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RESEARCH ARTICLE

MILK-CLOTTING PROTEASE ENZYME PRODUCING BACTERIA FROM SOILS IN ETHIOPIA

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ABSTRACT: Rennet (acidic proteases) are used to be traditionally extracted from animals as commercial sources of milk clotting (MC) enzymes for cheese production. However, the ever increasing demand of these enzymes necessitates search for alternative sources from fungi and bacteria. In this study, 49 bacterial strains were screened for milk-clotting (MCA) and protease (PA) activities *in vitro* using plate assay technique and further studied under Solid-state fermentation (SSF) and submerged fermentation (SmF). The result showed that 14 (29%) bacterial strains produced MCA on Skim milk agar forming clear zone diameter between 5.25 mm and 21 mm. Most of these MC bacterial strains were identified into the genus *Bacillus*; showing 100% sequence similarity with *Bacillus tequilensis* KCTC 13622, *Bacillus subtilis* subsp. *subtilis* NCBI 3610, *Bacillus paramycoides* NH24A2, *Bacillus siamensis* KCTC 13613. All of the strains induced MCA on SmF, and only 11 (79%) of the strains produced the enzyme under SSF. The strains produced enzymes on SmF ranging from 133.33 U/mL to 480 U/mL with MCA: PA ratio of 0.1–0.36. The MCA significantly increased during culture profiling upon partially optimized conditions with a maximum MCA production of 2533 U/mL and MCA/PA ratio of 14.05 recorded from *Bacillus subtilis* SMDFS 2B, which is a remarkable characteristic in the selection of commercially important rennet enzyme. Thus, *Bacillus subtilis* SMDFS 2B, together with *B. siamensis* 29B, can be further studied for enzyme purification under different optimization conditions to fully realize their potential for rennet enzyme production.

Key words/phrases: *Bacillus*, Milk-clotting activity, Milk-clotting protease, Submerged fermentation.

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INTRODUCTION

Milk-clotting is the basic step in the production of more than 2000 cheese varieties produced throughout the world (Nasr *et al*., 2016). These cheese products are categorized into three families namely; rennet coagulated, acid coagulated, and a combination of heat and acid coagulated cheese. Traditionally calf rennet, the milk-clotting enzyme obtained from the fourth stomach of suckling calves is the most widely used coagulant in cheese making all over the world (Amer *et al*., 2015).

The ever-increasing cheese production worldwide, the decrease in the supply of calf rennet, and ethical issues necessitated the search for other sources (Amer *et al*., 2015). Although various animals, and plants and can be alternative sources as milk-coagulants, microbial sources can be best rennin substitutes because of their rapid growth and inexpensive growth substrates (Dutt *et al*., 2008).

A search for microorganisms as substitute for rennet production has been started since the 1970s (Arima *et al*., 1970). At the beginning, the search focused on specific fungal groups such as *Mucor* sp. and *Aspergillus* sp. Today, various fungal species are known as important sources for aspartic proteases for most of cheese making enzymes (Baskar *et al*., 2014).

Apart from the fungi, bacteria are also considered as workhorses for the production of a wide range of biotechnologically important enzymes. They are more promising for they are diverse, and can be easily genetically improved to produce cheaper enzymes (El-Tanboly *et al*., 2013). *Bacillus* sp. is one of the most investigated microbial groups that can produce and secrete large quantities of extracellular enzymes.

Several *Bacillus* strains like *Bacillus licheniformis* (Akcan, 2012), *Bacillus subtilis* (Dutt *et al*., 2009), *Bacillus amyloliquefaciens* (Guleria *et al*., 2016), *Bacillus sphaericus* (El-bendary *et al*., 2007), and *Bacillus subtilis natto* (Wu *et al*., 2013) have been reported as the most effective producers of milk clotting enzymes. At present, microbial rennet is used for one-third of all the cheese consumed worldwide (Baskar *et al*., 2014), and is popular for the production of cheddar and cream cheese (Hang *et al*., 2016).

Ethiopia is one of the largest cattle producing countries in Africa. The total milk production in Ethiopia is 3.2 billion litres per annum, with per capita milk consumption of 19 kg/year as compared to 27 kg for other African countries and 100 kg for the world per capita consumption (CSA, 2017; Tadesse Mihret *et al*., 2017). The ever-increasing demand for dairy products

in urban areas is met with imported products worth about 1,548,000 USD from 2005–2009 (Zelalem Yilma *et al*., 2011).

All these shortcomings notwithstanding, there is a great potential for the development of the dairy industry including modern cheese production using rennet enzymes. The newly expanding dairy industry in Ethiopia is using commercial rennet enzymes imported from abroad with high prices for ripening and speedy processing of the product. Fekadu Alemu (2015) screened protease enzyme-producing bacteria from cheese on milk agar medium that have good potential in the production of protease enzymes for various activities. Daniel Yimer (2017) also screened various *Bacillus* spp for protease enzyme activity. However, there is still a dearth of information on milk-clotting protease enzymes important for cheese production. This necessitates the search for milk-clotting enzymes from microbes. Hence the present work was initiated to isolate potential bacteria endowed with milk-clotting protease enzymes from soils collected from different parts of the country.

