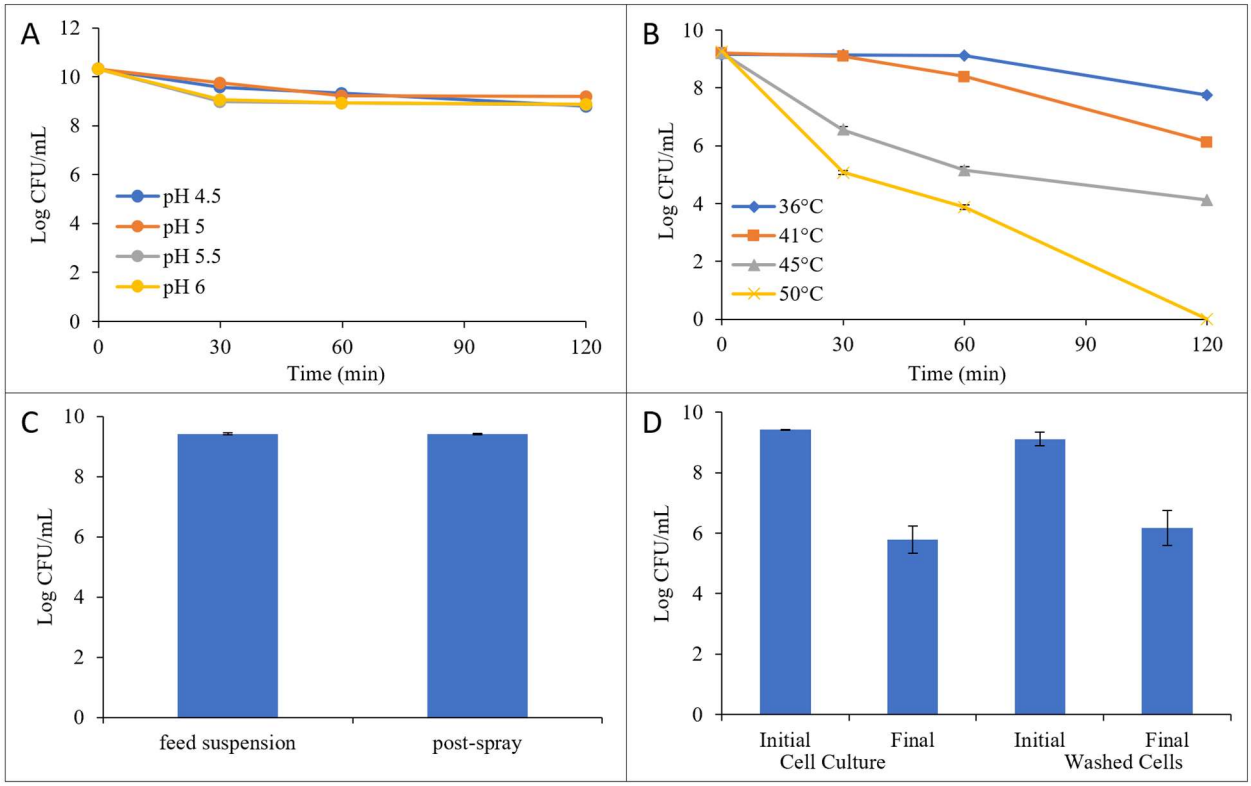

Figure A-1 Assessing sensitivity of *P. chlororaphis* to various stresses. (A) Effect of pH on the viability of *P. chlororaphis* in M9 minimal media without a carbon source and with pH adjusted using HCl or NaOH. (B) Effect of temperature on viability of *P. chlororaphis* suspended in the feed of what would be spray-dried at 36, 41, 45, and 50 °C. (C) Effect of nozzle spray shear generated from the nozzle pressure of the spray-dryer on the viability of *P. chlororaphis* in a 2% sigma alginate and 0.1% $CaHPO_4$ formulation before spraying (feed suspension) and immediately after spraying (post-spray). (D) Effect of desiccation on the viability of *P. chlororaphis* as a culture (with media components) and washed (media components washed out). Initial and final represent viability before drying and after rehydration.

While pH change and nozzle shear had little effect on lowering survival (**Figure A-1A** and **Figure A-1C**), temperature (**Figure A-1B**) and desiccation (**Figure A-1D**) were the most detrimental to viability. After reaching 45°C, the viability dropped by about 2 log CFU/ml after 30 minutes, and 5 log CFU/ml after 2 hours. At 50°C, there was a 4 log CFU/ml decrease after 30 minutes, and no survival after 2 hours. A typical spray run has an inlet temperature of 130°C and an outlet averaging 50°C. Bacteria are simultaneously dried and heated during spray-drying. Leaving culture and washed cells out at room temperature to dry then rehydrated to assess survival

demonstrated about a 4 log CFU/ml reduction in viability. To alleviate the temperature burden where the bacteria filled microcapsules are stored in the collection chamber, a water bath held around 33°C surrounding the collection chamber was used for all following spray-dry runs, and was found to be beneficial towards survival.

