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# Optimization of milk-clotting enzyme production by *Bacillus amyloliquefaciens* SP1 isolated from apple rhizosphere

Shiwani Guleria<sup>1\*</sup>, Abhishek Walia<sup>2</sup>, Anjali Chauhan<sup>3</sup> and C. K. Shirkot<sup>3</sup>

## Abstract

**Background:** Present study aims to isolate and optimize fermentation conditions of milk-clotting enzyme producing rhizobacteria *Bacillus amyloliquefaciens* SP1.

**Results:** A bacterium producing an extracellular milk-clotting enzyme (MCE) was isolated from the rhizosphere of the planted population of apple trees growing at Distt. Chamba of Himachal Pradesh, India. According to morphological, physiological, biochemical, and molecular characterization, isolate was identified as *B. amyloliquefaciens*. Single-factor testing was used to study the optimum physical conditions and nutritional parameters for production of the MCE per proteolysis activity which insures its usefulness as new source of milk coagulant for cheese making. The optimum conditions for production of the milk-clotting enzyme were: temperature, 30 °C; inoculum size, 1 %; and initial pH of the medium, 6.0. The maximum milk-clotting activity and milk-clotting activity per proteolysis activity were found using soyabean as nitrogen source and sucrose as carbon source.

**Conclusion:** These optimized conditions resulted in a 1.9-fold increase in production of the milk-clotting enzyme. This study reported a plant growth promoting rhizobacteria, i.e., *B. amyloliquefaciens* as source of milk-clotting enzyme which has potential as a calf rennet substitute.

**Keywords:** *B. amyloliquefaciens*, Milk-clotting enzyme, Protease, Optimization

## Background

The quality of cheese is significantly dependent on the type of milk-clotting enzyme, which is important in the process of milk coagulation and cheese maturation (Kumar et al. 2005). Calf rennet, one of the important enzymes, is traditionally used for cheese making all over world, but due to shortage of animal rennet supply, and ever increase of cheese production and consumption have stimulated the research for milk-clotting enzyme (MCE) from alternative sources to be used as calf rennet substitutes (Nouani et al. 2011).

However, various animals, plants, and microbial proteases have been suggested as milk coagulants (Zhang et al. 2011), but still attention has been focused on

production of milk-clotting enzymes from microbial sources (Silveira et al. 2005; Wu et al. 2013) due to their rapid growth, relative inexpensive growth substrate, and diverse properties. Microbial rennet-like milk-clotting enzymes are aspartic proteases that catalyze milk coagulation. Although, there are some shortcomings when compared with traditional calf rennet, even then microbial rennet has been accepted in markets of several countries. In the US alone, about 60 % of the cheese is manufactured using fungal enzymes (Hashem 2000). Many microorganisms that produce milk-clotting enzymes, i.e., *Rhizomucor miehei*, *Aspergillus oryzae*, *Enthothia parasitica*, and *Aspergillus flavo furcatis*, are widely used (He et al. 2011; Li et al. 2012; Alecrim et al. 2015). In the case of microbial rennets from *Mucor* sp., none of the available reports contains any indication that the use of the enzyme in cheese manufacture is unsafe (Tubessa and Delaimy 2003).

\*Correspondence: shg1988@gmail.com

<sup>1</sup> Department of Microbiology, Kurukshetra University, Kurukshetra, Haryana, India

Full list of author information is available at the end of the article

*Bacillus* sp. is one of the most investigated microbial groups, because they can produce varieties of biotechnological interesting substances. The capacity of *Bacillus* strains to produce and secrete large quantities of extracellular enzymes, including amylase, protease, and cellulases, has led them to be among the most important industrial enzyme producers. In the present study also, the production of milk-clotting enzyme from *B. amyloliquefaciens* has been reported. It is widely known that microbial enzyme production is greatly influenced by many factors, especially medium composition and cultivation conditions. Therefore, work on the optimization of parameters for milk-clotting enzyme is conducted using the “one-factor-at-a time” technique to find out high MCA with proteolytic activity. Because, an adequate substitute must have intense milk-clotting and proteolytic activities (PA) to minimize dissolution of the clot.

*Bacillus amyloliquefaciens*, which can produce milk-clotting enzyme, has been isolated from apple rhizosphere from northern Himalayas. The objective of present work is to investigate the possibility of use of *B. amyloliquefaciens* for the efficient production of rennin, such as enzymes. For cheese making, the present work was undertaken to investigate the pattern of milk-clotting and proteolytic activities of *B. amyloliquefaciens* in extracellular bacterial enzyme preparations under different conditions of culturing.

