



**PROBIOTIC EFFECT OF *BACILLUS COAGULANS*, MBTU-P1F2 FROM INFANT
FAECES WITH A KNOWN PROBIOTIC**

Honey Chandran C.¹, Summaya Jauhara² and Dr. Keerthi T. R.^{a*}

^aSchool of Biosciences, Mahatma Gandhi University, Kottayam.

*Author for Correspondence: Dr. Keerthi T. R.

School of Biosciences, Mahatma Gandhi University, Kottayam.

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ABSTRACT

Comparative evaluation of Probiotic potency of *Bacillus coagulans*, MBTU-P1F2 from infant faeces with a known probiotic *Bacillus clausii* from Enterogermina provides a new area for searching potent spore former from human intestinal flora. Aim of the study was to isolate and characterize spore forming bacteria from infant faeces and comparatively evaluate its probiotic potentials with a known probiotic (*Bacillus clausii*). The isolate were shown all the essential properties of probiotics such as tolerant to acidic pH, bile salts and viable in simulated gastric juice which would enable them to withstand the conditions prevailing in the gastro intestinal tract and molecularly identified as *Bacillus coagulans*. Comparative study with *Bacillus clausii* revealed that *Bacillus coagulans*, MBTU P1F2 showed more inhibitory against selected pathogens. Cell surface properties of the isolate revealed the presence of hydrophobic components on the cell surface and as in comparison, *Bacillus clausii* showed more hydrophobicity than MBTU-P1F2. The hydrophobic nature can be related to their ability to adhere to gastrointestinal mucosal epithelium. The studies further proved that they were autoaggregative and coaggregates the main causative agent of enteric fever, *Salmonella typhi*. MBTU-P1F2 showed capability to deconjugate bile salts (Bile Salt Hydrolase activity, have role in cholesterol removal) and was not detected in *Bacillus clausii*. The findings proved the probiotic potency of known probiotic *Bacillus clausii* as well as clear implications for future research in the field, to develop our isolate as probiotic for human or animal nutrition.

KEYWORDS: Probiotics, Human infant faeces, *Bacillus coagulans* MBTU-P1F2, Enterogermina, *Bacillus clausii*, Nutrition.

INTRODUCTION

Probiotics are commonly defined as mono or mixed cultures of live microbes that when applied to an animal or human, possess a beneficial effect on the health of host possibly by improving the balance of the indigenous microflora. The predominant species used as probiotic agents belongs to the group of LAB. The second largest group of microbes widely used as probiotics are the *Bacillus* species. Most extensively examined are *Bacillus subtilis*, *Bacillus clausii*, *Bacillus cereus* *Bacillus coagulans* and *Bacillus licheniformis*. *Bacillus* species have been used as probiotics for at least 50 years with the Italian product known as Enterogermina – registered 1958 in Italy as an OTC supplement.^[1] *Bacillus coagulans* known as “king of probiotics” is a lactic acid producing bacterial species within the genus *Bacillus*. The organism was first isolated and described as *Bacillus coagulans* in 1915 by B.W. Hammer. Many references to use of this bacterium in humans exist, especially in improving the vaginal flora, improving abdominal pain and bloating in irritable bowel syndrome patients and increasing immune response to viral challenges. One strain of this bacterium has also been assessed for safety

as a food ingredient. Spores are activated in the acidic environment of the stomach and begin germinating and proliferating in the intestine. Sporeforming *B.coagulans* strains are used in some countries as probiotics for patients on antibiotics.

Bacillus clausii is a rod-shaped, Gram-positive, motile and spore-forming bacterium which lives in the soil. *B. clausii* spores have been used in a European probiotic called Enterogermina, which stimulates GI tract immune system function by increasing production of secretory immunoglobulin A- indirectly acting as an antagonist to other bacterial pathogens that infect the gastrointestinal tract.^[2] *Bacillus clausii* sporulated strains are actually used in the treatment of gastrointestinal illnesses to restore intestinal flora because of their antibiotic resistance and ability to stimulate immune activity. The present study involves the isolation and probiotic characterization of lactic acid sporulating bacteria from infant faeces, and its comparative evaluation of probiotic potency with a known probiotic *Bacillus clausii* from Enterogermina available in the market. High tolerance of spore former with probiotic capabilities from intestinal

flora makes this isolate to develop a good candidates in neutraceutical as well as therapeutic application.