MATERIALS AND METHODS

Sample collection

Five hundred grams of soil (5–10 cm below the surface) were collected aseptically using a sterile polythene bag from the dairy farm area located at different parts of Ethiopia (Fig. 1 and Table 1). Soil samples were sieved (3–4 mm mesh), homogenized and stored at 4°C for further use.

Ten grams of each soil sample were mixed with 90 mL sterile distilled water, homogenizing by agitation for 20 min (Talantikite-Kellil *et al*., 2012). The homogenate were serially diluted to appropriate dilutions (10^5–10^6) from which, 0.1 mL was spread plated on Nutrient Agar media and incubated at 30°C for 48 h. Distinct colonies were subcultured, purified and stored at 4°C.

Primary screening of milk-clotting protease enzyme (MCP)

Primary screening for MCP was tested using Skim Milk Agar (Nestle TM) medium for the production of the clear zone (Saran *et al*., 2007). The detection medium (Skim Milk Agar Medium) was prepared from 20 g of skim milk, 20 g of agar agar each dissolved in 200 mL distilled water and 600 mL of 0.2 M phosphate buffer (K_2HPO_4 and KH_2PO_4) at pH 5.0. Isolates were grown overnight on nutrient broth and inoculated on the Skim-Milk Agar medium and incubated at 30°C for 48 h. The plates were examined for the formation of the clearing zone around colonies.

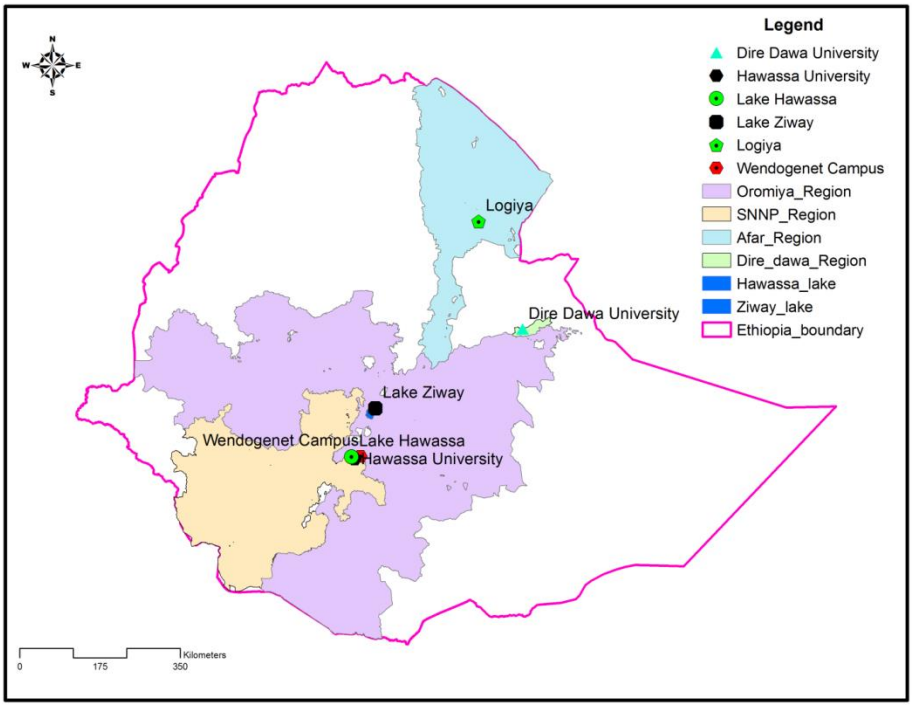

Fig. 1. Map of sample collection sites.

Table 1. Bacterial isolates recovered from different sampling sites.

S. No	Site	Type of sample	Number of samples	Number of isolates
1.	Hawassa	Soil and dung	3	9
2.	Wondogenet	Soil and dung	3	8
3.	Ziway	Soil	3	4
4.	Semera	Soil	3	15
5.	Dire Dawa	Soil	3	13
6.	Total	-	15	49

Characterization of bacteria

The milk-clotting protease enzyme-producing bacteria were characterized based on morphological and biochemical characteristics; Gram staining, catalase test, spore staining and API 50 CHB/E identification kits (Biomerieux, France) (Aruwa and Olatope, 2015). With regard to genomic analysis, selected bacterial isolates (14) were cultured in LB Broth with 1% glucose overnight at 37°C and 200 RPM. They were harvested by centrifugation at 4000×g for 30 min and the genomic DNA (gDNA) was extracted using Dneasy plant mini kit (50) QIAGEN. The DNA was finally suspended in 100 µl of elution buffer and quantified using NANO Drop. The total genomic DNA was kept at -20°C before use.

Species-level identification of strain was conducted by amplifying 16S *rRNA* gene using BC1 and BC2 primers. PCR was carried out in 25 µl reaction containing 2 µl of template DNA, 1.25 µl of each primer FW (5′-GCAAGTCGAGCGGACAGATGGGAGC-3′) and reverse primer RV (5′-AACTCTCGTGGTGTGACGGGCGGTG-3′), 0.5 µl of 10 mMdNTPs and 0.25 µl Phusion polymerase (Gene Bangalore) in 5 µl of 5 × Phusion buffer. Reaction was cycled 36 times at 98°C for 30 sec for initial denaturation, 98°C for 10 sec for denaturation, 58°C for 30 sec for annealing, 72°C for 1:30 min for extension and at 72°C for 7 min for final extension. The PCR products were analyzed on 1% agarose gel in 1 × TAE buffer, run at 100 V for 1 h (Guleria *et al*., 2016).