A.3 Alginates and protective additives to improve survival of *P. chlororaphis*

Two formulations with Sigma alginate and Hydagen alginate were produced, each with varying concentrations of additives. All concentrations were on a percent weight/weight basis, unless otherwise stated. Sigma CLAMs were composed of the following: 2% alginate, 1% succinic acid, 0.25% calcium hydrogen phosphate, with 2% lactose or trehalose, or 0.8% lactose or trehalose. Hydagen CLAMs: 1% alginate, 0.5% succinic acid, 0.125% calcium hydrogen phosphate, with 1% lactose or trehalose, or 0.4% lactose or trehalose. Spray-drying parameters remained constant (130°C inlet temperature, 100% aspirator, 45% spray rate, 40 mm nozzle pressure). The effect of using two different sources of alginates – Sigma low viscosity alginate and BASF Hydagen alginate and including soluble sugars in the CLAMs on bacterial survivability during spray drying was examined (**Figure A-2**). Lactose and trehalose at low and high concentrations were included in combination with each alginate.

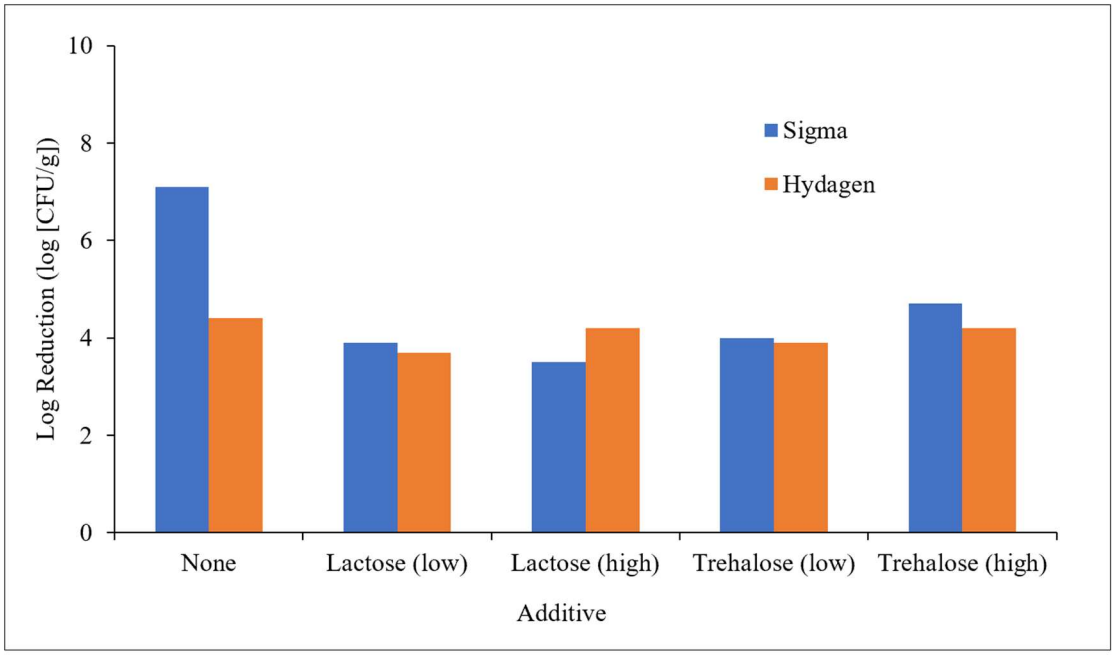

Figure A-2 Loss in *P. chlororaphis* viability during spray-drying of Sigma (2%) or BASF Hydagen (1%) containing no additive, a high concentration of additive, or a low concentration of additive. 'High' concentrations have a ratio of 1:1 alginate:sugar; 'low' concentrations have a ratio of 5:2 alginate:sugar.

Sigma alginate CLAMs with no additive ('None') (**Figure A-2**) had the largest loss in viability during spray-drying. Addition of lactose and trehalose at either concentration increased survival by about 3 log CFU/g on average for Sigma CLAMs. Interestingly, Hydagen CLAMs had on average 4 log CFU/g loss for all treatments, with and without additives. *P. chlororaphis* encapsulated in Hydagen CLAMs demonstrated comparable survival without amendments to Sigma CLAMs with lactose or trehalose.

A.4 Effects of increasing formulation solids on spray-drying process response variables

One formulation variation explored was the addition of starch in the CLAMs formulation to impact the moisture sorption properties. A response surface experiment was designed to study the impact of varying alginate and starch concentration in the CLAMs formulation on spray-dryer outlet temperature and moisture content and water activity of the resulting product. Surface plots

were created to show this effect (**Figure A-3**). These experiments were conducted without *P. chlororaphis* present in the formulation.