## Methods

### Sample collection

Rhizobacteria were isolated from the rhizosphere of the planted population of apple trees growing at four different locations, viz., Chamba, Shimla, Kinnaur, and Kullu of Himachal Pradesh, India. Five sites within each location and randomly ten trees from different apple orchards of each site were selected for sample collection. Samples were immediately stored at 4 °C in plastic bags loosely tied to ensure sufficient aeration and to prevent moisture loss until assaying of bacterial community structure.

### Strain isolation

One gram of each soil sample was serially diluted. Aliquots of 100 µl diluted soil samples were spread on screening plates with casein agar medium composed of 0.25 % yeast extract, 0.5 % casein, 0.1 % Glucose, and 1.5 % agar powder. The plates were incubated at 37 °C and observed daily for signs of clearing of agar around the colonies during incubation. The formation of clear zone around colony margin was picked as enzyme producers. The bacteria were maintained at 4 °C on the solid medium, and they were also stored in 15 % glycerol at –20 °C as cryoprotectant.

### Morphological characterization

Morphological characteristics of isolates, including colony morphology, Gram's reaction, cell shape, and presence of spores, were investigated (Sneath 1994).

### Metabolic fingerprinting

Metabolic fingerprinting was done using Bergey's manual of systematic bacteriology (Sneath 1994) and TM commercial kits, i.e., KB009 Hi carbohydrate kit (HiMedia, India).

### Molecular taxonomic characterization

Bacterial isolate was grown in nutrient broth at 35 °C overnight. Bacterial cells were harvested by centrifugation at 5000×g for 5 min, and DNA was isolated from these bacterial cells using the conventional method (Sambrook et al. 1989). The isolated DNA was finally suspended in 100 µl of elution buffer and quantified on 1 % agarose gel. The total genomic DNA was kept at –20 °C before use (Sambrook and Russel 2001). Species level identification of strain was conducted by 16S rDNA sequence comparison. PCR reaction was carried out in 20 µl reaction containing 50 ng of template DNA, 20 pmol of each primer fC1 (5'-GCAAGTC-GAGCGGACAGATGGGAGC-3') and reverse primer rC2 (5'-AACTCTCGTGGTGTGACGGGCGGTG-3'), 0.2 mM dNTPs, and 1 U Taq polymerase (Genei Bangalore) in 1 × PCR buffer. Reaction was cycled 35 times as 94 °C for 1 min, 50 °C for 30 s, and 72 °C for 90 s, followed by final extension at 72 °C for 10 min. The PCR products were analyzed on 1 % agarose gel in 1 × TAE buffer, run at 100 V for 1 h. Gels were stained with ethidium bromide and photographed. Amplified PCR products were eluted from the gel using gel extraction kit (Real genomic (Hi Yield0 TM Gel/PCR DNA Extraction Kit), and eluted fragment was then sequenced (Xcleris, India) using PCR primers.

### Sequence analysis

The sequence was aligned with corresponding sequences of 16S rDNA from the database using BLAST (Altschul et al. 1997). Multiple alignments were generated by the MULTALIN program (Corpet 1988). Phylogenetic tree was constructed with the help of ClustalW (Higgins et al. 1994). Tree was viewed with the help of TreeView (Page 1996).

### Liquid-state fermentation (LSF)

Liquid-state fermentation was carried out in Erlenmeyer flasks (250 ml) containing 25 ml of casein medium supplemented with skim milk autoclaved at 15 psi pressure for 20 min, and cooled and inoculated with 0.5 ml

bacterial suspension (OD 1.0 at 540 nm) (Walia et al. 2013). After mixing, the flasks were incubated at 35 °C for 3 days. The culture supernatant was obtained following centrifugation at 12,000 rpm for 20 min at 4 °C. The supernatant was used as the crude enzyme for assay of milk-clotting and proteolytic activities.

To investigate the factors affecting MCE per protease production, the cultivation was carried out in the medium and conditions indicated above except that the factors were varied by one-factor-at-a-time fashion.