From the amplified DNA, 15 µl of DNA was run on 1% agarose gel (#A8312, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) with 1X TPE buffer [stock concentration, 10X: 1 M Tris base (#A4281) 20 mMEDTA (#A3562), 225 mM phosphoric acid (#A0989) all products of Applichem GmbH, Darmstadt, Germany] at 85 V, 400 amps for 40 minutes. The gel was stained in ethidium bromide solution and viewed using the Gel documentation system. Amplified PCR products were purified using a PCR purification kit following the manufacturer's instruction and sequenced at Eurofins Genomics, Germany. The sequence was aligned with corresponding sequences of 16S *rRNA* from the EZBioCloud database (Yoon *et al*., 2017). Phylogenetic analysis of the sequences was performed using MEGA 7 software. The phylogenetic trees were inferred using the neighbour-joining method and bootstrap (1000 replicates) analyses. The evolutionary distances were computed using the Maximum Composite Likelihood method (Gontia-Mishra *et al*., 2013).

Secondary screening for enzyme activity using solid-state and submerged fermentation

For submerged fermentation (SmF), the bacterial strains were grown overnight in Nutrient broth and 3% inoculum size was inoculated to the culture medium (50 mL) containing (g/L): glucose, 16.2; wheat bran, 30; NaCl, 5; $MgSO_4 \cdot 7H_2O$, 5; KH_2PO_4, 2; and $CaCO_3$, 3 and pH 5.2 in Erlenmeyer flask (250 mL). They were incubated at 150 rpm for 96 h in an orbital shaker at ambient temperature (Ding *et al*., 2012).

For solid-state fermentation (SSF), a 3% inoculum size was inoculated into a culture media in 250 mL Erlenmeyer flasks. The medium contained;10 g wheat bran moistened with 30 mL of phosphate buffer (0.01 M, pH 6.0; 0.25% w/v casein) (Dutt *et al*., 2009). The flasks were incubated at 35°C for

72 h.

Enzyme extraction

Enzyme extraction was undertaken according to Garcia *et al*. (2005). Samples were collected and filtered through Whatman No. 1 filter paper and subsequently centrifuged at 10000 rpm for 10 min. In the case of Solid-state fermentation (SSF), samples were dispersed in 100 mL (1:10 ratio of Bran - solvent w/v) of distilled water, and vigorously shaken on a rotary shaker (MaxQ 2000 Open-Air Platform Shaker, Thermo fisher scientific, USA) at 240 rpm at a room temperature for 40 min and filtered by cotton cloth. The filtrate was then centrifuged (Heraeus Pico17/21 centrifuge, Thermo Electron Led, Germany) at 10000 rpm for 10 min at 4°C. The supernatant was used as a crude enzyme.

Assay for milk-clotting activity

The milk-clotting activity of the enzyme was undertaken according to Arima *et al*. (1970). Accordingly, 0.1 mL of the crude enzyme was added to 1 mL of reconstituted skim milk (Nestle TM) in 10 mL test tube pre-incubated at 35°C for 10 min. Reconstituted skim milk (Nestle TM) solution consisted of 10 g dry skim milk/100 mL, 0.01 M $CaCl_2$ (AppliChemTM). The appearance of the first clotting flakes was visually evaluated and quantified in terms of Soxhlet units (SU). The endpoint was recorded when discrete particles were discernible. The clotting time T (s), the period of time starting from the addition of crude enzyme to the appearance of the first clots and the clotting activity was calculated using the following formula:

$SU = (2400 * 5 * D) / (T * 0.5)$

Where T = clotting time (s) and D = dilution of crude enzyme

One SU is expressed as the quantity of enzyme required to clot 1 ml of a solution comprising 0.1 g skim milk powder and 0.01 M calcium chloride at 35°C within 40 min.

Assay of protease activity

The proteolytic activity was assayed according to Arima *et al*. (1970). Thus, 0.5 ml of the enzyme extract was added to 2.5 mL of 1% (w/v) soluble casein in 20 mM potassium phosphate buffer at pH 6.5, and the mixture was incubated in a water bath at 35°C for 10 min. After having added 2.5 mL of 0.44 M trichloroacetic acid to terminate the reaction, the mixture was filtered through Whatman No.1 (90 mm) filter paper. The filtrate was then mixed with (1 mL) volume of three times diluted 2 N Folin/phenol reagent

and 2.5 mL of 0.55 M sodium carbonate solutions and incubated at 35°C for 20 min to detect colour development and measure optical density (OD) with spectrophotometer (UV-VIS, Liantrinsat, and Model-CF728YW-UK) at 660 nm. One unit (1 U) of enzyme activity was defined as the amount of enzyme that liberated 1 µg of tyrosine per 1 mL in 1 min.

$$PA\ (U/mL) = \frac{\mu Tyr * V_t}{V_s * T * V_a}$$

Whereas PA is Protease activity; µTyr is µmole of tyrosine equivalent released; V_t is the total volume of assay in mL (5 ml of substrate plus 1 ml of Enzyme plus 5 ml of TCA); V_s is the sample volume (the volume of protease used for assay in mL); T is reaction time (time of incubation in minutes, 10 min); and V_a is the volume of assayed (the final volume of the product used in calorimetric determination) (Cupp-Enyard, 2008).