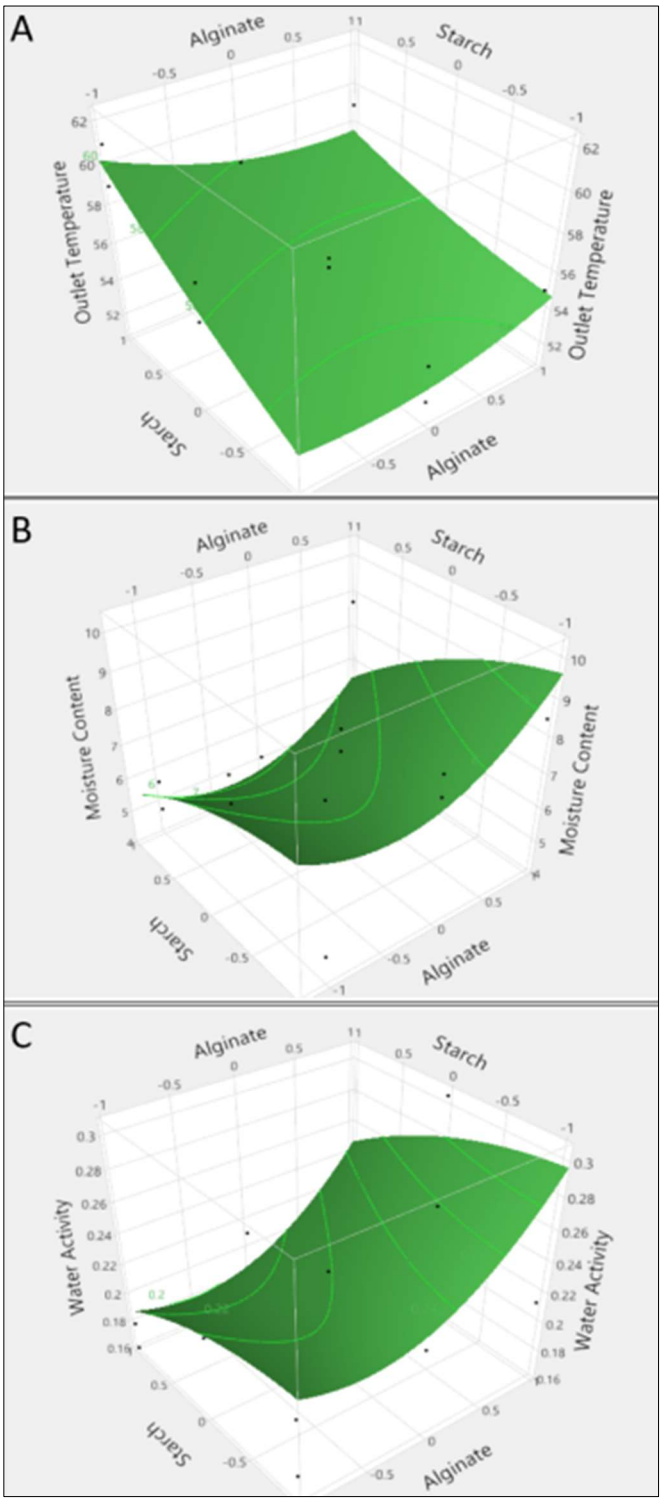

Figure A-3 Response surfaces illustrating effect of varying alginate and starch ratios in the CLAMs formulation on (A) outlet temperature (°C), (B) moisture content of powder (%, WB), and

(C) water activity of powder. Coded values at [-1, 0, 1] correspond to alginate concentrations % (w/w) of [0.5, 0.17, 1] and starch concentrations % (w/w) of [2, 4, 6]

As both alginate and starch concentration increase in the infeed, outlet temperature increases dramatically, with an overall change in temperature of up to 10°C (**Figure A-3A**). Water activity and moisture content of powders appear to decrease with increasing outlet temperature, while those formulations with higher alginate concentrations had overall higher moisture contents and water activities during which outlet temperature remained somewhat constant (**Figure A-3B** and **3C**). These results indicate that the outlet temperature significantly impacts moisture content and water activity immediately after the spray-drying process.

A.5 Moisture sorption isotherms of CLAMs

Spray-dried and freeze-dried CLAMs (1% Hydagen alginate, 0.5% succinic acid at pH 7, and 0.125% dicalcium phosphate) with 6% soluble potato starch and no starch were placed into desiccators at four levels to equilibrate to the relative humidity of each saturated salt solution (SSS). Four salt solutions were created to reach the targeted relative humidities at 25°C: LiCl (0.11); $KC_2H_3O_2$ (0.23); K_2CO_3 (0.43); NaCl (0.75). 1% Hydagen CLAMs were produced with different calcium levels to vary cross-linking: 0.25 (4:1), 0.125 (8:1), 0.1 (10:1), 0.05% (20:1) dicalcium phosphate. Starch and no starch Hydagen CLAMs were sent to Surface Measurement Systems for DVS (dynamic vapor sorption) analysis. Hydagen and Sigma CLAMs and alginates were sent to a BASF facility in San Diego for DVS and DDI (dynamic dewpoint isotherm) analyses.

We hypothesized that spray-dried powders with lower water activities while maintaining higher moisture contents favor shelf-stability of the encapsulated bacteria. Moisture sorption isotherms (MSI) illustrate the relationship between water activity and moisture content and were determined for standard CLAMs, soluble potato starch CLAMs, and two alginate types using three

methods: saturated salt solution (SSS), dynamic vapor sorption (DVS), and dynamic dewpoint isotherm (DDI).

