

# **Physicochemical Properties and Excipient Compatibility studies of Probiotic *Bacillus coagulans* Spores**

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## **Abstract**

The probiotic formulations are susceptible to loss in viability due to formulation, processing, storage and *in vivo* environment. The aim of the present study was to perform preformulation studies of probiotic *Bacillus coagulans* spores to aid designing of stable formulations. *Bacillus coagulans* spores were studied for hygroscopicity, resistance to compaction force, aqueous pH stability, and excipient compatibility. The spores were found to be moderately hygroscopic with a significant loss of microbiological assay at water activity value of more than 0.5. Progressive loss of viability from 95% to 58% was observed with increase in compaction force from 1000 to 4000 psi. Aqueous suspension of *Bacillus coagulans* spores in buffer solutions of pH 1.2 to 8 showed rapid degradation with maximal stability in pH 6.8. Excipient compatibility studies showed reduced assay with citric acid monohydrate, meglumine and sodium starch glycolate. The loss of activity seemed to be related to the moisture uptake, free and bound water present in the bulk.

## **Keywords**

Probiotic • Lactic acid bacteria • Preformulation • Drug Excipient interaction

## Introduction

Probiotics are live microbial feed supplements that can benefit the host by maintaining the balance of intestinal microflora [1]. Probiotics, as defined by the Food and Agricultural organization (FAO) of the United Nations and the World Health Organization (WHO) are "live microorganisms (bacteria or yeasts), which when ingested or locally applied in sufficient numbers confer one or more specified demonstrated health benefits for the host" [2]. Probiotics have been prescribed in the treatment of diarrhoea, lactose intolerance, irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) [3, 4]. They have also shown usefulness in the prevention of cancer, hypercholesterolemia and allergy. Accordingly, last few years have witnessed increased interest in the use of probiotic bacteria to restore and maintain normal bacterial flora of the human body.

Probiotic formulation should satisfy two requisites: first, it should be able to deliver adequate number of viable microorganisms to the target organ, and secondly, it should have a sufficiently long shelf life. A number of probiotic products are marketed in different dosage forms such as powders, tablets, capsules and food products such as yoghurt and ice creams. They are available in combination with various vitamins and antibiotics. However, despite the impressive list of usefulness of probiotics, physicians are reluctant to prescribe them. One of the reasons is the substantially low viable count against the label claim, resulting in compromised therapeutic benefit.

*Bacillus coagulans* (*B. coagulans*), commonly mislabeled as *Lactobacillus sporogenes*, [5] has a long history of use as a probiotic. Apart from dietary supplement, bacillus probiotics are used as a therapeutic product for the treatment of gastrointestinal and urinary tract infections. Patent Cooperation Treaty (PCT) patent application WO9854982 disclosed a method for the treatment of gastrointestinal tract (GIT) infection using *B. coagulans*. The therapeutic benefit is partly due to the ability of *B. coagulans* to secrete a bacteriocin, coagulin, which is active against a broad spectrum of enteric microbes [6]. Like other probiotic strains it also suffers wide variation between the actual content and the labeled claim of viable spores. To compensate for this, very high amount of overages are added, which not only increase the cost of production but also result in variable dose of the probiotic.

Problems in the stability of microorganisms commonly used in food industry have been documented [7]. Maggi and coworkers [8] studied the viability of lactobacilli incorporated in vaginal tablet. Similarly, the survival of probiotic vaginal lactobacilli in stock cultures has been assessed by J. Tomas *et al.* [9]. Although these studies were not carried out strictly as per the International Conference on Harmonization (ICH) guidelines for stability testing, they indicated significant fall in the probiotic content upon storage. Probiotics have been reported to be susceptible to pH conditions and moisture [10].