#### Assay for milk-clotting activity

Milk-clotting activity (MCA) was determined according to the method of Arima et al. (1970) and expressed in terms of Soxhlet units (SU). One SU is defined as the amount of enzyme which clots 1 ml of a solution containing 0.1 g skim milk powder and 0.00111 g calcium chlorides in 40 min at 35 °C. In brief, 0.5 ml of tested materials was added to a test tube containing 5 ml of reconstituted skim milk solution (10 g dry skim milk/100 ml, 0.01 M CaCl<sub>2</sub>) pre-incubated at 35 °C for 5 min. The mixture was mixed well, and the clotting time  $T$  (s), the time period starting from the addition of test material to the first appearance of clots of milk solution, was recorded and the clotting activity was calculated using the following formula:  $SU = 2400 \times 5 \times D/T \times 0.5$ ;  $T$  = clotting time (s);  $D$  = Dilution of test material.

#### Protease assay

Protease activity (PA) was measured according to the method described by Sigma with slight modifications (Enyard 2008). One milliliter of culture filtrate was added with 5 ml of 0.5 % (w/v) casein solution in tris-HCl buffer of pH 7.0 and incubated for 5 min at 50 °C. The reaction mixture was stopped by addition of 5 ml of trichloroacetic acid (110 mM). The reaction mixture was centrifuged at 10,000 rpm for 5 min and to 2 ml of supernatant 5 ml of 500 mM Na<sub>2</sub>CO<sub>3</sub> was added followed by 1 ml Folin-Ciocalteu reagent. The amount of tyrosine released was determined spectrophotometrically at 660 nm against the enzyme blank.

One unit of protease activity was equivalent to the amount of enzyme that required releasing 1 µg of tyrosine/ml/min under the standard assay conditions.

## Results

#### Isolation and screening of rhizobacteria

A total of 76 proteolytic bacteria were isolated from rhizosphere (RS) and endorhizosphere (ER) of apple trees of four different locations of Himachal Pradesh viz. Chamba, Shimla, Kinnaur, and Kullu. Forty colonies from Chamba, 15 colonies from Shimla, 14 producers from Kinnaur, and 7 isolates were from Kullu, respectively.

Among all, strain SP1, which was isolated from rhizosphere of the planted population of apple trees growing at District Chamba of Himachal Pradesh, India, showed maximum clear zone on casein agar plates after 48 h of incubation. In liquid fermentation, strain SP1 produced proteolytic enzyme and considerable amount of milk-clotting enzyme (data not shown).

#### Characterization of *B. amyloliquifaciens*

##### Phenotypic and metabolic characteristics of *B. amyloliquifaciens*

The strain SP1 was characterized initially according to morphological, physiological, and biochemical characteristics (Table 1). The isolated colonies on casein agar medium after 48 h of incubation were cream colour, irregular, undulate, wrinkled, and 0.5–0.8 mm in diameter. The morphological characteristics of the strain were as follows: the cells were Gram positive, rods, spore forming, and motile. The rods occurred singly or in chains.

The physiological and biochemical characteristics of the isolated strain SP1 are given in Table 1. The isolate is an aerobic and a catalase producing strain. The isolate tested positive for gelatine liquefaction and starch hydrolysis. The strain was positive for esculin hydrolysis and could utilize a wide array of carbohydrates [assessed using a KB009 Hi366 carbohydrate TM Kit (HiMedia, India)], including xylose, lactose, galactose, raffinose, trehalose, L-arabinose, glycerol, sorbitol, mannitol, erythritol, and melezitose as sole carbon source.

##### Phylogenetic analysis of *B. amyloliquifaciens*

Universal primers were used successfully to amplify 16S rDNA from bacterial isolate SP1, yielding an amplicon of the expected size, i.e., 1375 bp. The sequence of 16S rDNA from strain SP1 was then analyzed using the BLASTn analysis (<http://www.ncbi.nlm.nih.gov/blast>), and was found to have 100 % homology with several *B. amyloliquifaciens* reported from different parts of the world. The 16S rDNA sequence of strain SP1 was also compared with the corresponding sequences of different *B. amyloliquifaciens* reported from different parts of the world. Sequence analysis revealed that SP1 belongs to *B. amyloliquifaciens*, as it showed maximum homology (92 %) with *B. amyloliquifaciens* (accession no. Eu855195) (Fig. 1).

