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Full Length Research Paper

Isolation and identification of bacterial strain I33M producing milk-clotting enzyme: Optimization of culture parameters using response surface

Talantikite-Kellil, S[1], Nouani, A. [1,2]*, Saunier, M[3], Gillmann, L[3], Brahmi, A[4] and Fazouane, F[1]

[1]Food Technology Laboratory, University of Boumerdes, Algeria.
[2]Food Technology laboratory, ENSA, Algeria.
[3]lasba-Sonas (UPRES - EA 921 , Antenne IUT - Université d'Angers 49000, France.
[4]Department of Mathematics and Statistics - Moncton University, NB, Canada.

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A strain I33M which produces a milk-clotting enzyme was screened from Algerian soil near a dairy factory. This strain was identified as *Bacillus mojavensis* based on morphology and internal transcription spacer sequence. Sequencing analysis of 16S rDNA gene showed 100% identity of the tested strain with the *B. mojavensis* in the database. Phylogenetic analysis of this strain showed that it was most closely related to *Bacillus subtilis* strain. The optimum levels of these significant parameters to obtain the highest milk clotting activity and the lowest proteolytic activity were determined employing the response surface methodology (RSM), which revealed these as follows: wheat bran 7%, casein 0.094%, temperature 39°C, agitation size (rpm) 150. Among the various variables screened, agitation and temperature were most significant in submerged fermentation (SmF). The optimal value of milk clotting activity (MCA) is esteemed at 2.40.

Key words: Milk clotting protease, *Bacillus*, response surface methodology, sequencing analysis.

INTRODUCTION

The proteases constitute at least 65% of the total industrial enzyme market (Rao et al., 1998) and are also known as peptidyl_/peptide hydrolases (EC 3.4.21-24 and 99) which catalyze the hydrolysis of a peptide bond in a protein molecule such as casein for cheese making production (Beg et al., 2003).

In cheese technology, these extracellular proteases are obtained especially from microbial culture and throughout the world, many strains of clotting protease microorganisms are being produced industrially mainly fungi such as *Mucor miehei, Mucor pusillus, Endothia parasitica, Irpex lacteus, Aspergillus niger var. Awamori, Kluyveromyces lactis* and *Escherichiacoli* using solid state fermentation process (Olson, 1995; Chen et al., 2011).

*Corresponding author. E-mail: nouani2002@yahoo.fr.

It is well known that with the increase in world cheese production and consumption, attempts were made to find substitutes for this enzyme (Neelakantan et al., 1999). So far, most efforts for calf rennet substitute focus on finding new milk clotting-enzymes that could be used in cheese production and many microbial and plant proteases were identified and have been suggested as milk coagulants. Microbial proteases, especially from *Bacillus* sp. have traditionally held the predominant share of the industrial enzyme market of the worldwide enzyme sales with major application such as detergent product, waste treatment, food industry (Sepahy and Jabalameli, 2011). However, few studies have demonstrated the value of proteases as a substitute for rennet. Although, Ding et al. (2011) report that only few research work on bacteria are reported, wild bacteria with a high milk-clotting activity (MCA) in submerged fermentation show a great potential, due to shorter fermentation period, larger capacity of extracellular secretion and higher material utilization ratio.