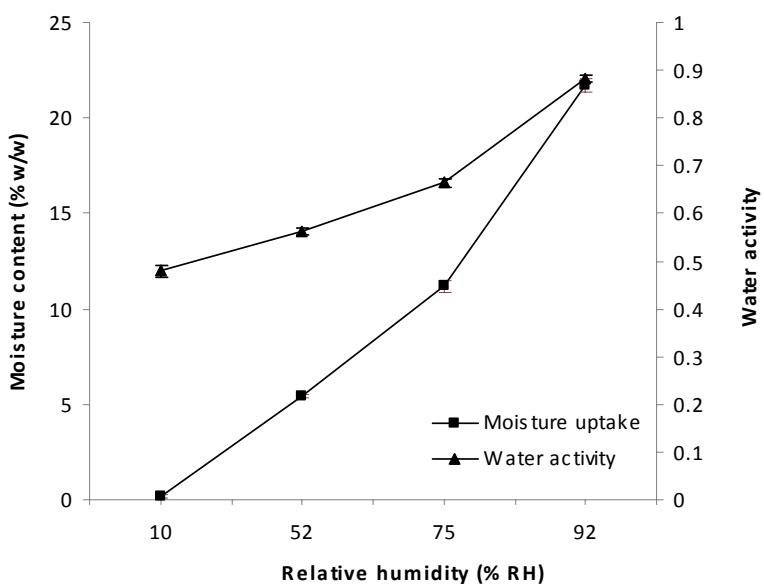
The effect of external stress parameters on *B. coagulans* can yield information on its formulation requirements and storage conditions. Thus, the specific aims of this research work were (1) to determine the effect of humidity, compaction pressure, and aqueous pH on viability of *B. coagulans*; and (2) to determine the compatibility of *B. coagulans* with commonly used pharmaceutical excipients.

## Results and Discussion

### **Moisture gain study**

Microorganisms can contain three types of water viz. free water, bound water and preserved water [11]. Free water consists of intercellular and part of intracellular water. Bound water is the part of intracellular water held by proteins, nucleic acids, membranes and contributes to structural organization of these substances. Preserved water is present in isolated pockets formed by lipids and other membrane components. Water content of microorganism increases on exposure to high humidity conditions. Sorption of moisture from the atmosphere is primarily a physical process which could be adsorption, absorption, or occlusion [12]. The free water content is deleterious to the stability of *B. coagulans* spores and provides realistic picture of moisture stability of probiotics than the total water content. The free water content can be measured by water activity ( $a_w$ ). To prevent undesired microbial proliferation,  $a_w$  value of 0.6 is generally accepted as the upper limit [13].

The initial assay, moisture content and water activity value of *B. coagulans* was  $3.8 \times 10^{11}$  CFU/gram, 7.1% w/w and 0.48, respectively. Moisture uptake by *B. coagulans* samples that is the difference in moisture content of initial and exposed samples, water activity and corresponding assay value of *B. coagulans* exposed to various RH conditions are summarized in Figure 1 and 2.

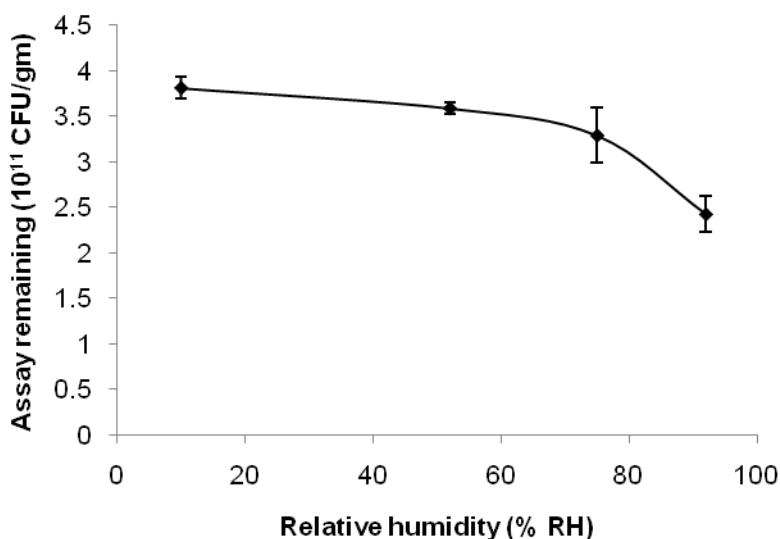


**Fig. 1.** Effect of different relative humidity conditions on moisture uptake and water activity of *B. coagulans* at 25°C. (Mean±S.D., n=3)

In the present study, there was no significant increase in  $a_w$  values below 52% RH. However, at 75% and 92% RH  $a_w$  values increased to 0.66 and 0.88, respectively. This made them susceptible to the growth of contaminant microbes and reduced the assay value to 82.3% and 60.6%, respectively. Petri plates with *B. coagulans* spores exposed to 92% RH showed white fungal growth. Role of water activity was also underlined in US

patent number 7122370 [14] which disclosed a probiotic composition with  $a_w$  value of not more than 0.028 and demonstrated improved shelf life stability.

