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Valorisationof microbial biodiversity: Characterization of a milk-clotting enzyme produced by *Bacillus velezensis FK6A* strain isolated from Algerian dairy farm soil in light of its use in the manufacture of hard cooked cheeses.

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ABSTRACT/RESUME

Abstract: *A strain known as FK6A, which produces a milk-clotting enzyme isolated from the soil of an Algerian dairy factory, was identified as Bacillus velezensis based on morphology and internal transcription spacer sequence. Phylogenetic and sequencing analysis of 16S rDNA gene showed a 100% identity match between the tested strainand the Bacillusvelezensis in the database* ***(Code MH368142).*** *This strain produced an active extracellular protease purified by partial purification using ammonium sulfate fractionation(40–80% saturation) followed by chromatography of molecular exclusion, which revealed the existence of tow active peaks. The optimal activity was observed at pH 5.7; 75°C and 40 mM of $CaCl_2$. The clotting enzyme has been shown to be a metalloprotease inhibited by EDTA. The highly proteolytic activity of the Bacillus velezensis extracts observed on the curds appears a few minutes after the appearance of a firm coagulum reflected by the appearance of an abundant serum.Thus, to solve this problem and to inhibit proteolytic activity, the curds were cooked after brewing during the manufacturing process.*

I. Introduction

Obtaining natural milk coagulants is a challenge, because the supply of rennet is limited compared to the dairy industry's rising demand for this enzyme. Natural coagulants of animal origin are costly, and for religious or dietary purposes, their use has been limited. This scenario has led to increased demand for new rennet replacements, promoting a search for new sources of proteases with coagulant properties [1, 2].

Microbial milk-clotting enzymes (MCE) have certain benefits over those derived from plants or animals, such as lower costs, greater biochemical diversity and easier genetic modification. Some MCE development work has now centered on solid state fungal fermentation such as *Mucor miehei, Rhizomucor pusillus* var., *Aspergillusoryzae* are already widely used in commercial cheese production [1]. Otherwise, as possible rennet alternatives, certain bacteria such as *Bacillus subtilis, Bacillus licheniformis, and Enterococcus faecalis*have been suggested [3]. Singh et al [4] and McGugan[5] suggested that bacterial MCE have contributed to distinguishing flavours in Cheddar cheese

production. In addition, different sources of microorganisms make a variety of cheeses with and organoleptic properties[6, 7]. The aim of this study was to elucidate the nature and characteristics of the novel milk clotting enzyme enzyme produced by FK6A (a soil borne *Bacillus velezensis* strain) The conditions for the fermentation of the solid state and the synthesis of enzymes were investigated and trials for the production of baked cheese are carried out.

II. Material and Methods

II. 1. Microorganism, culture conditions and isolation method

Fifty grammes of soil samples were collected near a dairy factory in the Boumerdes region (about 50 km to the north of Algeria). Under magnetic agitation, a small amount of soil was homogenized with 90 mL sterile physiological water for 20 min to create an initial dilution (10^{-1}). Sample dilutions were made for each sample, and 0.1 mL of the correct 10^{-9} dilution was applied to the plate count agar (PCA) medium containing 2% (w/v) actidione antifungal agent to prevent fungal growth and 12% (w/v) sterilized skimmed milk powder. For the enumeration of the caseolytic strain developing milk clotting protease the plates were incubated at 30°C for 48 h by the presence of a strong halo around the colony. Colonies with distinct differences in morphology such as color (Gram technique), shape, and size were selected and purified using the same medium to streak. All isolates were tested for the development of milk clotting, and microscopically examined before stock preparations. The gram-positive bacterial isolates known as Bacillus have been purified, and the selected strains displaying coagulation activities have been preserved on agar conservation.

II. 2. Identification of FK6Astrain

Talantikite et al [8], recorded the same strain identification method. Under optimized conditions, the PCR products were sequenced by Geno screen (Geno-screen Campus, Institut Pasteur de Lille 1, France) using Sanger technique. Since the sequencing results were collected, the 16S rDNA nucleotide sequence (724 bp sequence) of the strain was deposited in GenBank and matched with the 16S rDNA sequences available in the National Center for Biotechnology Information (NCBI) public databases(http://www.ncbi.nlm.nih.gov/) and LeBibi database (http:/umr5558-sud-str1.univ-lyon1.fr/lebibi/lebibi.cgi) that uses the simple local alignment search software (BLAST).