METHODOLOGY

Source of Organism

The test microorganism, *Bacillus coagulans* MBTU-PIF1 used for this study was isolated from infant faeces of up to six month old, *Bacillus clausii* Isolated from Enterogermina a probiotic product available in the market and the pathogens used in the study were obtained from MTCC & available in the culture collection of Microbial Biotechnology Laboratory, School of Biosciences, Mahatma Gandhi University, Kottayam. The test organism and *Bacillus clausii* were grown & maintained on LB agar medium.

Physiological and Biochemical characterization of Test Isolate

The isolate was studied for its basic biochemical characteristics by routine methods in addition also perform haemolysis and milk coagulation tests.

Molecular identification

DNA Isolation

Bacterial genomic DNA of the isolate was performed by the method of Sambrook *et al.*^[3]

Polymerase Chain Reaction (PCR)/Sequencing and phylogenetic analysis

PCR was carried by using universal primers. The sequence of the insert was determined using the automated DNA sequencing service provided at Sci Genomics Lab Pvt Ltd., Cochin, India. The BLAST program (www.ncbi.nlm.nih.gov/blast) was employed in order to assess the degree of sequence similarity. The phylogenetic tree was displayed using the Molecular Evolutionary Genetic Analysis (MEGA) version 6.0 program (<http://www.megasoftware.net>).

Comparative evaluation of MBTU – P1F2 with a known probiotic *Bacillus clausii*

In vitro Probiotic characterization

Acid tolerance test

Tolerance to acid was tested according to Khalil *et al.*^[4] with LB broth previously adjusted to pH values 2, 3 and 4.

Bile tolerance

Tolerance to bile was determined by bile broth assay with 0.3, 0.5 or 1% (w/v) of bile according to Walker and Gilliland.^[5]

Tolerance to simulated gastric juice to determine transit tolerance

Gastric juice was prepared with 0.3% pepsin, 0.5% NaCl at pH 2 and 3. Tolerance to simulated gastric juice was determined by the method of Charteris *et al.*^[6] with slight modifications.

Antagonistic action towards pathogens

Antibacterial activity of the isolates towards *Salmonella typhi*, *Klebsiella spp.* and *Bacillus subtilis* was studied by agar diffusion test.

Cell Surface properties: Hydrophobicity

This was determined according to Vinderola and Reinheimer.^[7] Hydrophobicity was performed by using xylene - a non polar solvent and chloroform an a polar solvent.

Auto aggregation

Optical Density of the isolates was adjusted at 600 nm as 0.5.4 ml of cell suspension was incubated at room temperature for 5hrs. 0.1 ml of the upper suspension was mixed with 3.9 ml of PBS and absorbance measured at 600 nm. Auto aggregation percentage were detected by the method of Iniguez-Palomares C.*et al.*^[8]

Coaggregation

Here cell suspension mixed with pathogen and absorbance measured at different interval for 5hrs. Results are expressed as the percentage reduction after 5 h in the absorbance of a mixed suspension compared with the individual suspension.

Tolerance to bile salt and bile salt hydrolase activity

Bile salts deconjugation assay: The isolates were spotted on MRS agar plates supplemented with 0.5% sodium salt of taurodeoxycholic acid (TDCA), incubated anaerobically at 37°C for 72 hr and observed for precipitation zone around colonies.^[9]

Statistical Analysis

Each experiment was independently replicated 3 times in a completely randomized design. All analysis and enumerations were done in duplicate. Statistical analysis was conducted using Student t test, values are expressed in mean \pm standard deviation.

RESULTS

Identification MBTU-P1F2

Colony characters

The result presented in Table 1 shows the colony characters, Gram's reaction and motility which are the primary screening tests to identify a new isolate.

Table 1: Colony characters.

Colony character	Gram reaction	Motility	Spore staining	Skim milk coagulation	Hae mysis
Small, round shaped creamish mucoid colonies with entire edge	Gram positive bacilli	Motile	Spore forming	+	-

Biochemical identification

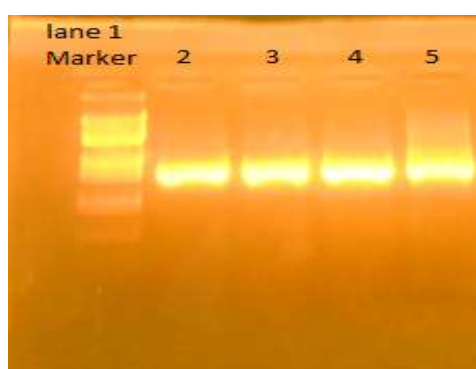
The result presented in table 2.