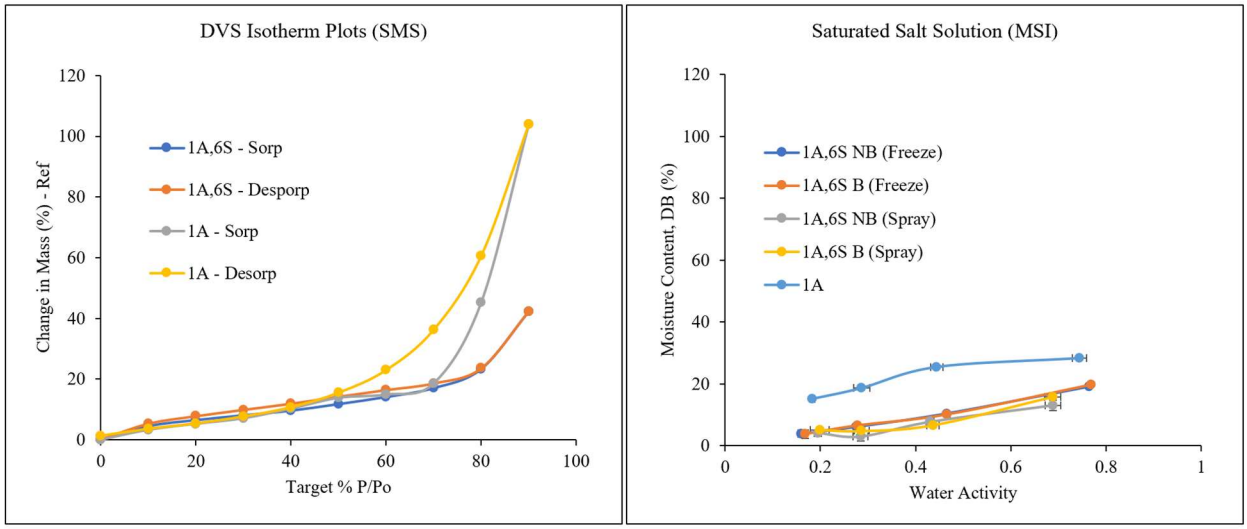

Figure A-4 Moisture sorption isotherms (MSI) generated by DVS method by Surface Measurement Systems (SMS) of two formulations one with no starch loading (1A) and one with 6% starch (1A,6S). MSI generated using the saturated salt solution (SSS) method with four salts of 1A (spray-dried) and 1A,6S, with (B) and without (NB) bacteria, both freeze-dried and spray-dried.

Using the saturated salt solution method to generate moisture sorption isotherms, there was no difference between starch CLAMs formed by spray-drying or freeze-drying (**Figure A-4**). The presence of bacteria also had no effect on the moisture properties. When starch was excluded from the formulation (1A), the SSS method indicated higher moisture content at all water activity levels. These microcapsules were primarily composed of alginate, whereas the starch CLAMs have six times the concentration of starch. Because alginate is more hygroscopic than starch, this may be possible. However, DVS isotherms of both starch and no starch CLAMs suggested no difference in each MSI until very high equilibrium relative humidity where there was an apparent "phase change" observed by SMS technicians. The DVS and SSS methods vary in that samples in the DVS are dried at ~100°C then exposed to and equilibrated to incrementally increasing relative humidity environments while the SSS method equilibrates separate sample aliquots in different

relative humidity chambers at ambient temperatures. Thus, one explanation for differences in moisture content of the CLAMs (1A) sample between DVS and SSS may be due to volatilization of more than just water from the samples during initial drying in the DVS; however, it is unknown what compounds may be removed or reacted upon to cause this change in mass.