The study indicates that addition of probiotic *B. coagulans* in a dietary supplement or a pharmaceutical formulation having  $a_w$  greater than 0.5 would result in fall in spore viability. Formulations with such high water content would require use of protective techniques such as microencapsulation using water resistant polymers, or waxy materials like stearic acid, to protect *B. coagulans* spores.



**Fig. 2.** Assay of *B. coagulans* spores after exposure to different relative humidity conditions for 7 days at 25°C temperature. (Mean±S.D., n=3)

#### **Effect of compaction**

The assay of *B. coagulans* powder used for preparation of 'cell pellets' was  $4 \times 10^{11}$  CFU/gram. The assay of blends used for preparation of 'diluent mixed cell pellets' containing LMH, DCP, MCC and corn starch, respectively were  $1.04 \times 10^{11}$ ,  $1.12 \times 10^{11}$ ,  $1.08 \times 10^{11}$  and  $1.01 \times 10^{11}$  CFU/gram, respectively. The *B. coagulans* microbiological assay of blends used for preparation of 'cell pellets' and 'diluent mixed cell pellets' were considered as 100%. Increase in compaction pressure reduced survival rate of *B. coagulans* spores. As shown in Table 1, there was an inverse relationship between the compaction pressure and the activity remaining. At relatively low compaction pressure of 1000 psi, assay values for 'cell pellet' and 'diluent mixed cell pellet' were reduced by about 5% but at higher compaction pressures the activity reduced significantly. These results were similar to the previous reports of Maggi *et al.* [8], Fazeli *et al.* [15] and Durand *et al.* [16] for other probiotic lactobacilli. A reduction in the viability of different strains of lactobacilli during manufacture of mono and double layer tablets of freeze dried cultures was reported by Maggi *et al.* Durand and Panes [16] demonstrated that different species of probiotic bacteria had different levels of resistance to compaction pressure.

In addition, results of the present study highlight that diluents can particularly protect against the adverse effect of compaction pressure. The degree of protection offered by various diluents was not statistically significantly different ( $p < 0.05$ ). Studies published by

Fazeli and coworkers [15] showed that tablets of *Lactobacillus acidophilus* containing hydroxypropyl methylcellulose, polycarbophil and sodium carboxymethyl cellulose underwent nearly 1 log cycle (~90%) reduction in the assay values after compaction. On the other hand, tablets containing carbomer exhibited significantly less reduction in the assay value.

In the present study, reduction in assay values was relatively less compared to that reported for other probiotic lactobacilli, which can be attributed to resistance of spore form of *B. coagulans* (as most of the other probiotic are vegetative cells) to compaction pressure [17]. High compaction pressures resulting in loss of viability of *B. coagulans* would partially explain the loss observed in tablet dosage form.

**Tab. 1.** Effect of compaction on the viability of cell pellets and diluent mixed cell pellets (Mean±S.D., n=3)

S. No.	Pressure (psi)	Assay of pellets (%)				
		Cell pellet	LMH	DCP	MCC	Corn Starch
1	1000	94.66±0.18	95.87±0.04	95.42±0.04	94.81±0.06	94.90±0.06
2	2000	75.24±0.12	77.69±0.06	74.92±0.06	76.38±0.06	76.14±0.03
3	4000	58.26±0.16	65.62±0.05	67.15±0.04	63.45±0.03	64.45±0.03

### **Excipient compatibility studies**

Probiotics must reach the site of action in active form and in sufficient concentrations to exert health benefits. Reduction in the probiotic assay during processing, storage and passage through the GIT, offers a major challenge to their formulation. This requisite, in turn, will be influenced by minimum therapeutic dose of probiotic. Reid and collaborators have reported that oral doses of higher than  $10^9$  viable cells per day are required to restore and maintain normal bacterial flora [18]. The effect of freeze drying and refrigerated storage conditions on different probiotics have been studied in the past [7, 19] and it has been reported that different excipients can exert cryoprotective effect on probiotics.