Table 2: Biochemical identification.

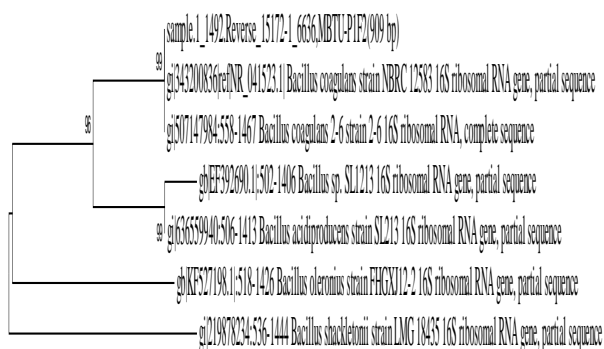
Tests	Indole production test	Methyl Red test	Voges Proskauer test	Citrate utilization test	Catalase test	Oxidase test
Isolate MBTU-P1F2	-	+	+	+	+	-

Molecular identification

Genomic DNA of MBTU – P1F2 was amplified with 16S rDNA primers by PCR method and result is shown in figure 1.

**Figure 1: Agarose Gel Electrophoresis of Amplified Product.****Phylogenetic tree and sequence similarities of MBTU – P1F2.**

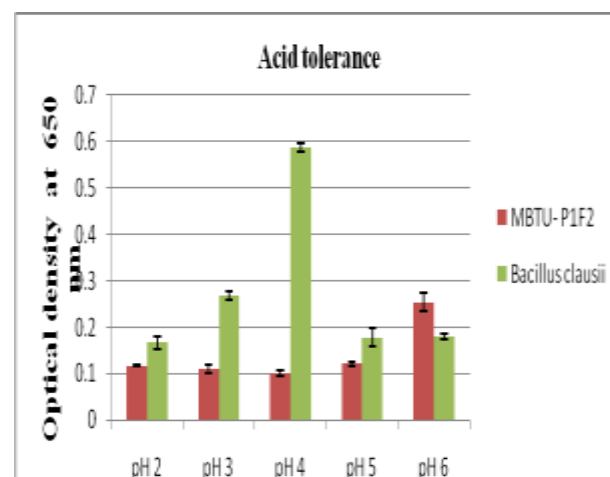
The figure displays the phylogenetic tree of MBTU-P1F2. The phylogenetic analysis revealed that the strain MBTU-P1F2 was closest to *Bacillus coagulans* and showed 99% identity. Phylogenetic tree of the isolated strain MBTU-P1F2, represented in figure 2.

**Figure 2: Phylogenetic tree of the isolated strain MBTU-P1F2.****Comparative evaluation of MBTU – P1F2 with a known probiotic *Bacillus Clausii***

The identified MBTU – P1F2 was characterized for their probiotic potency and compared it with a known probiotic *Bacillus clausii* isolated from Enterogermina.

Acid tolerance

Among the two strains, *Bacillus clausii* shows more acid tolerance than MBTU –P1F2. But at pH 6, MBTU –P1F2 (Figure 3) shows high tolerance than *Bacillus clausii*.

**Figure 3: Acid tolerance of MBTU-P1F2 and *Bacillus clausii*.****Bile tolerance test**

Resistance to bile salts is generally considered to be an essential property for probiotic bacteria to survive the conditions in the small intestine. Cells of *B. clausii* exhibited highest tolerance in 0.5% bile salt. MBTU – P1F2 are more tolerant to bile than *Bacillus clausii*.

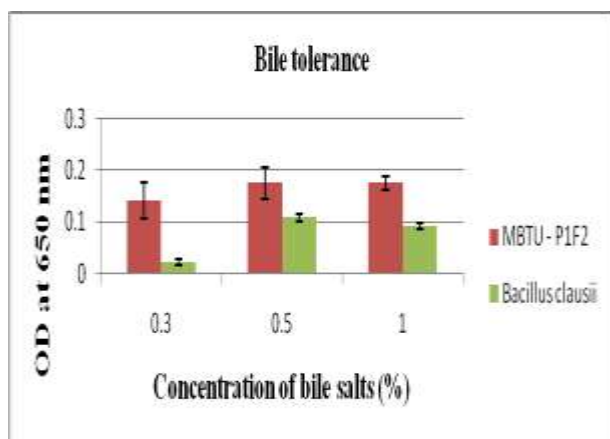


Figure 4: Bile Tolerance of MBTU-P1F2 and *Bacillus clausii*.