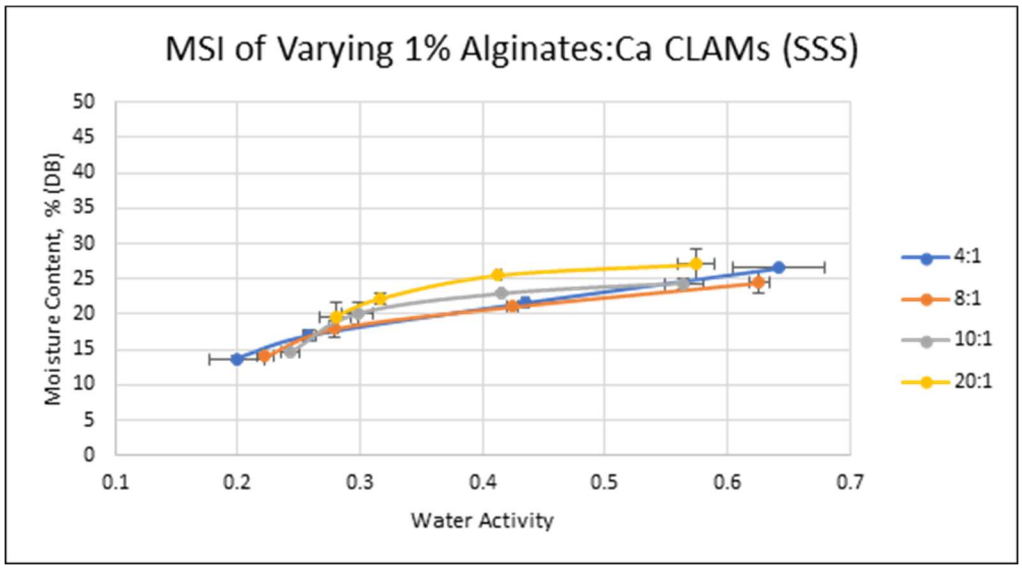

Figure A-5 Moisture sorption isotherms generated using SSS method of CLAMs prepared with 1% Hydagen alginates in the spray-dryer feed with four different alginate to calcium hydrogen phosphate ratios.

Varying the extent of cross-linking within CLAMs prepared with Hydagen alginates appeared to have little effect on the MSI using the SSS method (**Figure A-5**). Lowering the calcium concentration down to 0.05% (20:1) seemed to hardly increase the moisture content at the given water activity levels; to verify and confirm this difference, however, DVS or DDI would be the preferred method before making any conclusions.

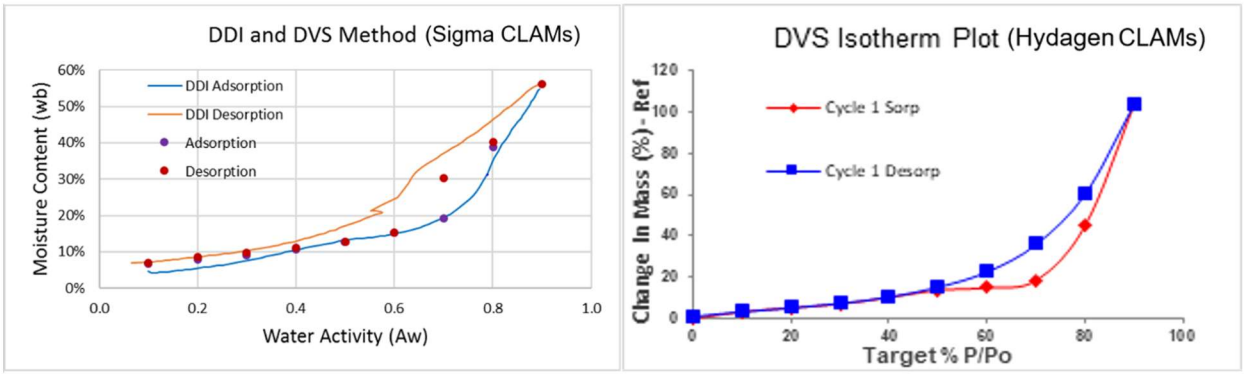

Figure A-6 Moisture sorption isotherms of Sigma CLAMs generated by BASF using DDI and DVS, and Hydagen CLAMs generated by SMS using DVS.

Comparing the MSI of CLAMs prepared with either Hydagen or Sigma alginates (referred to as Hydagen CLAMs and Sigma CLAMs, respectively), both have similar moisture contents at water activities below ~ 70% equilibrium relative humidity (**Figure A-6**). At higher relative humidities, the Hydagen CLAMs exhibited significantly higher moisture content. Hysteresis observed in both samples is likely due to irreversible caking of the powders at the higher moisture content. Photographs of the Sigma CLAMs samples taken by BASF technicians show a "dried cake" form of the powder after the process, which needed some force to break apart.

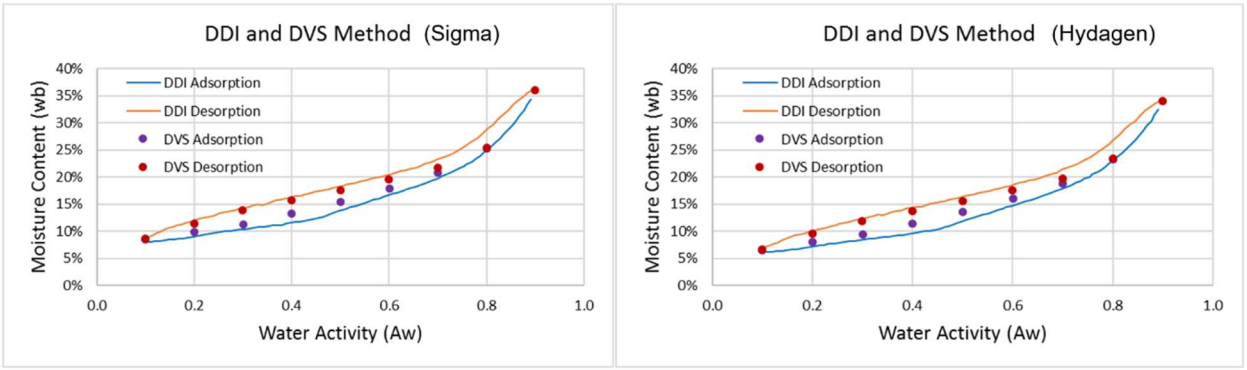

Figure A-7 Moisture sorption isotherms of alginates sourced from Sigma Aldrich and BASF (referred to as Sigma alginates and Hydagen alginates, respectively) generated by BASF using DDI and DVS methods.