In the present research work, the effect of some commonly used pharmaceutical excipients on the survival of *B. coagulans* spores was studied under isothermal stress conditions of  $40^\circ\pm2^\circ\text{C}$ / 75%±5% RH. Isothermal stress testing involves challenging drug-excipient mixtures in presence of moisture to degradation [20]. Under these conditions *B. coagulans* spores degraded by approximately 22% in 15 days. The inactive ingredients selected for the study included pH adjusting agents, encapsulating agents, diluents, disintegrants, glidants and lubricants. The *B. coagulans*:excipient blends were taken in ratio of 1:1, except for diluents and lubricants, where the ratio was 1:4 and 4:1, respectively. Initial assay of each blend was determined and considered as 100%. The assay of these blends determined at the end of study was expressed as percentage of the initial assay (Table 2).

Presence of interaction between *B. coagulans* and pH buffering agents- citric acid monohydrate and meglumine, was apparent from the change in color of the blend from light brown to dark brown. *B. coagulans* also showed loss of assay with these excipients. A high moisture uptake of 21.6% w/w and 24.36% w/w was observed for citric acid

monohydrate and meglumine containing mixtures, respectively coupled with water activity values beyond the limit of 0.6. However, loss in the assay of *B. coagulans* was less severe with trisodium citrate dihydrate. The latter had a moisture uptake of 11.4% w/w at the end of 15 days and the observed  $a_w$  of 0.409.

A series of microencapsulating agents were evaluated. Blends of all the tested encapsulating agents, except for sodium alginate, showed statistically insignificant differences in the assay as compared to the control. Sodium alginate, showed a significant reduction in assay value (6.5%). Amongst the three water soluble encapsulating agents evaluated (i.e. HPC, sodium alginate and HPMC), blend with sodium alginate showed maximum moisture uptake and highest water activity value. Marginally higher survival of *B. coagulans* was observed with carnauba wax, stearic acid and cellulose acetate. This can be attributed to the hydrophobic nature of these materials that led to a lesser moisture uptake.

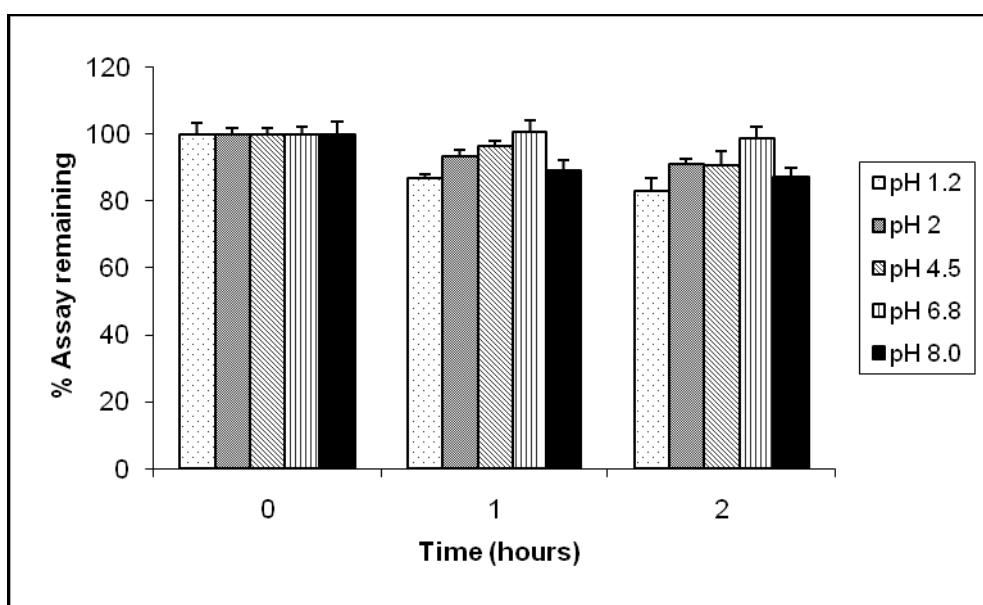
Most of the diluent blends demonstrated low moisture uptake tendency and thus maintained a low water activity. Within the diluents class best survival rate was observed in presence of DCP followed by LMH, MCC, and corn starch. In case of DCP, viability decreased by 17.8%, with water activity of 0.366 and moisture uptake of 4.3% w/w. Though, corn starch blend showed high moisture uptake of 18.3% w/w, it retained the assay value. This indicated that the corn starch though picks up moisture, is able to keep it in 'bound' form.