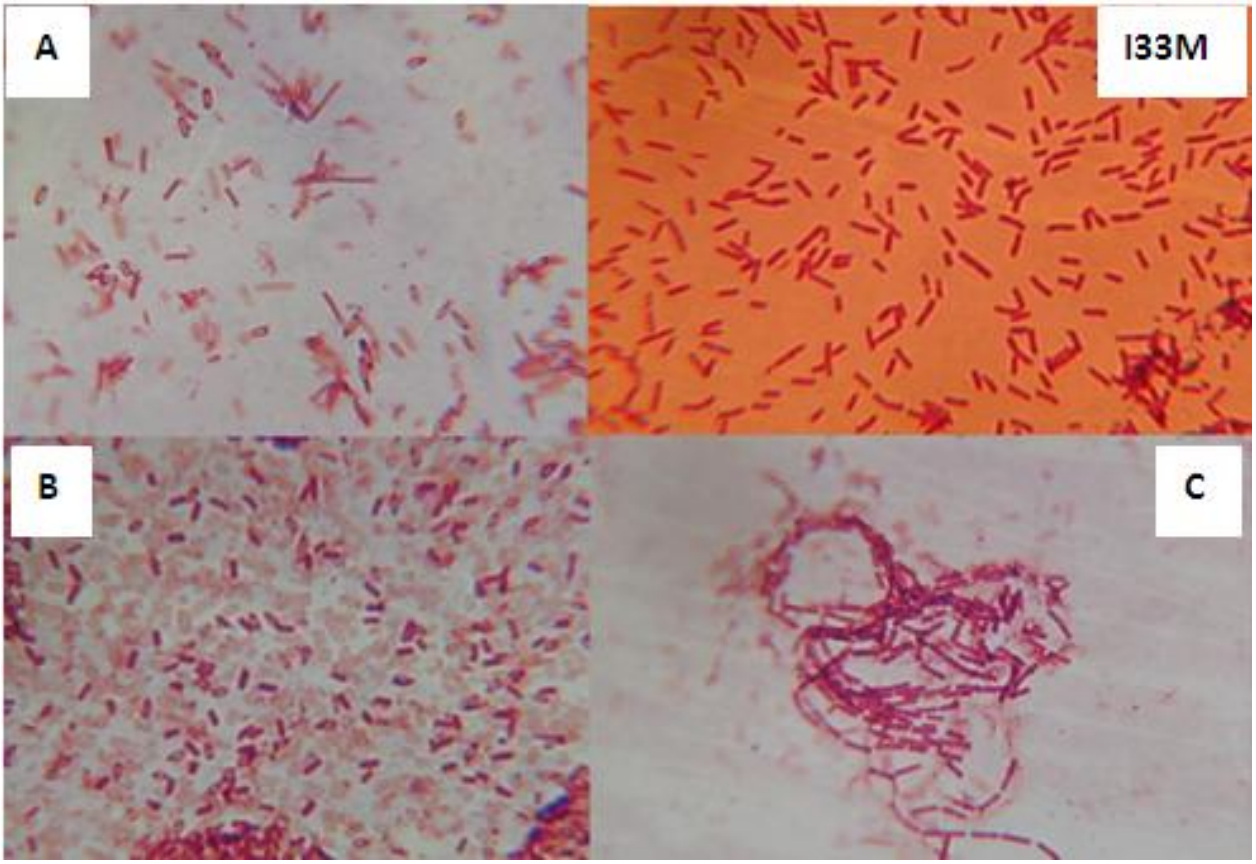

Figure 1. Photograph of microscopic observation of the strain I33M and other *Bacillus* strain Gram+ (A. B. C not studied).

Protease production is an inherent capacity of all microorganisms, however, only those microbes that produce substantial amounts of extracellular enzyme are of industrial importance such as dairy industry (Beg et al., 2003). Also, it is well known that extracellular protease production by microorganisms is greatly influenced by physical factors such as pH, temperature, time and by others factors (Thys et al., 2006) where submerged fermentation (SmF) has been traditionally used. The optimization of parameters becomes obvious and the RSM is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and searching for the optimum conditions (Chen et al., 2011).

Thus, our present study is a contribution of the achievement of a milk clotting enzyme from the isolation of a bacterial strain local. Statistical methods have been applied for optimization of protease production (Gangadharan et al., 2008). A statistical approach has been employed for identifying significant variables influencing protease production under submerged fermentation (SmF). The levels of the significant variables were further optimized using response surface methodology.

MATERIALS AND METHODS

Microorganism, culture conditions and Isolation method

Fifty grams of soil samples were collected near a dairy factory in the region of Boumerdes, Algeria. A small amount of soil was homogenized under magnetic agitation for 20 mn with 90 mL sterile physiological water to make an initial dilution (10^{-1}) (Davet and Rouxel, 1997). Serial dilutions were made for each sample and 0.1 mL of the appropriate dilution to 10^{-9} was spread on plate count agar (PCA) medium containing 2% (w/v) actidione antifungal agent to prevent fungal growth and 12% (w/v) sterilised skimmed milk powder. The plates were incubated at 30°C for 48 h for the enumeration of the caseolytic strain producing milk clotting protease by the presence of a clear halo around the colony (Figures 1 and 2). Colonies with distinct morphologies differences such as color (Gram technique), shape and size were selected (Table 1) and purified by streaking using the same medium. Of the 68 strains selected, 52 were Gram positive and 16 were Gram negative. All isolates were checked for milk clotting production and examined microscopically before stock preparations. The Gram positive bacterial isolates identified as *Bacillus* was purified and the selected strains are those with a clotting activity and are maintained on agar conservation.

Protease production in shake flask cultures, medium composition and inoculum

According to Rao and Mathur (1976), the basal minimal medium

Figure 2. Caseolyse area of some strains tested. Strain I33M and other *Bacillus* strain Gram positive (A. B. C not studied).

Table 1. Experimental range and levels of the independent variables used in RSM in terms of actual and coded factors.