We examined the MSIs of the alginates from two commercial sources, Sigma-Aldrich and BASF (referred to as Sigma alginates and Hydagen alginates, respectively) used in the CLAMs formulations (**Figure A-7**). Sigma alginates seemed to have higher moisture contents at all water

activity levels, although the differences are not significant. Both confirm the behavior seen in DVS of both CLAMs, except for the very high moisture content of Hydagen CLAMs at the highest equilibrium relative humidity levels.

A.6 Starch amendments and bacterial survival

Soluble potato starch at varying quantities (2-6%) was incorporated into the standard CLAMs formulation of 1% Hydagen alginate, 0.5% succinic acid at pH 7, and 0.125% dicalcium phosphate. Spray-drying was carried out using the standard parameters (130°C inlet temperature, 100% aspirator, 45% spray rate, 40mm nozzle pressure) unless otherwise specified. One experiment lowered inlet temperature to 120°C, while another increased spray rate to 65%.

Three separate experiments (**Figure A-8**, **Figure A-9**, and **Figure A-10**) were carried out with varying soluble potato starch loadings added to the standard CLAM's formulation to examine its impact on bacterial survival as an amendment. The first experiment(**Figure A-8**) used standard spray parameters – 130°C inlet temp and 45% spray rate –, while the second and third experiments (**Figure A-9** and **Figure A-10**) exploited the lower infeed moisture content to lower inlet temperature and increase spray rate while still producing a dry, collectable powder.

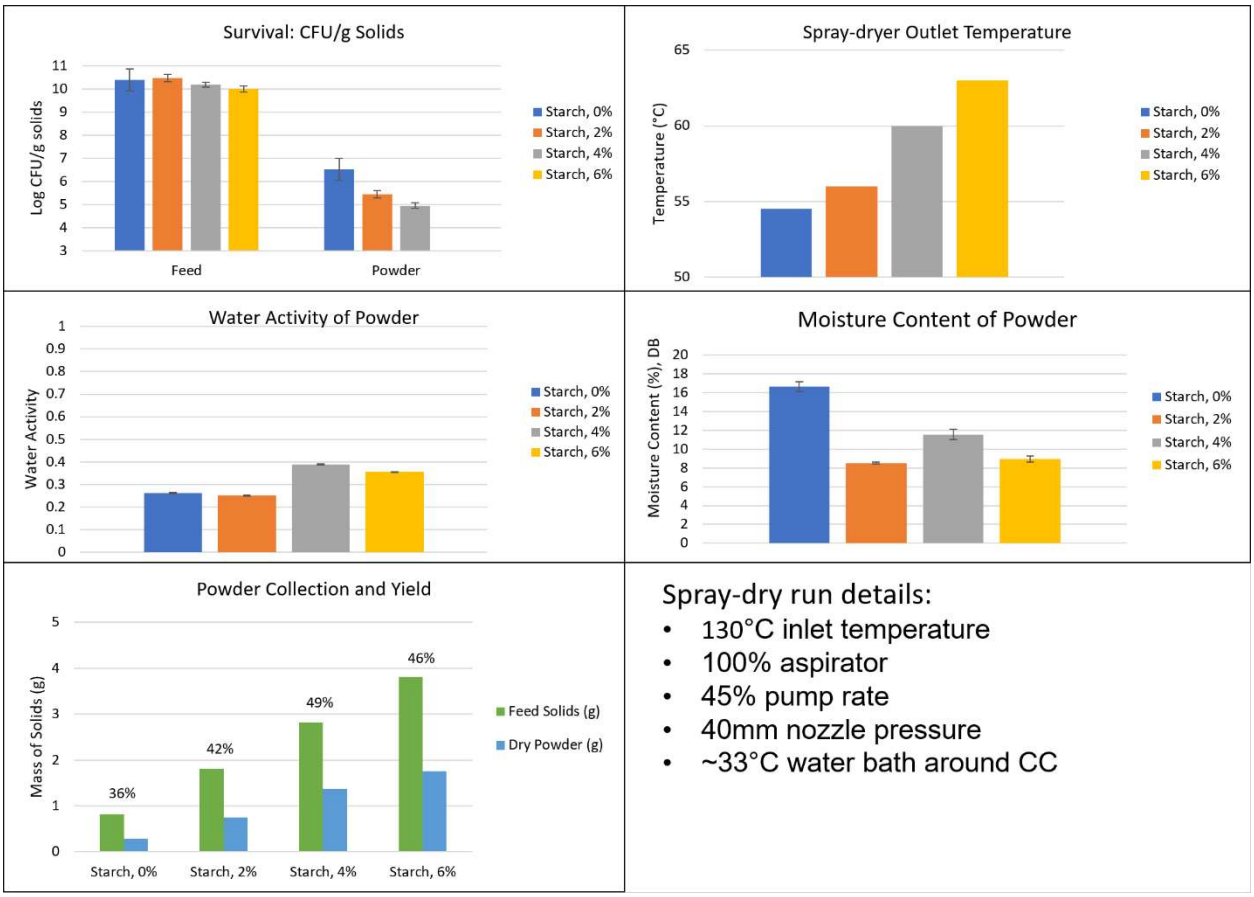

Figure A-8 Spray-drying with *P. chlororaphis* using standard parameters. Formulation includes a range of starch concentrations from 0 to 6% (w/w).