Cultivation profile of the selected bacterial species

The cultivation profiles of the selected milk clotting (MCA) and protease producing (PA) bacterial strains were determined using standard media ((g/L): glucose, 16.2; wheat bran, 30; NaCl, 5; $MgSO_4 \cdot 7H_2O$, 5; KH_2PO_4, 2; and $CaCO_3$, 3 and pH 5.2) at 35°C and 150 rpm in incubator shaker for 96 h under automated laboratory (Downstream Processing Laboratory), Department of Life Sciences and Chemistry, Jacobs University, Bremen, Germany. The crude enzyme was extracted in 24 h interval for 4 days.

Determination of soluble carbohydrate and protein

The total protein of the crude extract was determined using Bicinchoninic acid (BCA) methods (Walker, 2002). The working reagent was prepared by mixing 24.5 mL of BCA (Thermo ScientificTM) reagent A with 0.5 mL BCATM Reagent B into 50 mL beaker. The protein content was calorimetrically determined (Eppendorf Biophotometer, Model #61318, Marshal Scientific, Germany) at 562 nm against a standard curve prepared from known concentrations of bovine serum albumin (BSA).

Total soluble sugars of the crude extract were determined using the dinitrosalicyclic calorimetric method. The absorbance was recorded using a spectrophotometer (Eppendorf Biophotometer, Model #61318, Marshal Scientific, Germany) at 562 nm with glucose as standard (Gusakov *et al*., 2011).

Data analysis

Data analyses were performed using SAS software version 9 (Inc. Cary NC USA). The experiments were carried out in duplicate. Analysis of variance (ANOVA) and means comparisons was done by Duncan's multiple range tests.

RESULTS

Primary screening for protease producing bacteria by plate assay techniques

A total of 49 bacterial isolates were recovered from different soil samples, of which, 14 isolates (29%) showed clearing zone ranging from 5.25 up to 21 mm (Fig. 2 and Table 2). All the 14 bacterial isolates were gram-positive, rod-shaped, catalase-positive and produced spores. They displayed cream colour, irregular, undulate, wrinkled colony characteristics with 0.5–1 mm in diameter (data not shown). Based on their biochemical and genetic characters, all the 14 bacterial isolates were classified under the genus *Bacillus*. The molecular characterization revealed that 7 isolates showed 100% similarity with *Bacillus tequilensis*, whereas 4 isolates, 2 isolates and one isolate showed ≈100% similarity with *B. subtilis B. siamensis* and *B. paramycoides* (Fig. 3 and Table 3).

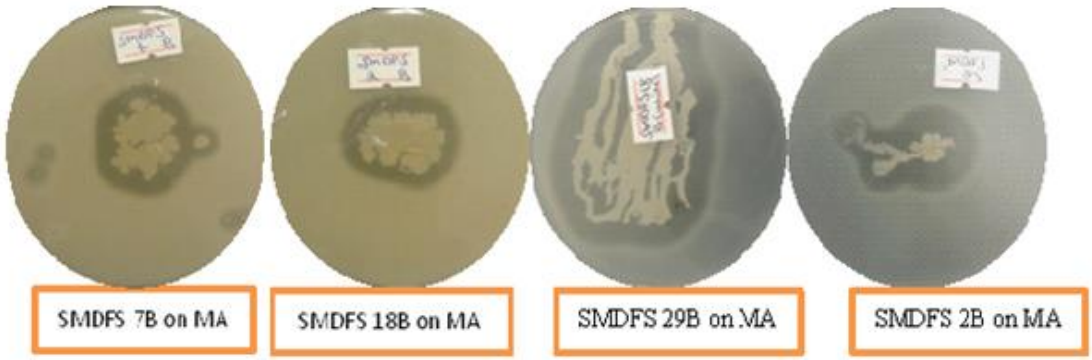

Fig. 2. Primary screening for protease enzyme production from bacteria on Skim milk Agar.

Table 2. Clear zone diameters of bacterial isolates that were grown on Skim milk Agar (SMA) at 30°C for 48 h.

No.	Isolates	Clear zone diameter (mm) Mean±SD
1.	SMDFS 2B	11.00±1.00bc
2.	SMDFS 3B	8.00±1.00ced
3.	SMDFS 5B	7.00±1.50ced
4.	SMDFS 6B	6.00±1.00de
5.	SMDFS 7B	11.00±2.00bc
6.	SMDFS 8B	6.00±2.50de
7.	SMDFS 11B	10.00±1.00bcd
8.	SMDFS 12B	5.25±0.25e
9.	SMDFS 14B	9.00±3.00ced
10.	SMDFS 15B	6.00±1.00de
11.	SMDFS 16B	6.00±0.00de
12.	SMDFS 18B	9.75±1.75bcd
13.	SMDFS 29B	21.00±1.00a
14.	DRDFS 13B	13.50±0.50b

SMA: Skim-milk Agar; CZD: Clear zone diameter, SD: standard deviation, Mean: is average of two measurements, Different letters (a, b, c,d) designate significantly different means as determined by Duncan multiple mean comparison test ($P<0.05$).