Disintegrants, are hygroscopic in nature [12], thus their blends show a high moisture uptake and a high water activity (about 0.6). Maximum reduction in the assay of *B. coagulans* was observed with disintegrants. Crospovidone and croscarmellose sodium showed an activity loss of approximately 8% more and sodium starch glycolate showed an activity loss of nearly 15% more than the control.

The glidants and lubricants evaluated are water insoluble materials with small particle size. For CSD blend a moisture uptake of 11.2% w/w was observed but still the water activity remained very close to the initial value. This indicated a preferential water uptake by silicon dioxide that is kept in 'bound' form. The ability of CSD to coat the spore surface and act as a barrier to the environment can be attributed to its high surface area ( $200 \text{ m}^2/\text{gm}$ ) [21]. The CSD blends showed low initial and final water activity, even though the total moisture content increased significantly. Similarly, blend containing talc was able to retain water in bound form and reduction in the assay was less pronounced.

Correlating the moisture uptake and water activity of blends it can be summarized that, with the initial water activity values below 0.5, a moisture uptake of about 10% w/w led to only a second decimal increase in water activity thus the assay was maintained in the range of control sample. At moisture uptake of about 15% w/w, water activity increased beyond 0.5 with corresponding approximate 8% reduction in the assay. For blends with moisture uptake of 20% or higher, water activity values were close to 0.6, with reduction of assay by about 15%.

The worst affected blends in terms of assay in the compatibility study were that of citric acid monohydrate, meglumine and sodium starch glycolate. This study indicated that the water insoluble, hydrophobic excipients such as cellulose acetate, carnauba wax, stearic acid reduced the moisture uptake tendency of blend and hence maintained the viability of *B. coagulans* spores. It was also observed that certain excipients like corn starch, talc and colloidal silicon dioxide had the ability to retain moisture in 'bound' form. Thus the study provides rationale for selection of suitable excipients for probiotic formulations.



**Fig. 3.** The aqueous pH stability profile of *B. coagulans* spores. (Mean±S.D., n=3)

#### Aqueous pH stability profile of *B. coagulans*

For *B. coagulans* to be beneficial to the host they should colonize in the colon. Following ingestion, *B. coagulans* must survive the transit through the gastric environment and reach the colon in quantities large enough to facilitate colonization. The germicidal effect of gastric juice is mainly attributed to its low pH [22].

As seen in Figure 3, pH of aqueous media had a significant effect on activity of *B. coagulans*. Viability of *B. coagulans* was reduced in acidic as well as in alkaline pH environment. *B. coagulans* showed optimum stability at pH 6.8 condition. Study on the spore forming lactic acid bacillus by Hyronimus and coworkers [23] had similar observations for acidic pH environment and found that the spore forming lactic acid bacillus are sensitive to low pH environment. Other lactobacilli like *Lactobacillus acidophilus*, a common probiotic, was found to be extremely sensitive to low pH environment. Kim and others [24] showed *Lactobacillus acidophilus* ATCC 43121 was almost completely destroyed within 1 hour of incubation in pH 1.2 environment. Similar results were reported by Durand and Panes [16].

**Tab. 2.** Moisture content, water activity and assay remaining in blends used for compatibility study, before and after 15 days storage at temperature of 40°C/ 75% RH condition. (Mean±S.D., n=3)