To trace out the evolutionary patterns of the test isolate and to determine the relationship with other selected sequences at NCBI, a phylogenetic tree was also constructed using the neighbor-joining (J) method of mathematical averages (UPGMA) with quite high statistical support by the bootstrap estimates for 1,000 replications. The resulting phylogenetic tree (Fig. 1) also

**Table 1 Differential phenotypic and metabolic characteristics of the *B. amyloliquifaciens***

Characteristics	<i>Bacillus amyloliquifaciens</i>
Colony morphology	Irregular undulate wrinkled
Gram character	+
Sporulating	+
Motile	+
Gelatin liquefaction	+
Starch hydrolysis	+
Catalase	+
Cellulolytic	–
Oxidase	–
Urease production	–
H <sub>2</sub> S production	–
<i>Utilization of</i>	
Lactose	+
Xylose	+
Maltose	–
Fructose	–
Dextrose	–
Galactose	+
Raffinose	+
Trehalose	+
Melibiose	–
Sucrose	–
L-Arabinose	+
Mannose	–
Inulin	–
Sodium gluconate	–
Glycerol	+
Salicin	–
Dulcitol	–
Inositol	–
Sorbitol	+
Mannitol	+
Arabitol	–
Erythritol	+
α-Methyl D-glucoside	–
Rhamnose	–
Cellobiose	–
Melezitose	+
α-Methyl D-mannoside	–
Xylitol	–
ONPG	–
Esculin hydrolysis	+
D-Arabinose	–
Citrate utilization	–
Malonate utilization	–

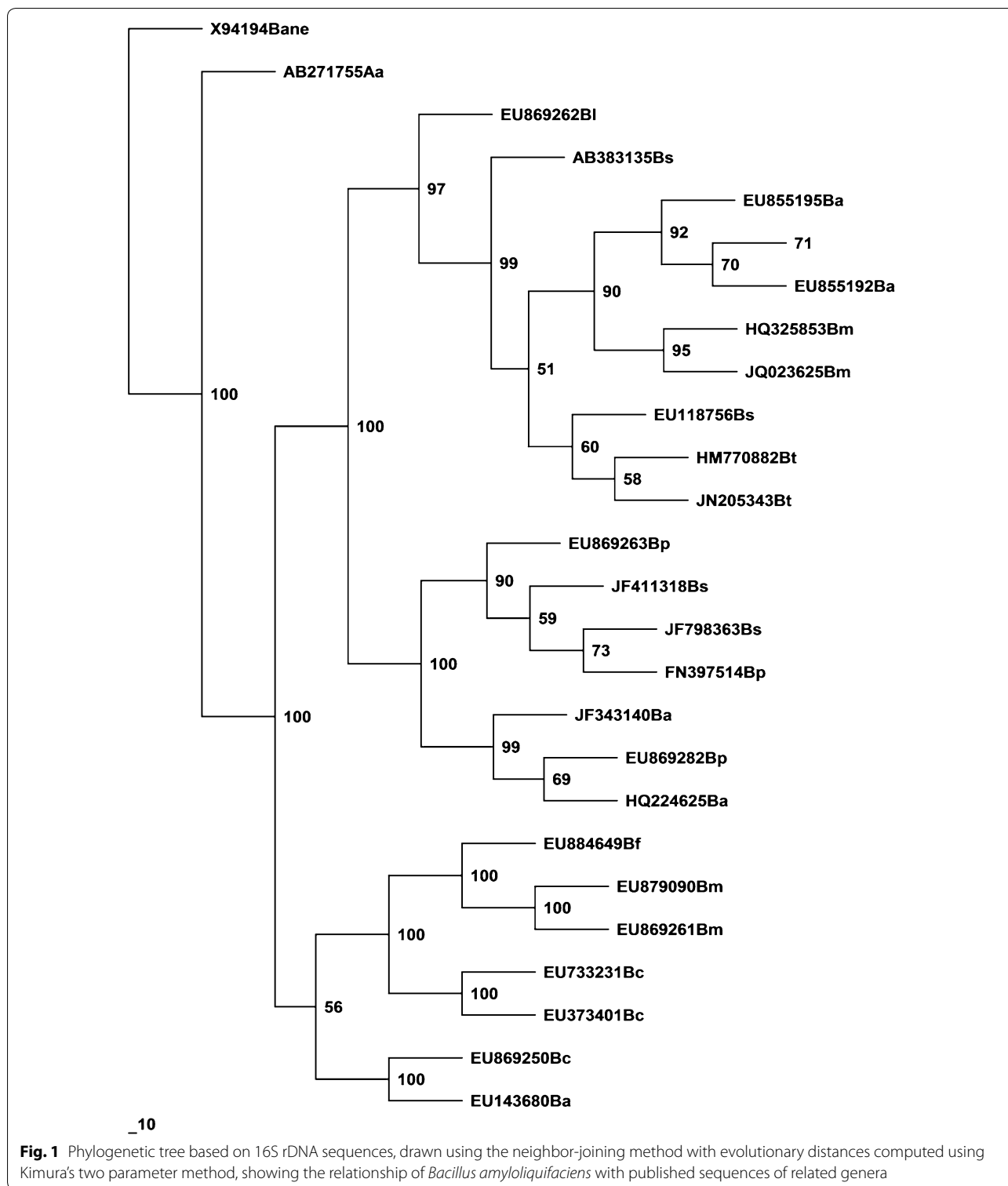
verified SP1 as *B. amyloliquifaciens* clustered closely with *B. amyloliquifaciens* with high boot-strap value (92 %). The 16S rDNA sequence of the strain has been deposited in the GenBank database under accession number KF923792. Based on above morphological, biochemical, and molecular characterization, the strain SP1 was identified as *B. amyloliquifaciens*.