Tolerance to simulated gastric juice

MBTU – P1F2 and *B. clausii* shown resistance to simulated gastric juice, MBTU – P1F2 showed highest tolerance than *B. clausii* at pH 3, whereas at pH 2, *B. clausii* exhibited more tolerance than vegetative cells of MBTU – P1F2. The results are shown in figure 5. The potential probiotic bacteria were found viable in simulated gastric juice.

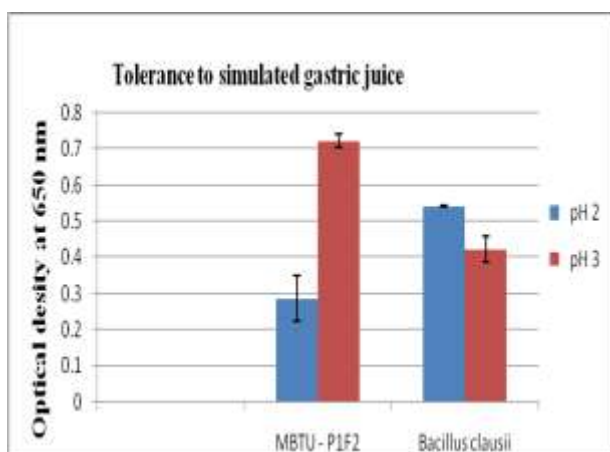


Figure 5: Tolerance to simulated gastric juice.

Cell Surface Properties: Hydrophobicity

Microbial adhesion to solvents (MATS) was used to evaluate the hydrophobic/ hydrophilic cell surface properties of MBTU-P1F2 and *B. clausii*. The results indicated that the both of them exhibited high affinity to xylene than chloroform indicates more hydrophobic surface nature. Among the two strains, *Bacillus clausii* exhibits more hydrophobic nature (85.2%) than MBTU – P1F2 (36.4%) The results are plotted in the figure 6.

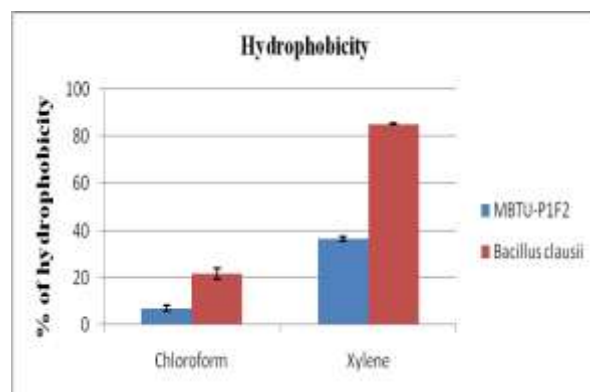


Figure 6: Hydrophobicity.

Auto aggregation

Auto aggregation is the cell - to - cell adherence between bacteria of the same strain suitable for commercial purposes. Vegetative cells of MBTU – P1F2 showed high auto aggregation nature than *B. clausii*. Auto aggregation ability found to increase periodically during incubation periods of 1-5 hrs. Results are expressed in figure 7.

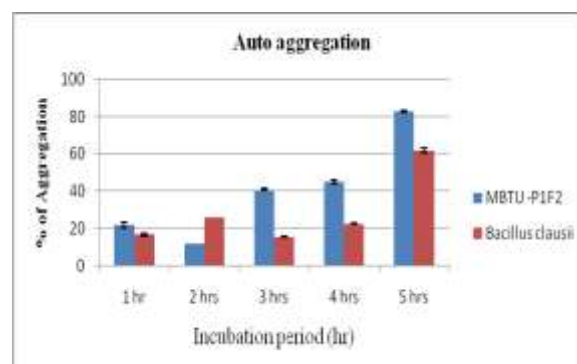


Figure 7: Autoaggregation.

Co-aggregation

Co- aggregation is the cell to cell adherence between genetically different strains. Co aggregation ability of cells of *B. clausii* (53.45%) is more as compared with MBTU – P1F2 (17.536%). Figure 8 showed coaggregation capacity of the isolates towards *Salmonella typhi* which is an important defence mechanism against pathogens.