Bacterial survival was observed to be greatest in the no starch formulation, decreasing as the starch loading increased (**Figure A-8**). Outlet temperature increased significantly with increasing starch concentration, which may explain the decrease in survival. With an outlet temperature of 63°C, there was no detectable survival in the 6% starch formulation. Water activity was slightly higher with more starch, however, there was a notable difference in moisture content when starch was included in the formulation. The formulation without starch exhibited a significantly higher moisture content at a lower water activity, consistent with the moisture sorption properties of CLAMs (**Figure A-4**).Overall, the addition of starch in the CLAMs formulation did not improve survival of the bacteria during spray drying.

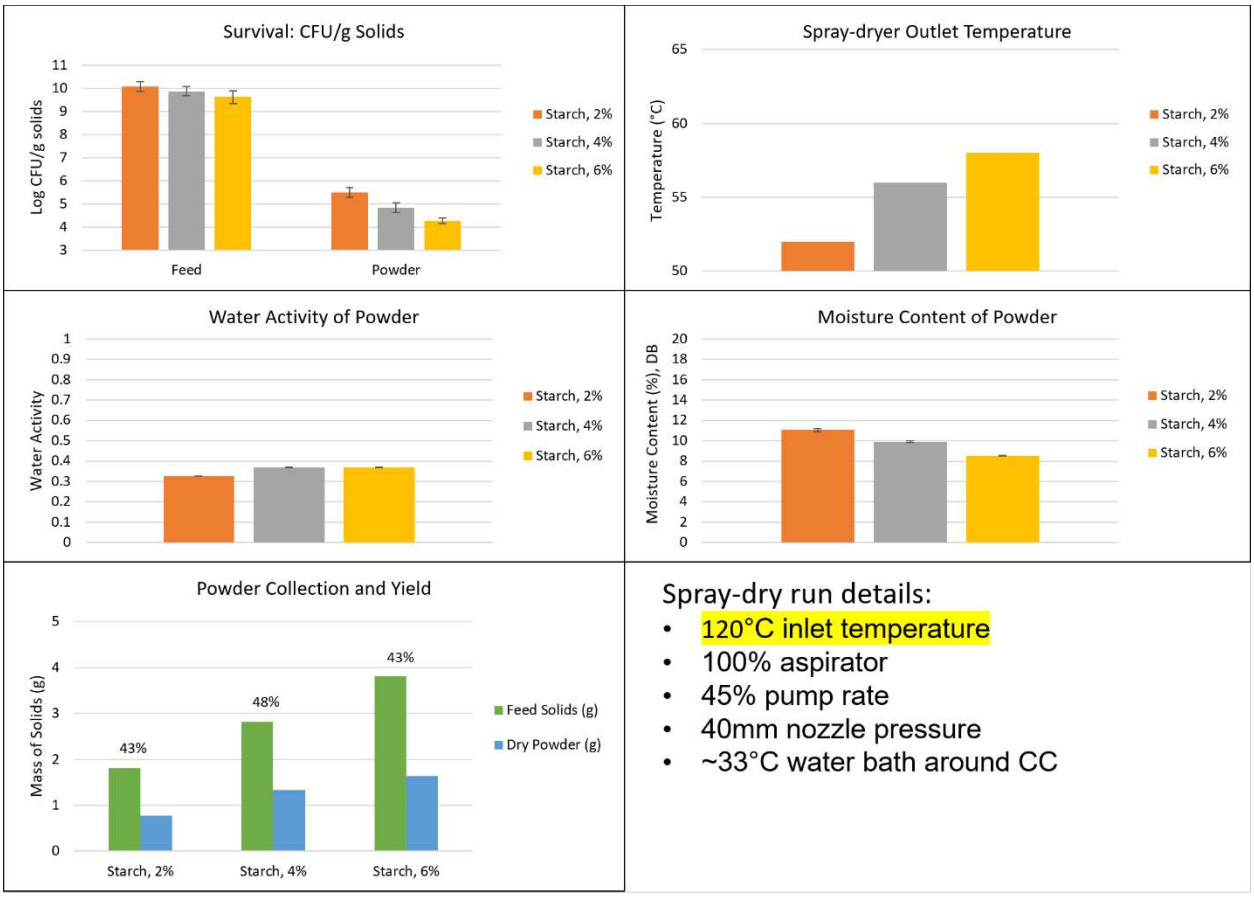

Figure A-9 Spray-drying with *P. chlororaphis* with a lower inlet temperature. Starch concentration ranges from 2-6% (w/w).