#### Effects of carbon sources on the production of milk-clotting activity

To determine the impact of carbon sources on the production of MCA by *B. amyloliquifaciens*, the bacteria were cultivated in the casein broth medium except that the carbon source, i.e., glucose was replaced with the sucrose, maltose, lactose, starch, and cellulose in the same amount. As shown in Fig. 2, when the lactose, sucrose, starch, and cellulose were used as the carbon source, low MCA was detected in the medium. In contrast, sucrose (75 SU/ml) and glucose (80 SU/ml) produced high levels of MCA. However, maximum proteolytic activity (2050 µg/ml/min) was observed when maltose was in production medium and lowest, i.e., 1630 µg/ml/min and 1740 µg/ml/min protease activity was observed in the case of glucose and sucrose. It is noticed that although using glucose as a carbon source produced higher MCA than using sucrose, the proteolytic activity was also significantly higher when glucose was used instead of sucrose. The ratio of MCA/PA is higher for sucrose than for glucose. Therefore, sucrose appears to be a more suitable carbon source for the production of MCE by *B. amyloliquifaciens* SP1.

#### Effects of nitrogen sources on the production of milk-clotting activity

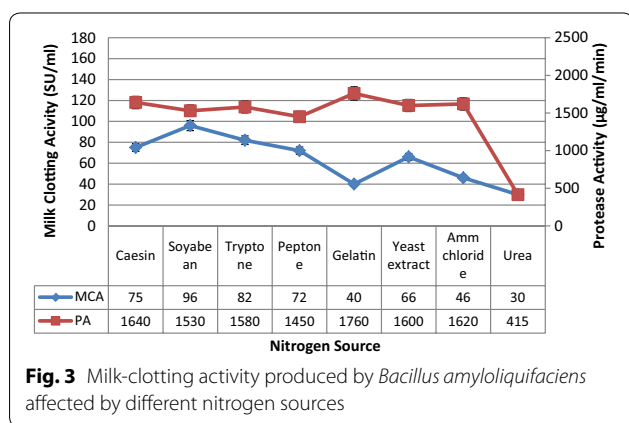
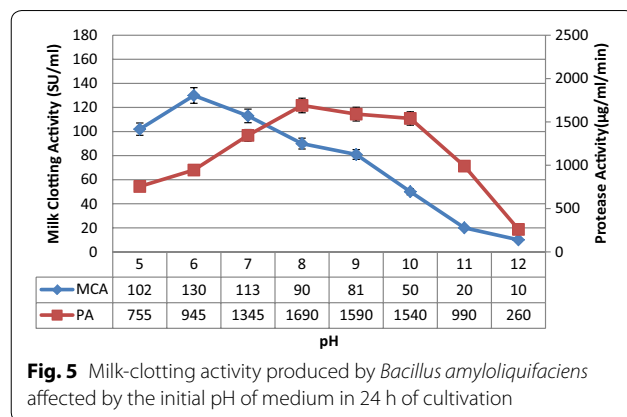
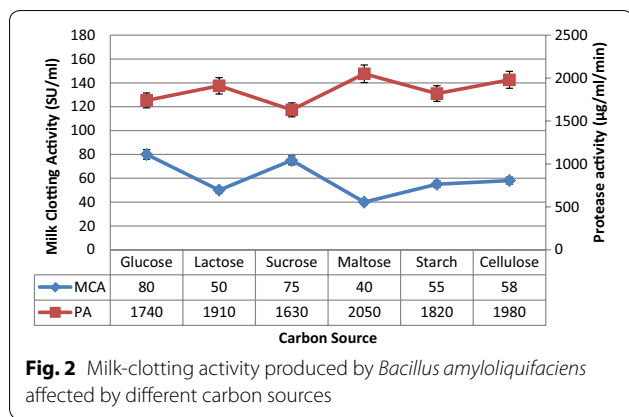
To investigate the effect of nitrogen sources on the MCE production, experiments were carried out with different organic and inorganic nitrogen sources, namely casein, soyabean, tryptone, peptone, gelatin, yeast extract, urea, and ammonium chloride (NH<sub>4</sub>Cl). The medium with the presence or absence of caesin was further supplemented with various nitrogen sources at 0.5 % level. The bacteria were incubated at 37 °C with shaking at 120 rpm for 48 h. The results are shown in Fig. 3. It appears that the presence of inorganic nitrogen sources (urea and ammonium chloride) is not critical for the production of milk-clotting activities by *B. amyloliquifaciens*, but organic nitrogen sources (casein, soyabean, tryptone, peptone, and yeast extract) favored high enzyme yield. Similarly, maximum protease activity (1760 µg/ml/min)