<b>S.No.</b>	<b>Category</b>	<b>Ingredients</b>	<b>Moisture content (% w/w)</b>		<b>Water activity</b>		<b>% Assay remaining</b>
			<b>Initial</b>	<b>Final</b>	<b>Initial</b>	<b>Final</b>	
1	Control	<i>B. coagulans</i>	7.2	18.6	0.503	0.528	77.9±0.09
2	pH adjusting agents	Citric acid monohydrate	5.3	26.9	0.421	0.624	61.8±0.12**
3		Trisodium citrate dihydrate	8.2	19.6	0.377	0.409	76.6±0.06
4		Meglumine	4.9	29.3	0.417	0.628	63.2±0.06**
5	Encapsulating agents	HPC	4.2	14.9	0.462	0.481	78.3±0.09
6		Sodium alginate	4.7	21.8	0.415	0.540	71.4±0.14
7		HPMC	3.9	14.9	0.453	0.477	78.1±0.03
8		HPMCP	4.2	14.8	0.450	0.469	78.3±0.08
9		Carnauba wax	3.4	8.5	0.422	0.433	81.2±0.12
10		CA	3.6	10.7	0.388	0.411	79.8±0.06
11		Stearic acid	3.7	10.5	0.414	0.431	80.8±0.16
12	Diluents	MCC	3.2	11.0	0.409	0.442	78.3±0.07
13		LMH	4.1	11.4	0.379	0.405	78.6±0.09
14		Corn starch	3.7	22.0	0.394	0.483	75.8±0.04
15		DCP	3.8	8.1	0.353	0.366	82.2±0.12
16	Disintegrants	SSG	6.9	34.7	0.439	0.559	63.2±0.16**
17		Crospovidone	5.9	21.1	0.421	0.503	70.2±0.09
18		CCS	6.5	23.2	0.448	0.528	69.9±0.07*
19	Glidants	CSD	2.6	13.8	0.379	0.385	80.8±0.18
20		Talc	3.2	18.8	0.398	0.447	77.1±0.05
21	Lubricant	Magnesium stearate	2.6	16.1	0.389	0.426	78.6±0.08

HPC: Hydroxypropyl cellulose; HPMC: Hydroxypropyl methylcellulose; HPMCP: Hydroxypropyl methylcellulose phthalate; CA: Cellulose acetate; MCC: Microcrystalline cellulose; LMH: Lactose monohydrate; DCP: Dibasic calcium phosphate dihydrate; SSG: Sodium starch glycolate; CCS: Croscarmellose sodium; CSD: Colloidal silicon dioxide.

Values in the column followed by no asterick, \* and \*\* sign indicate three groups that have statistically significant differences between them. Values with a same sign indicate that they are not statistically significant different from each other (p< 0.05).

The different species of spores seem to exhibit different levels of resistance to acid environment and thus entail individual characterization of each species. pH stability profile showed that *B. coagulans* was sensitive to extreme acidic and basic environments. These observations can also indicate the solid state stability of *B. coagulans* in products where presence of acidic or basic excipients can influence the pH of the microenvironment. *In vivo* the typical pH condition in the fasted stomach is between 1 to 2. Upon ingestion of meal gastric pH at first increases and then returns back to the fasting pH values within 3–4 hours [25]. Some of the reported protective strategies to address the pH instability are microencapsulation by enteric polymers like cellulose acetate phthalate [26], coating of

probiotics with lipidic excipients like waxes [16], mixing with resistant starch [27] and symbiotic microencapsulation by emulsion spray drying technique [28].

## Experimental

### *Microorganism*

The probiotic bacteria used in this work were spores of *Bacillus coagulans* (Uni Sankyo Limited, Hyderabad, India). These spores are widely used in the production of probiotic powders and tablets. *B. coagulans* batch had microbiological assay in the range of  $3.8 \times 10^{11}$  to  $4.2 \times 10^{11}$  spores per gram and powder was used as supplied by the manufacturer.

### *Chemicals*

PNY medium for microbiological assay of *B. coagulans* was purchased from HiMedia Labs, Mumbai, India. Pharmaceutical excipients corn starch, stearic acid, sodium alginate, talc, and citric acid monohydrate were purchased from Loba chemie Ltd. (Mumbai, India). Hydroxypropyl methylcellulose (HPMC) and hydroxypropyl methylcellulose phthalate (HPMCP) were purchased from Colorcon Asia Pvt. Ltd. (Mumbai, India), lactose monohydrate from Lactose India Ltd. (Mumbai, India), dicalcium phosphate dihydrate (DCP) from Gangwal Chemicals (Mumbai, India) and sodium starch glycolate from JRS Pharma (NY, USA). Microcrystalline cellulose (MCC) and croscarmellose sodium were sourced from FMC Biopolymer (Brussels, Belgium), cellulose acetate from Eastman Chemical Ltd. (Singapore) and crospovidone from ISP technologies (NJ, USA). Carnauba wax and meglumine were purchased from Sigma Chemical Co. (USA). Hydroxypropyl cellulose, magnesium stearate, colloidal silicone dioxide were purchased from Shin-Etsu Chemicals (Japan), Sinai Pharma (India), and Degussa Ltd. (UK), respectively. Trisodium citrate was supplied by Merck Specialties Pvt. Ltd. (Mumbai, India). All other chemicals used were of analytical grade.