Variable	Range of level					
	Actual	Coded	Actual	Coded	Actual	Coded
Wheat bran (%w/v)	3	-1	5	0	7	+1
Casein (%w/v)	0.02	-1	1.01	0	2	+1
Temperature (°C)	25	-1	37.5	0	50	+1
Agitation (RPM)	100	-1	150	0	200	+1

(pH 6.8) used for milk clotting protease production, contained ($g.l^{-1}$): Wheat bran (50), yeast extract (3), glucose (4), casein (2), Na_2HPO_4 (3). The production medium (50 ml) in a 250 ml Erlenmeyer flask) was inoculated with 10^7 to 5.10^8 bacteria per milliliter (Mac Farland3) and incubated at 30°C on a rotary shaker with agitation (110 rpm) for 24 h (Poza et al., 2003) in a IKA® KS 4000i control shaker. After incubation, the culture broth was filtered and then centrifuged at 4,000 g for 30 min at 4°C in a cold centrifuge (Jouan GR 422). Total protease yield and milk clotting activity was determined in the cell-free supernatant. Strain that showed the highest milk-clotting activity and the lowest proteolytic activity was selected for further studies and identified using 16S rDNA sequencing. The selected strain is called I33M.

In the next stage, response surface methodology is used to study the interactive effects of four variables, that is, wheat bran, casein, temperature and agitation for improving total protease production by the strain I33M

Response surface methodology

The present work was based on the central composite design (CCD) utilized to obtain the experimental data, which would fit an empirical, full second-order polynomial model representing the response surfaces over a relatively broad range of parameters as reported by Dutta et al. (2004). Each factor in the central composite design (CCD) was studied at three different levels, low (-1), medium (0) and high (+1). For this purpose, the response surface approach was used and a set of experiment was performed. The range and levels of experimental variables investigated are presented in Table 2. Four independent variables were used to approach the

Table 2. Experimental designs by using four independent variables showing observed and predicted values of milk clotting activity of strain IM33 (*B. mojavensis).*

Run order	Coded level				
	Wheat bran	Casein	Temperature	Agitation	Enzyme activity (IMCU/ml)
1	-1	0	0	0	1.82
2	0	0	0	0	2.70
3	0	0	1	0	2.27
4	0	0	0	0	2.13
5	1	0	0	0	2.86
6	0	0	0	0	2.33
7	0	-1	0	0	2.86
8	1	1	-1	-1	0.01
9	0	0	0	0	2.38
10	0	0	-1	0	0.24
11	1	1	1	1	0.01
12	1	-1	-1	1	0.05
13	-1	1	-1	-1	0.51
14	-1	-1	1	-1	-
15	0	0	0	-1	0.15
16	-1	1	1	1	0.42
17	-1	-1	1	1	0.12
18	1	-1	1	-1	-
19	0	0	0	0	2.44
20	1	-1	1	1	-
21	0	1	0	0	1.33
22	-1	1	1	-1	0.03
23	-1	-1	-1	1	0.06
24	-1	-1	-1	-1	0.02
25	0	0	0	0	2.17
26	0	0	0	1	0.42
27	-1	1	-1	1	-
28	1	1	-1	1	0.01
29	1	1	0	-1	0.42
30	1	-1	-1	-1	0.01
31	-1	0	0	0	1.79
32	0	0	0	0	2.63
33	0	0	1	0	2.22
34	0	0	0	0	2.38
35	1	0	0	0	3.33
36	0	0	0	0	2.70
37	0	-1	0	0	2.86
38	1	1	-1	-1	0.01
39	0	0	0	0	2.78
40	0	0	-1	0	0.21
41	1	1	1	1	1.67
42	1	-1	-1	1	-
43	-1	1	-1	-1	0.01
44	-1	-1	1	-1	-
45	0	0	0	-1	0.21
46	-1	1	1	1	0.01
47	-1	-1	1	1	0.04
48	1	-1	1	-1	-
49	0	0	0	0	2.17

Table 2. Continued.

50	1	-1	1	1	0.33
51	0	1	0	0	1.39
52	-1	1	1	-1	0.01
53	-1	-1	-1	1	0.06
54	-1	-1	-1	-1	0.02
55	0	0	0	0	2.13
56	0	0	0	1	0.17
57	-1	1	-1	1	-
58	1	1	-1	1	0.01
59	1	1	0	-1	0.02
60	1	-1	-1	-1	0.01

interaction among different factors (2^4 CCD with four factors was applied) including 6 centre points and a set of 60 experiments was carried out. The central values (zero level) chosen for experimental design were: wheat bran 5% (w/v), casein 1.01% (w/v), temperature 37.5°C and agitation 150 rpm.