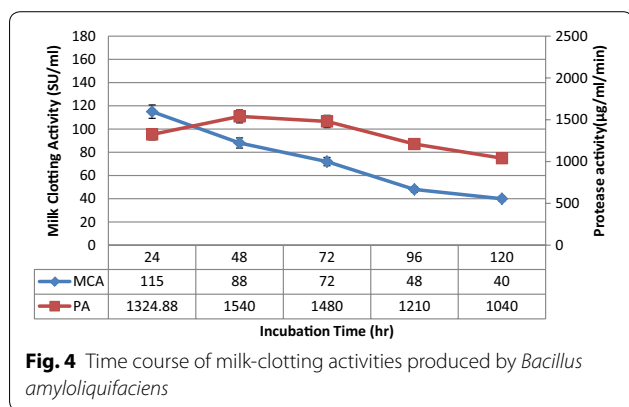
observed when gelatin was used as nitrogen source. In the present study, it was revealed that soyabean as nitrogen source produced maximum MCA (96 SU/ml) and MCA per PA.

**Time course of milk-clotting activity produced**

Figure 4 shows the time course of patterns of MCE and protease production for *B. amyloliquifaciens* at 37 °C, the highest MCA (115 SU/ml) with minimum



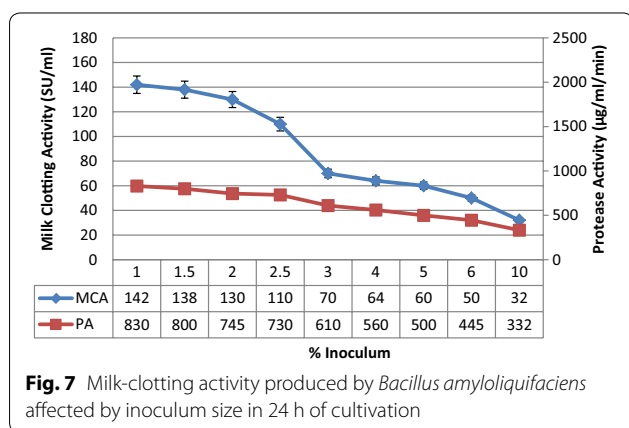
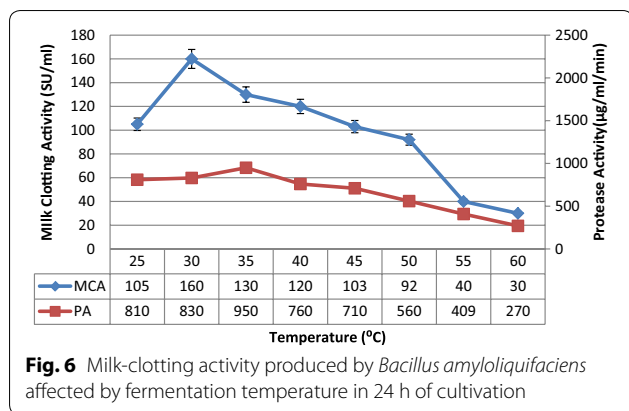
**Production of milk-clotting activity affected by the initial pH of medium**  
 Figure 5 shows the MCE and protease production by *B. amyloliquifaciens* in the medium, which was adjusted to different initial pH values (pH from 5 to 12) and cultivated at 37 °C with shaking at 120 rpm for 24 h. It was observed that the MCE production reached their maximum (130 SU/ml) when the initial pH of medium was adjusted to 6 with maximum MCA/PA (130/945). Further decrease in the initial pH values resulted in the reduced MCE and protease production. However, further increase in pH of medium decreased MCA production, but protease activity increased upto pH 8.0, and thereafter, decrease was observed.