Table 3. Identification of the milk-clotting protease producing bacteria using genetic characteristics.

Isolates	Accession Number	Similarity	Species
SMDFS 2B	MN715837	100	*Bacillus subtilis* subsp. *subtilis* NCBI 3610
SMDFS 3B	MN715838	100	*Bacillus tequilensis* KCTC 13622
SMDFS 6B	MN715840	100	*Bacillus subtilis* subsp. subtilis NCIB 3610
SMDFS 7B	MN715841	100	*Bacillus tequilensis* KCTC 13622
SMDFS 8B	MN715842	100	*Bacillus tequilensis* KCTC 13622
SMDFS 11B	MN715843	100	*Bacillus tequilensis* KCTC 13622
SMDFS 12B	MN715844	100	*Bacillus subtilis* subsp. *subtilis* NCIB 3610
DRDFS 13B	MN715845	100	*Bacillus tequilensis* KCTC 13622
SMDFS 14B	MN715846	99.89	*Bacillus subtilis* subsp. *subtilis* NCIB 3610
SMDFS 16B	MN715848	100	*Bacillus tequilensis* KCTC 13622
SMDFS 18B	MN715849	100	*Bacillus tequilensis* KCTC 13622
SMDFS 5B	MN715839	100	*Bacillus paramycoides* NH24A2
SMDFS 15B	MN715847	100	*Bacillus siamensis* KCTC 13613
SMDFS 29B	MN715850	100	*Bacillus siamensis* KCTC 13613

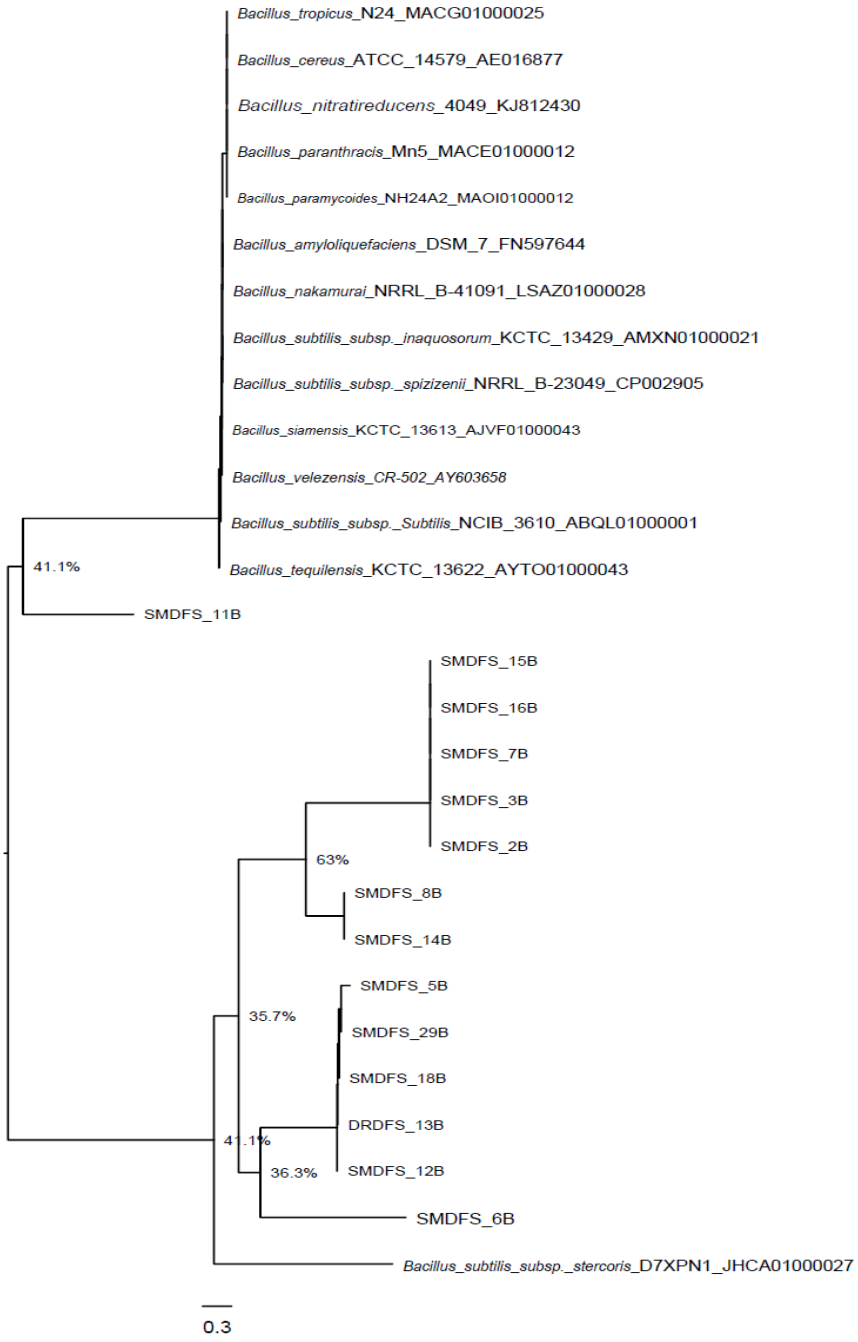

Fig. 3. Phylogenetic tree of 14 bacterial strains.