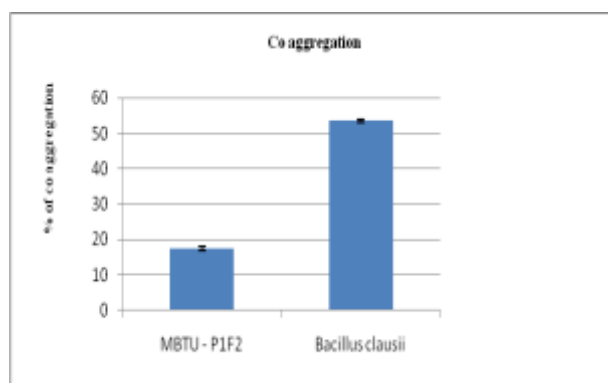


Figure 8: Co- aggregation.

Bile Salt Deconjugation Assay

Vegetative cells of MBTU – P1F2 shown fine precipitated white granules around the colonies indicating bile salt hydrolase activity (Figure 9). *B. clausii* doesn't have any precipitation around the colonies. Our isolate is capable of deconjugating bile acids which in turn would lead to reduction of serum cholesterol level.



Figure 9: Bile salt deconjugation of MBTU-P1F2.

Anti bacterial activity

Culture supernatant of both MBTU – P1F2 and *B. clausii* selectively inhibit the indicator pathogens and the culture supernatant of MBTU – P1F2 found to be more antagonistic towards *Salmonella typhi*. The results are given in figure 10. Table 3 represents the zone diameter with CFS of MBTU- P1F2 and *Bacillus clausii*.

Table 3: Zone Diameter with CFS of MBTU-P1F2 & *Bacillus clausii*.

Pathogens	MBTU-P1F2	<i>Bacillus clausii</i>
<i>Klebsiella pneumoniae</i>	10 mm ± 0.02	9mm ± 0.086
<i>Bacillus subtilis</i>	10 mm ± 0.134	10 mm ± 0.055
<i>Salmonella typhi</i>	14 mm ± 0.091	12 mm ± 0.023



Figure 10: Antagonist effect of MBTU-P1F2 (SJ1) and *Bacillus clausii* (SJ2) vegetative cells against *Klebsiella*, *Salmonella typhi* and *Bacillus subtilis*.

DISCUSSION

Members of Lactobacillus are well-recognized as safe probiotics, available in commercially probiotic products. Recently several studies have been carried out by the researchers around all over the world on the use of spore formers as probiotics.^[4,10] Selections of sporeformers are comparatively easy, because they are abundant in nature. In the present study, bacteria were isolated from infant faeces of up to 6 month old. On the basis of Gram's staining, motility, colony characteristics, skim milk coagulation and haemolysis, the isolate was chosen for primary probiotic characterization. Tolerance to acid, bile and simulated gastric juice is an essential criterion

for the selection of probiotic strains.^[11] Based on primary probiotic characterization, the isolate (MBTU-P1F2) that shown high tolerance to acid, bile and gastric juice. With reference to colony morphology, biochemical tests as outlined in the Bergey's manual of bacteriology, the bacteria 'MBTU-P1F2' was more similar to *Bacillus coagulans*. Accurate species labeling is important in the field of probiotics because it was important in quality control efforts, confidence in product labeling, and for safety considerations. To establish the relatedness of the strain at the genetic level, 16S rRNA genes of 'MBTU-P1F2' were sequenced to analyze species similarity. PCR product was sequenced by using an automated sequencer and phylogenetic analysis showed that MBTU-P1F2 has phylogenetic relationship with *Bacillus coagulans*, a lactic acid producing sporulating bacterium. *Bacillus coagulans* is a gram positive, motile, sporulating lactic acid forming bacteria. It is known as the "king of probiotics". Because of its capability to produce various antimicrobial metabolites, it is used as a therapeutic agent for the treatment of diarrhea, vomiting and other gut associated problems.^[11]