II. 3. Protease production in shake flask cultures medium

different rheological

II. 3.1. Protease production in shake flask cultures, medium composition and inoculum:

For fermentation, basal medium (pH 6.8) used in the milk clotting protease production, containing (g/L): wheat bran (50), yeast extract (3), glucose (4), casein (2), and Na_2HPO_4 (3). The medium (50 mL) placed in an Erlenmeyer 250 mL flask was inoculated with $15x10^8$ CFU/mL (Mac Farland 4.42) and incubated for 24 h in a control shaker at 30°C on a rotary shaker with agitation (110 rpm). The contents of the incubated Erlenmeyer flasks are first washed onto a gaze band under a sterile host, placed on a funnel, to recover the crude enzyme extract. At 4°C, the filtrate then undergoes centrifugation at 4000 rpm for 30 mi.[9] The supernatant recovered forms the crude enzymatic extract.

II. 4. Analytical procedure

II. 4. 1. Milk clotting activity

Milk clotting activity (MCA) was measured with slight modifications of the procedure described by Shieh et al [10].Skim milk powder was reconstituted by dissolving 12.5 g $CaCl_2$ solution in 100 mL of 0.5 g/L (pH 6.5). The clotting assay was performed by mixing 10 mL of this substrate and 1 mL of sample which is based on the visual assessment of the first clotting flakes' appearance. The clotting activity was expressed as Soxhlet Units (SU). One SU is defined as the amount of enzyme that coagulates 1 mL of a solution that contains 0.12 g skim milk powder and 0.11 mg $CaCl_2$ at 35°C in 40 min. The clotting activity was determined with the following formula:

$$US=2400\times10\times D/T$$

Where: *T*=clotting time and *D*=dilution of crude extract.

II. 4. 2. Proteolytic activity essay

Proteolytic activity was determined using the method reported by Silva et al [11]and Shieh et al [10]. The reaction mixture consisted of 0.4 mL 0.5% casein (Sigma) (w/v) in distilled water, 0.4 mL of 0.2 mol/L acetate buffer at pH5.5, and 0.2 mL of crude enzyme. The reaction occurred in a bath at 35°C and at 30 min, the reaction was stoppedby adding 1 mL of 10% trichloroacetic acid (TCA). 2 mL of the above filtrate was added by 5 mL NaOH (0.28 N) solution and 1.5mL Folin-Ciocalteau phenol solution: water (1:2). After the mixture was kept at 35°C for 15 min, optical density (OD) at 660 nm was measured. The proteolytic activity was expressed in terms of units corresponding to OD_{660nm} (UV-Visible Spectrophometer, model V560, Shimadzu, Japan).

The ratio (R) was determined by dividing the milk clotting activity (MCA) by the proteolytic activity by the formula **R=MCA (SU)/PA (POD_{660nm}).**

II. 4.3. Protein concentration assay

The protein content was determined according to Lowry method [12] using crystalline bovine albumin as standard.

II. 5. Purification of the crude enzymatic extract

II. 5. 1. Partial purification of the milk-clotting enzyme $(NH_4)_2SO_4$ fractionation

The crude enzyme extract is exposed to an ammonium sulphate precipitation at three stage saturation (w/v): 40%, 60%, 80%. According to the 4°C ammonium sulphate precipitation table, 4.86 g, 7.80 g and 11.22 g are added to 20 mL of enzymatic extract. The precipitate collected at 10,000 g at 4°C for 20 min after centrifugation was suspended in the acetate buffer (0.01 M, pH5.5) and dialyzed overnight against repeated adjustments of the same buffering.