**Production of milk-clotting activity affected by cultivation temperature**  
 It is known that the production of MCE by microorganisms was profoundly affected by cultivation temperature. The effect of cultivation temperature on MCE production by *B. amyloliquifaciens* in the medium was studied at pH 6 and at various temperatures (25–60 °C, at 5 °C increments) (Fig. 6). Although strain could grow in broad temperature range (25–50 °C), but maximum MCA was detected at 30 °C with low proteolytic activity (830 µg/ml/min) after cultivation for 24 h with shaking at 120 rpm. Increasing or lowering the cultivation temperature resulted in the reduction of the MCE production by test organism, and the MCA disappeared completely at 50 °C.

proteolytic activity of 1324.88 µg/ml/min was reached after the bacteria were cultivated for 24 h, but significantly reduced MCA was observed in the culture filtrate with extended cultivation time. However, protease activity was increased upto 48 h (1540 µg/ml/min), thereafter decreased.

**Effect of inoculum size on production of milk-clotting enzyme**  
 An inoculum size of 1 % is found to be optimal for MCE production by *B. amyloliquifaciens* with 142 SU/ml and maximum MCA/PA ratio (Fig. 7). Further increase in inoculum size resulted in decrease in MCA production.



**Discussion**

Many bacteria, especially several species belonging to *Bacillus*, are known to produce variety of extracellular enzymes, and they have a wide range of industrial applications (Wim 2006). Bacteria belonging to *Bacillus*, such as *B. licheniformis*, *B. subtilis* and *B. subtilis natto*, have been suggested as promising microbial rennet producers (Dutt et al. 2009; Shieh et al. 2009; Ding et al. 2011; Zhang et al. 2013). In this study, we characterized a strain of *B. amyloliquifaciens* capable of producing milk-clotting enzyme and proteolytic activity (Guleria et al. 2014). Milk-clotting enzymes are invariably accompanied by proteolytic activity. In a program for the screening of bacteria for MCA, it was of interest to determine their proteolytic activity (exocellular). According to the morphological, biochemical, and 16S rDNA sequencing, isolate was identified as a strain of *B. amyloliquifaciens* (Table 1; Fig. 1).

To find out an adequate substitute to commercial rennet, in the present work, optimization of such culture conditions and medium components was done, where intense milk-clotting activity and low proteolytic activity were observed. From results shown in Fig. 2, on the

basis of high MCA/PA ratio, sucrose was considered as best C-source. The results shown here are in agreement with those found for many other microorganisms in that sucrose (0.5 %) was the most favorable carbon source for *Bacillus subtilis natto* (Shieh et al. 2009) and *Penicillium oxalicum* (Hashem 1999) to produce MCE. Glucose, on the other hand, was not favorable for *P. oxalicum* (Hashem 1999), and did not influence the enzyme production for *Nocardiopsis* sp. (Cavalcanti et al. 2005). Instead of glucose and sucrose, Odeniyi et al. (2009) found galactose, lactose, and trehalose as best C-sources for MCE production, whereas Merheb-Dini et al. (2010, 2012) used wheat bran as substrate.

Addition of organic nitrogen sources to the medium showed maximum production with soyabean (96 SU/ml) followed by tryptone (82 SU/ml) and peptone (75 SU/ml). However, the addition of inorganic nitrogen sources, such as ammonium chloride and urea (46 and 30 SU/ml), respectively, causes a decrease in milk-clotting activity (Fig. 3). Our results are similar to the earlier reports that showed that inorganic nitrogen sources (sodium nitrate, urea, and ammonium nitrate) gave poor production of MCE, but organic nitrogen sources (wheat bran, wheat four, and skim milk powder) favored good enzyme yield (Thakur et al. 1990; Merheb-Dini et al. 2010, 2012; Alecrim et al. 2015). Depending on the peptide nature and level, MCE synthesis may be induced or repressed by proteinaceous materials. It is noted that the nitrogen sources used in the seed culture in this study was also complex organic compound (soyabean) that might explain the ample MCE production by *B. amyloliquifaciens*.