In the present study, the probiotic potency of of MBTU - P1F2 was evaluated and compared with *Bacillus clausii*., a known probiotic from Enterogermina (a probiotic product available in the market as spore suspension). To survive and colonize in the GIT, microorganisms should express tolerance to acid and bile salts.^[12] Probiotics delivered through the feed system have to survive during transit through the upper GIT.^[13] Therefore, incubation MBTU-P1F2 and *Bacillus clausii* with a pH of 2.0 was an attempt to mimic the conditions that a probiotic would have to survive as it passes through the stomach. In order to reach the small intestine they have to pass through the stressful conditions of stomach. Bile salts are synthesized in the liver from cholesterol and are secreted from the gall bladder in to the duodenum in the conjugated form in volumes ranging from 500-700ml per day.^[14] The relevant physiological concentrations of human bile range from 0.1-0.3% (w/v).^[15] MBTU-P1F2 and *Bacillus clausii* showed different tolerant capabilities towards acid and bile. Resistance to bile salts up to 0.4% helps the isolate in proper colonization without being inhibited by the presence of bile salts in the small intestine. MBTU-P1F2 and *Bacillus clausii* also showed resistance to simulated gastric juice. The cell surface properties, such as, hydrophobicity, auto aggregation and co-aggregation are important attributes which help in the attachment to various substrata that explain the probiotic nature of the microorganism.^[10] The present study revealed both of the strains exhibits hydrophobicity in varying manner, increased hydrophobic nature may be due to the presence of hydrophobic proteins.^[16] Many previous studies^[17] on the physicochemistry of microbial cell surfaces have shown that the presence of glyco-proteinaceous material at the cell surface results in higher hydrophobicity, whereas hydrophilic surfaces are associated with the presence of polysaccharides.

Aggregation studies suggest that, both the isolates are capable for auto aggregation. Auto aggregation was found to be more in MBTU-P1F2 than *Bacillus clausii*. The aggregation ability together with cell surface hydrophobicity could be used for preliminary screening in identifying potentially adherent bacteria with probiotic properties suitable for commercial purposes. To quantify inter bacterial adherence a co-aggregation assay was developed, which established co aggregation between selected isolates and enteric pathogen, *Salmonella typhi*. Spencer and Chesson.^[18] suggest that co-aggregation between native bacteria and pathogens has been considered as a way to exclude pathogenic bacteria from their host. Reid et al.^[19] evaluated co - aggregation of LAB with pathogens is an important host defense mechanism against infection. In the present study, we could find out that *Bacillus clausii* has significant co-aggregating ability with *Salmonella typhi* than the co-aggregating ability of MBTU-P1F2. Cholesterol is the precursor of primary bile salts that are formed in the liver and are stored as conjugated bile salts in the gall bladder for secretion in the GIT.^[20] Our isolate *Bacillus coagulans*, MBTU-P1F2 showed capability to de conjugate bile salts, but this capability not detected in *Bacillus clausii*. Antagonistic principles reveal the nature of antagonistic substances released by the isolates against enteric pathogens. Both the isolates found to produce inhibitory substances against *Salmonella typhi*, *Bacillus subtilis* and *Klebsiella*. Hyronimus et al.^[21], found that *B. coagulans* can produce inhibitory compounds against broad spectrum of enteric pathogens.

In the present study, a comparative evaluation of probiotic potency of lactic acid forming *Bacillus coagulans*, MBTU-P1F2 isolated from infant faeces with a known probiotic strain *Bacillus clausii* from Enterogermina. This study established characteristics of *Bacillus clausii* as potential probiotic as well as equal competence of our isolate as a candidate probiotic.

This study also reveals, our isolate is equally good as *Bacillus clausii*, interestingly in some aspects it shown better than *B. clausii*. Our isolate *B. coagulans* as spore forming LAB group having probiotic capabilities isolated from infant feces, have better survivability and can withstand technological parameters than traditional probiotics makes the isolate a good future in probiotic research. Our overall investigation booster for the further detailed study to develop our isolate as probiotic with respect to therapeutic and nutraceutical.

CONCLUSION

In the present study, out of the 7 isolates obtained from infant faeces, we selected a spore forming lactic acid bacteria namely, MBTU-P1F2 based on basic probiotic characteristics. Biochemical characterization and molecular identification, identified this isolate as *Bacillus coagulans*. The comparative evaluation of probiotic potency of MBTU-P1F2 performed with *B. clausii*, a known probiotic isolated from a probiotic

product available in the market with the trade name Enterogermina. The comparative study justify the potency of *B.clausii* as well as concluded that our isolate is equally good as *B.clausii*. Our isolate MBTU-P1F2 has able to produce BSH, which helps to deconjugate bile salts, this helps to lower the cholesterol level, which is an remarkable property than *B.clausii*. Besides the basic probiotic properties both the isolates produced antimicrobial metabolites against *Klebsiella pneumoniae*, *Salmonella typhi* and *B. subtilis*. This study provided a strong information to go for further steps to develop our isolate, MBTU-P1F2 as potent probiotic.

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