II. 5. 2. Size-exclusion chromatography

The concentrated extract was pre-equilibrated with an acetate buffer (0.01 M, pH 5.5) on the Sephadex G-75 column (30 cm x 1.5 cm). The 1.5 mL enzyme fractions were eluted with the same buffer at 36 mL/h and tested for enzyme activity and protein content. To further analysis, active fractions of the enzymes were pooled and lyophilized.

II. 5. 3. Molecular weight determination

The molecular weight of the protease from *B. velezensis*FK6Awas determined upon purification of the active fraction on Sephadex G-75. The column was measured using a mixture of established molecular weight

standard proteins

including BSA (67 kDa), ovalbumin (40kDa), carbonic anhydrase (30kDa), and trypsin (23.8kDa).

II. 5. 4. Effect of inhibitors on enzyme activity

The effect of the enzyme inhibitors was evaluated using the Raposo and Domingos[13]technique. The residual activity was calculated after preincubation of the crude enzyme extract at 37°C for 30 min with inhibitors under the conditions defined by He et al [14] Different protease inhibitors including particularly a metalloprotease inhibitor (ethylene-diaminetetraacetic acid (EDTA) at 5mM, an aspartic protease inhibitor (pepstatin A at 10 µM and 20 µM and 2-mercaptoethanol at 5mM, were tested.

II. 6. Biochemical properties of purified enzyme

II. 6. 1. Optimum pH and temperature

The optimum temperature determined in the 30 to 85°C range was examined at pH 6.3 of the skim milk substratum. The pH effect on the milk coagulation enzyme expressed in relative activity (%) was evaluated by adding 0.75 mL of suitable skim milk buffer solutions to make the substrate at different pH (5.5 to 8).

II. 6. 2. Effect of $CaCl_2$ concentration on milk clotting

The effect of $CaCl_2$ concentration on milk coagulation was measured by increasing concentrations of the solution of calcium chloride (0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, and 0.08 M).

II. 6. 3. Effect of enzyme concentration

The optimum raw extract concentration was determined by observing the shortest time of milk coagulation at 0.2, 0.4, 0.6, 0.8, and 1 mg/mL.