Secondary screening of milk-clotting protease production using SmF and SSF

The bacterial strains were further checked for milk-clotting (MCA) and Protease (PA) activities under submerged (Sm) and solid-state (SS) Fermentations (Table 4). All strains induced MCA under SmF, and all, but three strains; *B. subtilis* SMDFS 6B, *B. tequilensis* SMDFS 3B and *B. paramycoides* SMDFS 5B showed MCA under SSF. They performed better in MCA on SmF fermentation systems ranging from 133.33 U/mL to 480 U/mL and PA ranging from 425.28 up to 1521.6 U/mL with the highest MCA/PA ratio of 0.36 and the lowest ratio of MCA/PA of 0.10. Although most strains produced protease enzymes under SSF, they showed the lowest MCA/PA activity of 0.01–0.09. However, *B. siamensis* 29B produced the highest activity of 381 U/mL with MCA/PA ratio of 0.4 under solid-state fermentation

The bacterial strains also showed significant differences in MCA amongst species ranging from the highest range of 338.3 U/mL up to 480 U/mL produced by *B. subtilis*, followed by *B. tequilensis* with a range of 100.7 U/mL up to 436.51. They also showed a similar pattern of MCA/PA ratio ranging from 0.27–0.36 by *B. subtilis* and 0.1–0.36 by *B. tequilensis*.

Table 4. MCA and Protease activity of bacterial strain in SmF (incubated at 150 RPM for 96 h in an orbital shaker) and SSF (incubated at 35°C for 72 h) at ambient temperature.

Strain	**SmF**			**SSF**		
	MCA (U/mL) Mean ± SD	**PA (U/mL) Mean ± SD**	**Ratio MCA/PA**	**MCA(U/mL) Mean SD**	**PA (U/mL) Mean ± SD**	**Ratio (MCA/PA)**
B. subtilis 2B	480.77±19.23a	1330.30±78.44ab	0.36	57.14±0.00d	974.07±8.75d	0.06
B. subtilis 6B	429.81±23.03b	1521.60±53.16°	0.28	NDf	1003.89±6.16d	ND
B. subtilis 12B	338.30±9.53c	1227.55±11.35b	0.28	25.20±0.20e	676.82±37.60e	0.04
B. subtilis 14B	363.97±11.03c	1347.16±35.01ab	0.27	56.61±0.53d	1341.32±72.61b	0.04
B. tequilensis 3B	200.35±8.35e	780.55±8.43c	0.26	NDf	229.50±25.28f	ND
B. tequilensis 7B	436.51±7.94b	1222.04±186.71b	0.36	98.98±1.02b	1779.25±4.86a	0.06
B. tequilensis 8B	250.98±15.69d	1317.66±33.39ab	0.19	79.34±0.66c	1247.00±244.73bc	0.06
B. tequilensis 11B	344.62±24.62c	1437.60±86.55ab	0.24	7.99±0.10f	593.19±12.32e	0.01
B. tequilensis 13B	133.44±3.71f	492.38±50.24d	0.27	97.62±2.38b	1085.25±35.01cd	0.09
B. tequilensis 16B	358.93±16.07c	1252.51±7.13b	0.29	99.38±0.62b	1604.86±25.61a	0.06
B. tequilensis 18B	100.70±8.39g	425.28±18.15d	0.24	98.78±1.22b	1057.05±63.21cd	0.09
B. siamensis 15B	124.39±1.94fg	1202.26±42.46b	0.10	9.45±0.15f	892.71.±9.08d	0.01
B. siamensis 29B	201.40±16.78e	888.81±90.76c	0.23	381.82±18.18a	952.02±21.07d	0.40
B. paramycoides 5B	137.15±22.85f	732.57±17.50c	0.19	NDf	561.75±3.57e	0.00

ND: Not determined, MCA: Milk-clotting activity (U/mL), PA: protease activity (U/mL), Ratio: is the ratio of MCA/PA, SD: standard deviation, Mean: is average of two measurements, Different letters (a, b, c, d..) designate significantly different means as determined by Duncan multiple mean comparison test ($P<0.05$).

Cultivation profile of potential bacterial species in submerged fermentation

The cultivation profile of the selected bacterial species was studied under partially optimized conditions in submerged fermentation. All strains showed significantly higher milk-clotting activity of which, *B. subtilis* SMDFS 2B displayed the highest milk-clotting activity of 2533 U/mL with a MCA/PA ratio of 14.05 upon the third day of incubation. In fact, all other strains also showed an increase in MCA/PA ranging from 4.06 up to 10.26 after 4 days of incubation. This indicates a 10–40 fold increase in the milk-clotting per protease activity compared to the previous screening (Table 5).

The strains also showed slight, but not significant variations in the pH of the culture during the process of fermentation compared to the blank. Thus, *B. subtilis* SMDFS 2B reduced the pH from 5.93 to pH 5.34; whereas *B. subtilis* SMDFS 14B slightly raised the pH to 6.63. This shows that the optimum activity of the enzyme derived from the *Bacillus* spp was within the acidic pH of 5.5 to 6.2. On the other hand, the concentrations of soluble carbohydrates showed a five-fold decrease in 4 days of fermentation; and with a concomitant sharp (2–4 fold) increase in total protein, and specific activity at their optimum incubation times.