At the standard spray drying parameters, outlet temperatures were reaching low 60's (°C) severely compromising *P. chlororaphis* survival (**Figure A-8**). By decreasing the inlet temperature to 120°C, there was a few degrees decrease in outlet temperatures compared to an inlet temperature of 130ºC (**Figure A-9**). Bacterial survival at the 2-6% starch loadings, however, did not improve despite the slightly lower outlet temperatures. Moisture content and water activity also remained unchanged (**Figure A-9**).

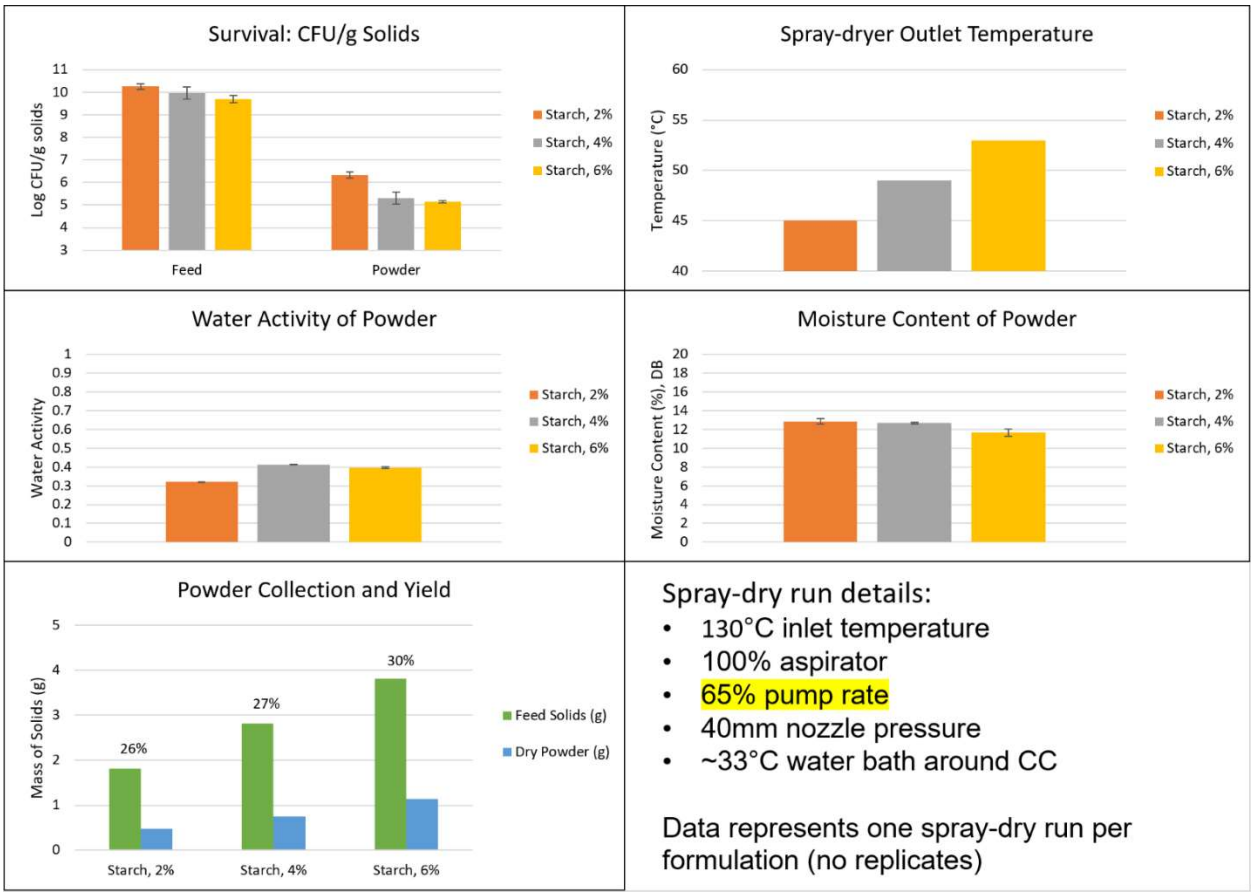

Figure A-10 Spray-drying with *P. chlororaphis* with a higher spray rate. Starch concentration ranges from 2-6% (w/w).

Increasing the spray rate was another option to counter the high outlet temperature with standard spray parameters (**Figure A-8**). Increasing the spray rate to 65% from 45% reduced the outlet temperature more than lowering the inlet to 120°C, and there was slightly improved survival for all formulations (2-6% starch) (**Figure A-10**). This may be an outcome from lower overall outlet temperatures than both previous experiments, or from a shorter residence time within the evaporation chamber during drying. Lower outlet temperatures resulted in slightly higher moisture contents in all cases, which was expected based from the response surface data (**Figure A-3**). One downside, however, was the reduction in powder yield.

Results from the three experiments with this starch amendment suggest that soluble potato starch does not improve bacterial survival at these levels. In all three experiments, there was a

general trend of decreasing survival as outlet temperature increased, confirming that the outlet temperature plays a role in bacterial survival during the spray-drying process.