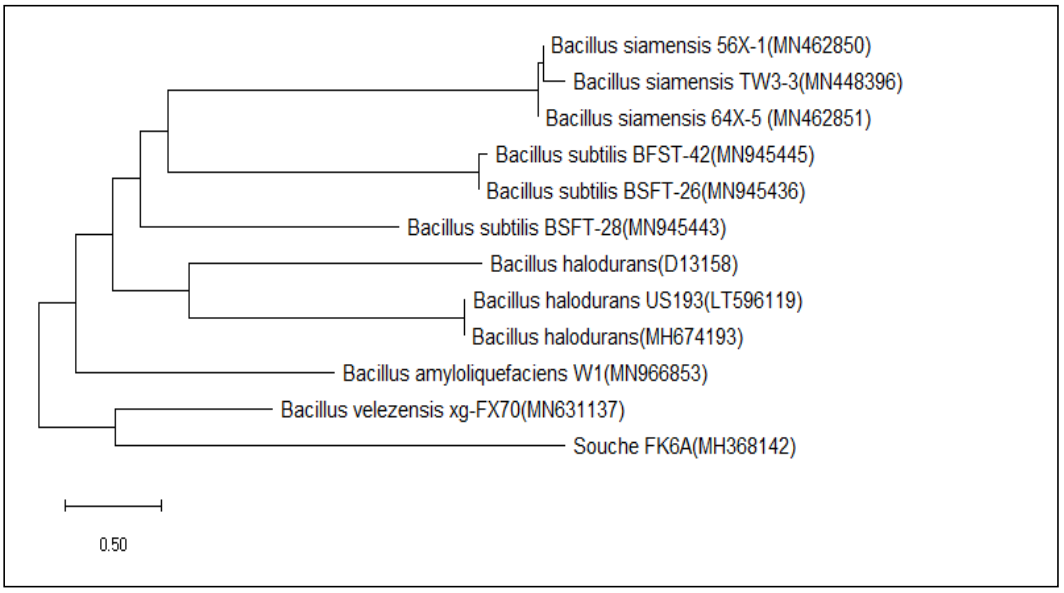
III. Results and Discussion

III. 1. Identification of the strain

For further characterization, the PCR product of strain FK6A was examined by electrophoresis, purified and then sequenced by Geno- screen (Campus de l'Institut Pasteur de Lille, France). In addition to biochemical and morphological identification, the 16S rDNA gene was successfully amplified from the genomic DNA of the strain FK6A **(figure 1a, b).** The nucleotide database has shown 100% homology with *B. velezensis* strain. Therefore, our strain was designated *B. velezensis* FK6A with accession number *MH368142*and the data bases allowed us to elaborate a phylogenetic dendrogram for FK6A and related strains **(figure 2)** according to the Neighbor Joining method by MEGA 6 software.

CGTCCTATCTCTAGGATTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAA
ACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTC
CCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCCTAACACTTAGCACT
CATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGC
GTCAGTTACAGACCAGAGAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCT
ACACGTGGAATTCCACTCTCCTCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACCCTCCCCGGTTGA
GCCGGGGGCTTTCACATCAGACTTAAGAAACCGCCTGCGAGCCCTTTACGCCCAATAATTCCGGACA
ACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTAC
CGTCAAGGTGCCGCCCTATTTGAACGGCACTTGTTCTTCCCTAACAACAGAGCTTTACGATCCGAAAA
CCTTCATCACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCC
TCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACC

Figure 1 (a). *16S rDNA sequence of strain FK6A with query length of 724 bp*



(b) **.** *Phylogenetic tree derived from analysis of 16S rDNA sequence of strain FK6A*

III. 2. Production of raw enzymatic extract

Based on the preliminary study of fermentation conditions, the production profile of enzymatic activity by the strain of *Bacillus velezensis* FK6A was found to be of interest to submerged fermentation and to grow and secrete coagulating enzymes in a short period of time (36 h). After fermentation accompanied by cold centrifugation, a raw enzyme extract wasrecovered by 76.6 mg/mL of protein content. A relatively lower protein concentration (11.14 mg/mL) was obtained from *Bacillus velezensis* FK6A compared to that found from *Bacillus mojavensis* P47 M in a previous study [15]**.**

It should be mentioned also that the content reflects the total protein. Separation of the various proteins during purification will measure the concentration of required coagulating proteins. The raw extract coagulating force is in the range of 122.05 to 3.29 US and appears to be lower compared to *Bacillus amyloliquefaciens D4* (4645.16 US [14]*Bacillus subtilis* B1 (782US) **[7]**), and *Bacillus sp.*P45 (239US) [16].

The proteolytic activity obtained (0.362±0.001) is relatively high, but similar to that obtained from *Bacillus subtilis*varnatto (0.440±0.009) and *Rhizopusoligosporus* (0.392±0.001) by Shieh et al [10]**.** The AC/AP obtained by the REE is also in the order of 338.12 and indicates a high proteolytic activity.

III. 3. Raw enzymatic extract purification and molecular weight estimation

Ammonium sulphate is used in the raw extract for hydrolysis and precipitation of proteins. The

gradient of precipitation used is within the precipitation of saturated enzyme proteins (80%) (**Table 1**) is consistent with that reported by Khan et al [17], Esawy and combet blanc[18] and Won Park et al [19], with enzymatic extracts of *Mucorpusillus* , *Bacillus licheniformis* 5A1 and *Bacillus sp* k-295G-7 respectively.

On Sephadex filtration gel G75, the chromatographic profile of the raw coagulating extract shows three (3) distinct absorption peaks (**Figure 2**). Clotting tests have confirmed that the first peak is inactive, while the 2nd and 3rd peaks havean enzymatic activity from the 10th to the 18th fraction, with 17.6 mL as total elutionvolume. The other fractions, as reported in *Bacillus amyloliquefaciens* by Ding et al [20] and in *Bacillus sphaericus*[21], represent non-active proteins concentration range of 40 to 80%. Total probably derived from the fermentation environment. The purification parameter results were presentedin **Table 2**. The fraction coagulating force is in the range of 16.45US, coagulating frequency is 0.068UP, and protein content is 76.6 mg/ml**.** Gel filtration chromatography is well developed as an effective technique for estimating the molecular mass of proteins.