DISCUSSION

In this study, we screened milk-clotting protease-producing bacteria on the skim-milk agar medium and reported that 29% of the strains (14 strains) were capable of producing the enzymes. The selected strains also differed in their effectiveness to produce enzymes evident from the diameter of the clearing zone on the medium ranging from 5.25 up to 29 mm. Rupali (2015) also reported a similar pattern of clearing zone formation of 11–29 mm by protease-producing bacteria isolated from soil samples in India.

Based on genetic characteristics, our strains showed 99.89–100% similarity with four species; *Bacillus tequilensis*, *Bacillus subtilis*, *Bacillus paramycoides*, and *Bacillus siamensis* of which strains from *Bacillus tequilensis* were dominant followed by *B. subtilis*. Many bacteria, especially species belonging to *Bacillus*, are well known to produce a variety of extracellular enzymes, with a wide range of industrial applications including rennet (milk-clotting enzymes) (Wim, 2013).

Table 5. Cultivation profile of six potential bacterial isolates in submerged fermentation incubated at 35°C and 150 rpm in incubator shaker for 96 h (ND: Not determined, MCA: Milk-clotting activity (U/mL), PA: protease activity (U/mL), Ratio: is the ratio of MCA/PA, SD: standard deviation, Mean: is average of two measurements. Different letters (a, b, c, d) designate significantly different means as determined by Duncan multiple mean comparison test (P<0.05).

Isolates	Day	pH of extract Mean±SD	Total carbohydrate (mg/mL) Mean±SD	Total protein (mg/mL)	MCA (UmL) Mean±SD	PA (U/mL) Mean±SD	Specific activity MCA(U/mg)	Ratio MCA/PA
Blank		5.96±0.01	14.48±0.21	6.32±1.39	NDl	47.05±2.72gfh	-	-
B. subtilis SMDFS 2B	1	5.91±0.00	8.15±0.02	12.74±0.75	579.07±20.93de	107.44±0.19def	45.45	5.39
	2	5.66±0.01	7.28±0.00	23.85±1.80	2000.00±0.00b	142.88±1.46cde	83.86	14.00
	3	5.47±0.00	6.56±0.09	28.68±0.30	2533.34±1.33a	180.25±0.77c	88.33	14.05
	4	5.34±0.00	5.56±1.01	17.67±0.33	873.02±15.88c	140.80±6.43cde	49.41	6.20
B. subtilis SMDFS 6B	1	5.31±0.00	7.25±0.19	5.28±0.50	NDl	65.21±0.74fgh	ND	ND
	2	5.12±0.00	2.36±0.94	9.99±1.45	29.07±0.03kl	97.19±0.79efg	2.91	0.3
	3	6.11±0.01	1.78±0.06	11.51±0.02	46.56±0.27kl	249.81±4.01ab	4.05	0.19
	4	6.12±0.00	1.26±0.14	15.12±0.56	589.16±10.85de	128.86±1.01cde	38.96	4.57
B. tequilensis SMDFS 7B	1	5.55±0.01	9.75±0.12	12.52±0.05	115.25±0.42ijk	158.52±3.23cd	9.21	0.73
	2	5.61±0.03	5.55±0.32	13.74±2.09	202.53±0.00ghi	238.10±34.67b	14.74	0.85
	3	5.72±0.00	5.11±0.23	28.30±2.70	273.53±2.34g	156.03±1.12cde	9.66	1.75
	4	5.69±0.00	2.07±0.03	31.07±3.38	285.76±3.41g	70.33±0.59fgh	9.19	4.06
Blank		5.99±0.00	16.34±0.28	7.13±0.99	NDl	23.29±0.27h	ND	ND
B. tequilensis SMDFS 12B	1	6.10±0.00	12.44±0.49	17.26±0.45	157.55±5.17hij	107.44±8.59def	9.12	1.47
	2	5.51±0.00	7.20±0.08	18.44±0.76	461.93±13.33f	204.69±7.26ab	25.05	2.26
	3	5.86±0.00	5.81±0.04	15.51±1.71	259.46±0.00g	87.44±0.21efg	16.73	2.97
	4	5.48±0.01	3.85±0.05	11.27±1.01	270.44±2.28g	96.73±0.24fgh	23.99	2.80
B. subtilis SMDFS 14B	1	5.93±0.00	11.44±0.17	11.80±0.21	400.00±0.00f	107.42±8.19def	33.90	3.72
	2	6.29±0.00	11.27±0.26	15.01±1.26	545.53±6.20e	294.35±3.20a	36.34	1.85
	3	6.63±0.00	11.23±0.08	16.33±1.80	548.74±9.41e	162.88±75.79cd	33.56	3.37
	4	6.16±0.00	7.70±0.39	13.66±0.09	658.03±18.03d	64.12±0.89fgh	48.17	10.26
B. tequilensis SMDFS 16B	1	6.12±0.00	8.77±0.06	6.19±0.60	13.56±0.23l	157.60±9.44cd	2.19	0.09
	2	6.07±0.01	8.19±0.16	11.94±1.03	82.41±0.22jkl	235.70±12.84b	6.90	0.35
	3	5.86±0.01	6.02±0.01	15.82±4.86	202.54±0.86ghi	48.12±2.40gfh	12.80	4.21
	4	5.14±0.01	4.71±0.16	9.96±0.34	231.89±1.12gh	31.52±1.45h	23.28	7.36