Time course for the MCA production by *B. amyloliquifaciens* was studied in the liquid-state fermentation. The maximum enzyme production and MCA/PA ratio were obtained after 24 h of incubation, and it is obvious that prolonged incubation time after 24 h decreases the MCA production (Fig. 4). However, the reason(s) for the decline remains to be investigated. Although similar results of fast MCE production by *B. subtilis* (natto), Takahashi and *Thermomucor indicae* were observed by Wu et al. (2013) and Merheb-Dini et al. (2012). However, optimal production of *Nocardiopsis* sp. and *Mucor circinelloides* was observed after 56 h and 5 days, respectively (Thakur et al. 1990).

Effect of initial pH of the medium on the MCE production has been illustrated. The MCE production by *Bacillus licheniformis*, *A. rouxii* (Yu and Chou 2005) and *Bacillus amyloliquifaciens* (Zhang et al. 2013) is the highest in the medium with an initial pH value at 7 and 7.57, respectively. While maximum MCE production by *Fusarium subglutinans* (Ghareib et al. 2001) and *Bacillus subtilis* (Dutt et al. 2009) were reported with an initial

pH of 6.0. It was clear from Fig. 5 that *B. amyloliquifaciens* SP1 may also capable of producing alkaline protease (1690 µg/ml/min at pH 8.0), but at pH 6.0, higher amount of MCA/PA was observed. The observations shown in this study along with the reports of previous investigators suggest that the optimal initial pH, i.e., 6.0 of the medium for MCE production may vary depending on the culture medium and the test organism (Fig. 5).

The enzyme production by *B. amyloliquifaciens* at 25–50 °C temperature range revealed that there is an increase in MCA production from 25 °C (105 SU/ml) to 30 °C (160 SU/ml) with corresponding increase in MCA/PA ratio (Fig. 6). The optimal MCE production temperature found in the present study is in contrast with those of previous reports in that it was 30 °C for *A. rouxii* (Yu and Chou 2005), *Mucor* J20 (Hosoi and Kiuchi 2004) and 37 °C for *B. amyloliquifaciens* (Zhang et al. 2013), while it was 42 °C for *M. miehei* (Thakur et al. 1990), and sudden decrease was observed with further increase in temperature.

Inoculum size is the key factor that strongly influences microbial growth and activity in submerged fermentation. The results represented in Fig. 7 showed that the optimum MCE production (142 SU/ml) with low proteolysis, i.e., 830 µg/ml/min, is obtained when the inoculum size is 1 %. However, optimal MCE production of *Bacillus subtilis* was observed with 3 % inoculum size (Dutt et al. 2009). A decrease in enzyme production was noted when the inoculum size increased, which is due to the shortage of nutrients available for the larger biomass and faster growth of the culture (Sathya et al. 2009).

## Conclusion

After exploring rhizosphere of apple trees of trans Himalayas, 76 protease producing isolates from RS and ER of apple trees were obtained. In conclusion, this investigation revealed a potential MCA producer, i.e., *B. amyloliquifaciens*, and its operational and nutritional factors have been studied for the production of MCE. High MCA (142 SU/ml) and MCA/PA ratio can be obtained when the bacteria was cultivated in the medium containing sucrose as C-source (0.1 %) and soyabean as N-source (0.25 %) at pH 6.0 and 30 °C for 24 h at 1 % inoculum size. These optimized conditions resulted in a 1.9-fold increase in the production of the milk-clotting enzyme. Hence, this high amount of MCA and MCA/PA ratio insures its usefulness as new source of milk coagulant for cheese making.

## Authors' contributions

SG carried out all the experimental work. AW and AC helped in the analysis of data. CKS guided all research work and participated in the design of study. All authors read and approved the final manuscript.

## Author details

<sup>1</sup> Department of Microbiology, Kurukshetra University, Kurukshetra, Haryana, India. <sup>2</sup> Department of Microbiology, DAV University, Jalandhar, Punjab 144012, India. <sup>3</sup> Department of Basic Sciences (Microbiology Section), Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan, HP 173230, India.

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## Competing interests

The authors declare that they have no competing interests.

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