Tableau 1.*Precipitation of the crude enzyme extract with ammonium sulphate*

Saturation rate $(NH_4)_2SO_4$ (%)	**Enzyme activity in supernatant (SU)**	**Enzyme activity in pellet (SU)**
40	17,51	10,2
60	15,13	18,7
80	-	21,76

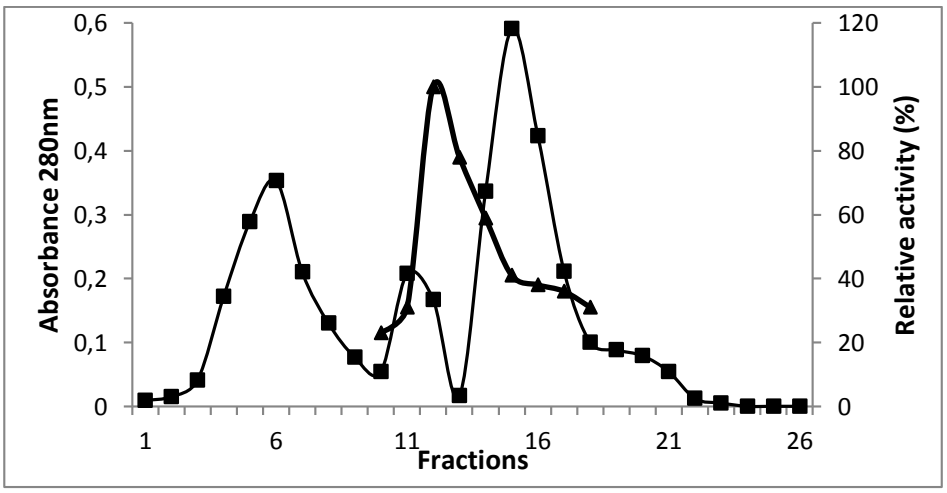


Figure 2. *Size-exclusion chromatography through Sephadx G-75 of a crude extract of B. velezensis FK6A strain after precipitation with 40- 80% saturated ammonium sulphate (Column 1,5 × 30 cm, Acetate buffer 0,01 M, pH 5,5 , Flow rate: 36 ml/h, fraction of 2,2 ml) (■ Absorbance 280 nm, ▲ Relative activity (%)*

Table 2 .*Purification steps of milk clotting enzyme from B. velezensisi FK6A*

Purification steps	Protein (mg/ml)	Total activity (SU)	Specific activity (U/mg protein)	Purification fold	Recovery (%)
Crude extract	76.6	122.05	1.59	1	100
$(NH_4)_2SO_4$ (40% - 80%).	2.34	30.76	13.14	8.26	25.20
Size exclusion Sephadex G-75	0.268	16.45	61.38	38.60	13.48

Our findings (**Figure 3**) indicate about 46 kDa of molecular weight assessed by molecular exclusion. This mass appears to be greater from *Bacillus licheniformis* strain USC13 (35 kDa) reported by Ageitos et al [6]With *Bacillus subtilis* MTCC 10422 and using the same method, Kumari et al. [22]observed a mass of 27 kDa. PreviouslyYasuda et al [23] published similar findings by filtration gel followed by electrophoresis, showing a 29 kDa monomeric band. Our results are in a range of 27 to 44 kDa and consistent with the PM of several proteases from *Bacillus* sp. as reported by Peng et al [24]

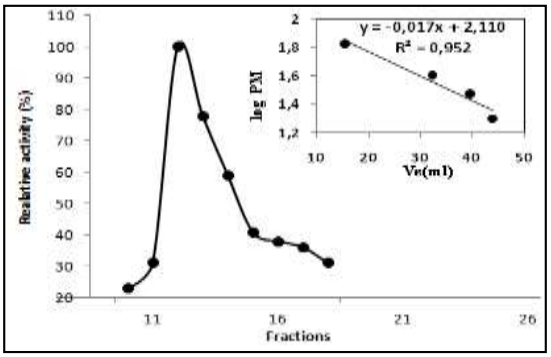


Figure 3:*Size-exclusion chromatography through Sephadx G-75 of a purified extract of B. velezensis FK6A strain (active fraction) and molecular weight determination of the milk-clotting enzyme (in box). Molecular-weight standards: 1: Bovin serum albumin (67 kDa), 2: Ovalbumin (40kDa), 3: Carbonic nhydrase (30kDa), 4: trypsin (23.8k Da) (a).***III. 4.**