Several studies showed that most promising bacterial species implicated with rennet production were isolated from soil, and belong to the genus *Bacillus*; *B. coagulans*, *B. stearothermophilus* and *B. lichenformis*, *B. cereus*, *B. circulans*, *B. laterosporus*, *B. pumilis*, *B. brevis*, *B. sphaericus* and *B. macerans* (Sidra *et al*., 2006), *B. amyloliquefaciens* (Guleria *et al*., 2016), *B. licheniformis*, *B. subtilis* and *B. subtilis* natto (Akcan, 2012; Ding *et al*., 2011).

Although large clearing on the casein medium is a very good indicator of protease production, it does not necessarily corroborate with production under fermentation conditions (Singh and Bajaj, 2015). Thus, several medium components, physiological factors, type of fermentation and operational parameters influence the biochemical behaviour of the microbial strains and subsequent metabolite production pattern.

Apart from that, milk-clotting enzymes are invariably accompanied by producing other proteases. Thus, both milk-clotting activity (MCA) and total proteolytic activity (PA) were recorded to determine the MCA: PA ratio. Thus, the ratio of MCA/PA is a very good indicator for the selection of milk-clotting proteases from the overall protease enzyme production. However, it is desirable to have more milk-clotting activity than proteolysis for cheese production. Low MCA/PA implies the presence of other non-specific proteolytic enzymes that are active after milk coagulation and further degrade casein fractions. This results in in lower cheese yield and bitter flavour due to extensive non-specific peptide bond attack (Yegin *et al*., 2010).

To this end, the milk clotting bacterial strains were tested under SmF and SSF conditions. Thus, the milk-clotting activity recorded in submerged fermentation was higher than solid-state fermentation. This implies SmF performs better than SSF for the production of milk-clotting enzymes from bacteria. Shieh *et al.* (2009) also showed that milk-clotting and proteolytic activities of *Bacillus subtilis* (natto) strains were more than twice effective in MCA/PA ratio under SmF than the ones extracted from SSF. However, our study showed *B. siamensis* SMDFS 29B induced better MCA than PA activity with a ratio of 0.4 compared to the 0.36 ratio of the activity displayed by all other species under solid fermentation.

Although it is established that bacteria and fungi show higher enzyme activities under SmF and SSF, respectively due to various advantages, it is not common to find higher enzyme activity by bacteria under SSF, and by

fungi under SmF, provided that the cultures are agitated to enhance the level of oxygen (Ding *et al*., 2011; Handel and Fraile, 1984; Yegin *et al*., 2011).

The secondary screening with SmF and the enzyme profiling of the bacterial strains gave a better insight on the effectiveness of the operational parameters and the performance of the bacterial strains in relation to milk-clotting activity. Under the circumstances, the bacterial strains induced up to 480.77 U/mL and 2533 U/mL MCA during the secondary screening and partly controlled enzyme profiling, respectively. This was comparable and even higher than those obtained from *B. subtilis* (120.31 U/mL), *B. amyloliquefaciens* SP1 (160 SU/mL) and *B. subtili*s (581.8 U/mL) in submerged fermentation (Dutt *et al*., 2008; Guleria *et al*., 2016; Wehaidya *et al*., 2016). However, the MCA per PA index of 0.36 in the preliminary screening and the 14 fold increase during enzyme profiling was much lower than the ones reported by the same authors MCA/PA of 219–592.

The study also showed that total protein concentration was increased while soluble carbohydrate decreased during the fermentation process. This implies that enzyme production was supported by glucose consumption, which was almost exhausted at the time of maximum enzyme production (Yegin *et al*., 2012). The effect of fermentation time also had a profound effect on the production of milk-clotting protease, and all strains, except *Bacillus subtilis* SMDFS 2B, showed maximum activity upon 4 days of fermentation. This strain also showed the maximum milk-clotting to protease (MCA/PA) activity of 14 which was 2–3 times higher than the other strains. The strains also worked within pH 5.5–6.6 similar to different *Bacillus* sp. characteristics of acidic proteases used for cheese production (Guleria *et al*., 2016).

Several studies showed that one-variable-at-a-time optimization of the process of enzyme production by bacteria is necessary to fully realize the potential of acidic enzyme activity (Ding *et al*., 2011; Shieh *et al*., 2009; Wehaidya *et al*., 2016; Zhang *et al*., 2013). These include optimization under different media, fortification with additional carbon and nitrogen sources, under specific pH and temperature. These, together with enzyme purification, could enhance milk-clotting enzymes and reduce excess production of proteases and narrow down MCA/PA ratio.

CONCLUSION

The present study revealed that submerged fermentation best suits the bacterial species for better milk-clotting activity. The *Bacillus subtilis* SMDFS 2B best performed under submerged fermentation and partially

controlled experiments. *B. siamensis* SMDFS 29B was also an interesting strain performing best under SSF. These two strains have the potential for commercial production provided that they are tested under different optimal nutrient, environmental and operational conditions.

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