### *Bacterial enumeration*

The method used for microbiological assay of *B. coagulans* spores by the manufacturer was reproduced in our laboratory. *B. coagulans* were enumerated in the PNY medium. Serial dilutions of *B. coagulans* were prepared in normal saline. Dilution tubes were allowed to stand in the water bath at temperature of 70°C for 30 minutes and then cooled immediately to about temperature of 45°C. These heat treated cultures were plated by pour plate technique. The plates were incubated at temperature of 37°C for 48 hours. The procedures were carried out in triplicate using aseptic techniques. The colony forming units (CFU) of bacteria were counted and % viability was determined.

$$\% \text{ Viability} = \frac{\text{CFU before exposure to treatment}}{\text{CFU after exposure to treatment}} \times 100$$

### *Moisture gain study*

Accurately weighed amounts of *B. coagulans* powder were exposed to relative humidity (RH) conditions of 10, 52, 75 and 92% obtained by using saturated aqueous solutions of lithium chloride, magnesium nitrate hexahydrate, sodium chloride and potassium nitrate respectively, in vacuum sealed desiccators at temperature of 25°C. After 7 days, samples

were analyzed for microbiological assay by serial dilution method, moisture content by Karl Fischer titration (Karl Fischer autotitrator, Metrohm, Herisau, Switzerland) and water activity using water activity meter (Hygrolab III, Rotronic AG, Switzerland).

### **Effect of compaction on *B. coagulans***

The *B. coagulans* spores powder and blends of *B. coagulans* with pharmaceutical diluent in 1:3 ratio were compressed in a hydraulic press (Model 3912, Carver Inc., India) with flat faced punches of diameter 8 mm at 1000, 2000, and 4000 psi pressure. Lactose monohydrate (LMH), dibasic calcium phosphate dihydrate (DCP), microcrystalline cellulose (MCC) and corn starch were the diluents used in this study. The resultant compacted pellets of probiotic powder were labeled as 'cell pellets' and those of *B. coagulans*:diluent were labeled as 'diluent mixed cell pellets'. The initial powder blends and the compressed pellets were analyzed for microbiological assay to assess the effect of compaction on the viability *B. coagulans* spores.

### **Excipient compatibility studies**

Binary blends of *B. coagulans* spores powder with excipients were prepared and stored in dark stability chamber maintained at temperature of  $40^{\circ}\text{C} \pm 2^{\circ}\text{C}$  and  $75\% \pm 5\%$  RH conditions. Samples were withdrawn after 15 days and analyzed for microbiological assay, moisture content, water activity and physical changes. *B. coagulans* spores powder was exposed to similar conditions and was used as control for comparison.

### **Aqueous pH stability profile of *B. coagulans***

The stability of *B. coagulans* was evaluated in aqueous media of pH range from 1.2 to 8.0 to cover the pH environments encountered by *B. coagulans* in the GIT. 1% w/v aqueous suspension of *B. coagulans* was prepared in 0.1N HCl (pH 1.2), 0.01N HCl (pH 2.0), buffers of pH 4.5 acetate, 6.8 and 8.0 phosphate at temperature of  $25^{\circ}\text{C}$ . Suspensions were stirred at 750 rpm and analyzed at 0, 1 and 2 hours for microbiological assay.

## **Conclusions**

*B. coagulans* spores were found to be sensitive to the conditions encountered in processing of pharmaceutical and food products. *B. coagulans* spores were moderately hygroscopic and exposure to RH conditions of 75% and above was detrimental to its viability. Impact of mechanical stress showed loss in spore viability, which also indicates its sensitivity to high shear processes. The aqueous pH stability profile showed a rapid degradation with maximal stability of *B. coagulans* spores at pH 6.8. The spores were found to be compatible with the excipients evaluated, with noted exception of citric acid monohydrate, meglumine and sodium starch glycolate. The physicochemical profiling of *B. coagulans* spores presented in the study provides understanding of the material attributes critical to product design in terms of selection of formulation ingredients, process conditions and pack suitability.

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## Authors' Statement

### Competing Interests

The authors declare no conflict of interest.

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