Effect of some inhibitors on coagulating activity

Two protease inhibitors were tested in order to continue the biochemical characterization of the raw *Bacillus velezensiss* FK6A extract and to classify the category of coagulating proteases. **Table 3** shows that pepstatin A inhibits less than 13.7% of the enzyme at a concentration of 0.1 mmol/L and 48.36% at a concentration of 0.2 mmol/L. Thus, pepstatin A is not to be considered as a possible inhibitor. However, with EDTA at 5 and 10 mmol/L, coagulating activity is completely inhibited (100%), suggesting that the enzyme studied belongs to the metalloprotease class with the divalent metal ions at their active site. This group, includes mainly microbial proteases from *Bacillus amyloliquefaciens* D4 [14]**,***Bacillus amyloliquefaciens* JNU002 [20], *Bacillus subtilis* YB-3 (**[25]** and *Paenibacillus spp*. BD3526 [26]

Tableau 3 . *Effect of inhibitors on milk clotting activity of purified extract from Bacillus velezensis FK6A*

Inhibitors	***Concentration (mM)***	***Residualactivity (%)***
Control	0	100
Pepstain A	0,1 0,2	86,30 51,64
2-mercaptoethanol	5	36
EDTA	5	0

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III. 5.Optimal conditions for coagulating activity

III. 5. 1. Influence of temperature and pH on raw enzymatic extract (REE) activity

The optimal milk temperature on the coagulating activity of the REE of *Bacillus velezensis* FK6A strain was tested at temperatures ranging from 30 to 85°C by a level of 5°C. The findings recorded in **Figure 4A** indicate that enzymatic activity rises (2.40 ± 0.36 UP) with maximum at around 75°C. There is a significant decrease in coagulating activity after 75°C, and complete enzyme inhibition at 85°C.The optimum temperature obtained by the enzyme extract is similar to that reported by *bacilluslicheniformis* 5A5 strain [27] and close to that reported in *Bacillus subtilis* B1 (70° C) **[7].**

The effect of milk pH on the coagulating activity of the raw enzyme extract was measured at a pH interval of 5.7 to 8.1, with a pallier of 0.2. As shown in **Figure 4B**, 5.7 is the optimal pH of casein hydrolysis with a coagulating levels of 0.7±0.01 UP. Beyond this pH value, the coagulating activity slowly decreases until total inhibition at pH 8.1. This agrees with the optimum coagulating function of the enzyme obtained from the strain *Bacillus amyloliquefaciens* D4 with 5.5 as optimal pH and 8 as pH inactivating pH. In rennet activity, Mahaut et al [28] indicated an optimum at pH 5.8 and inactivation at pH 7.5 and complete denaturation at pH 8.

III. 5. 2. Effect of $CaCl_2$ concentration

Glantz et al [29]found that the calcium ion concentration in milk is linked to its gelling properties. Higher concentration in this element contributes to shorter gelling times and improved resistance and firmness of gels. The results shown in the **Figure 4C** indicate that the protease coagulating activity decreases with concentration of $CaCl_2$ until an optimum value of 40 mM.The same finding was reported by Talantikite et al [8] in a related analysis on *Bacillus mojavensis* I33M coagulase.

III. 5. 3. Effect of raw enzyme extract concentration

The milk clotting activity of rennet gradually increases with its concentration [30, 31]. This is in accordance with our findings (**Figure 4D**) with a steady increase in activity as the extract concentrations increase.

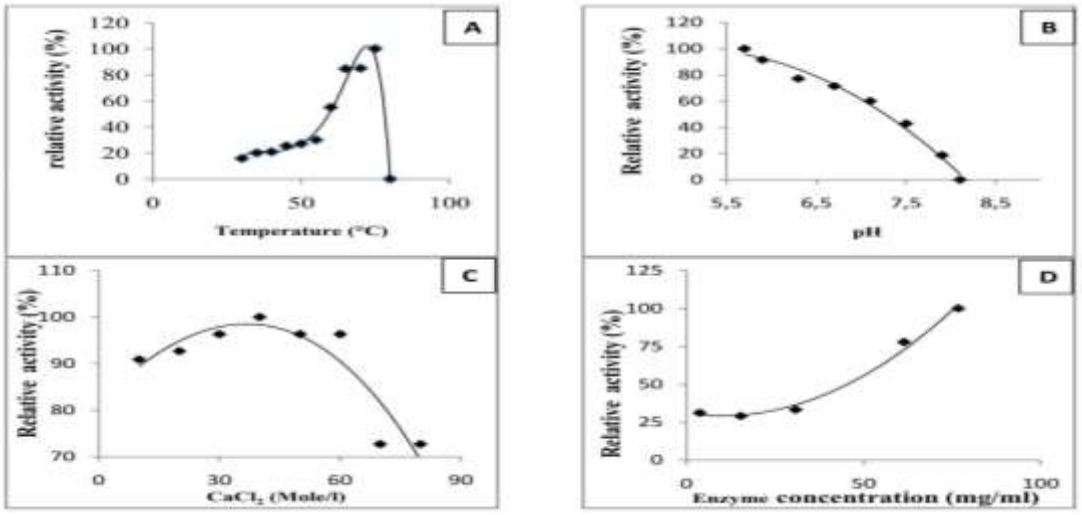

Figure 4. *Physico–chemical properties of milk-clotting activities of the enzymatic extract from Bacillus velezensis FK6A* ***(A****: Temperature,* ***B****:pH,* ***C****:$CaCl_2$,* ***D****:Enzyme concentration)*

IV. Conclusion

This strain known to generate proteolytic enzymes was not the target on its ability to coagulate milkof any research in the reviewed literature. We took an interest in this aspect by applying more rigorous methods, and by showing the originality of the concept of our subject through the results obtained.Therefore, the proteolytic activity of *Bacillus velezensis*FK6A extracts found on the curds occurs a few minutes after the formation of a firm coagulum resulting in the presence of an abundant serum, during the technique

studies. Nevertheless, in order to solve the problem caused by The REE high proteolytic activity and totally inhibit the general proteolysis, we directed the development towards a form of hard cooked (**Figure5).**

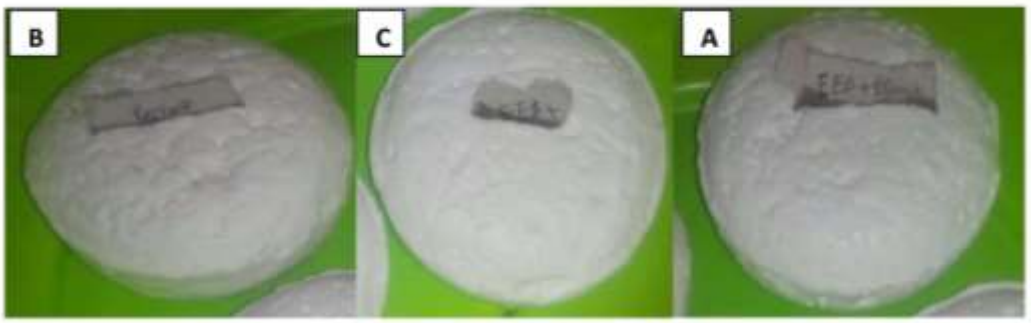

Figure 5. *Appearance of cooked cheese dough making by commercial rennet* ***(A),*** *(B) crude extract of Bacillus velezensis FK6A* ***(B)*** *and Bacillus velezensis + rennet (50/50)* ***(C)***

All the results of our study show that the protease of significantcoagulating potential and provides new complementary research avenues for development and milk coagulating microbial enzymes studied has production of milk coagulating microbial enzymes.

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