Results were analyzed by the experimental design module of the *STATGRAPHICS plus V 5.1software* (Statsoft, USA). The model permitted evaluation of the effects of linear, quadratic and interactive terms of the independent variables on the dependent variable. The statistical significance of the regression coefficients was determined by Student's t-test and the second order model equation was determined by Fisher's test. Three-dimensional surface plots were drawn to illustrate the main and interactive effects of the independent variables on milk clotting protease production (Myers and Montgomery, 2002; Thys et al., 2006).

For the validation of CCD, only two factors, temperature and agitation were taken, and remainder of the factors were fixed on the basis of the data obtained from the first set of experimental design.

Analytical procedures

Milk clotting activity assay

Milk clotting activity (MCA) was measured according to the procedure described by international dairy federation (IDF) standard (IDF, 2002). Skim milk powder was reconstituted by dissolving 12.5 g in 100 ml of 0.5 g/L of $CaCl_2$ solution (pH 6.5). The clotting assay was carried out by mixing 10 ml of this substrate with 1 ml of sample. Clotting activity was expressed in international milk clotting units (IMCU)/ml

Proteolytic activity

Proteolytic activity was determined according to the study of Murado and Siso (1993). One millilitre of the enzymatic extracts was added to 1 ml of 2% (w/v) alkali soluble casein in 0.02 M sodium-citrate buffer (pH 5.2). The reaction mixture was incubated at 35°C in a water bath for 10, 30, 60, 90 and 180 min, and the reaction was terminated by adding 5 ml of 12% (w/v) trichloroacetic acid. After 15 min, the precipitates formed were removed by filtration through Whatman no. 1 filter paper. Five millilitres of 0.5 M sodium carbonate solution was added to 1 ml of the aforementioned clear filtrate. The mixtures were incubated at 35°C for 10 min, when 0.5 ml of Folin–Ciocalteu reagent was added. The reaction was incubated for 20 min at 35°C for colour development. The optimal density at 660 nm expresses the rate of tyrosine released during hydrolysis.

Protein estimation

Protein concentration was measured by the method of Lowry et al. (1951), with crystalline bovine albumin as the standard.

Identification of strain I33M

Macroscopy and microscopy were performed on Petri dishes with plate count agar (PCA) on colonies of 24 h. For sequencing analysis, the genomic DNA was extracted from the strain by the standard chloroform isoamyl alcohol method using PrepMan ultra sample preparation reagent kit (Applied Biosystems) and purified on plates NucleoFast (Macherey Nagel). The amplification of the 16S rDNA was performed through PCR technique, using Taq DNA polymerase, genomic DNA as a template, and 3 forward and 5reverse universal primers. The sequences of these primers used are as follows: F1 (5'-AGAGTTTGATCCTGGCTCAG-3'), R1 (5'-GTATTACCGCGGCTGCTGGCAC-3'), F2 (5'-CTCCTACGGGAGGCAG-3') and R2 (5'-GACACGAGCTGACGACA-3'). PCR products were sequenced by Sanger technique using optimised conditions performed by Geno screen (*Geno-screen Campus de l'Institut Pasteur de Lille 1, rue du Professeur Calmette – 59000 Lille – France*). Receiving the sequencing results, 16S rDNA nucleotide sequence of the strain was deposited in GenBank, and aligned with the 16S rDNA sequences available in public data bases in National Center for Biotechnology Information (NCBI) (Available at: http://www.ncbi.nlm.nih.gov/), and LeBibi database (http://umr5558-sud-str1.univ-lyon1.fr/lebibi/lebibi.cgi) using basic local alignment search tool (BLAST) software.

Bacterial growth and protease production in a fermentor

The final validation of the statistical approach to protease production from *B. mojavensis* was carried out in a 2 l bioreactor (Sartorius Biostat A+, Switzerland) with a working volume of 1.5 L. The optimized medium (pH 6.8, 7% (w/v) wheat bran and 0.094% (w/v) casein) containing 0.3%(w/v) yeast extract and 0.3% (w/v) Na_2HPO_4 was sterilized *in situ* at 120°C for 30 min. The medium was inoculated aseptically with 60 ml of inoculum (Mc Farland 3) (9. 10^8 CFU / ml). The fermentation was carried out at 39°C for 50 h with uncontrolled pH and the impeller speed was initially adjusted to 150 rpm. Samples were withdrawn periodically at an interval of 2 h and analyzed for protein and protease production (as described previously) and biomass estimation was determined using the technique described by Thys et al. (2006). The nutrient agar plates loaded by bacterial suspension were incubated for 24 h at 37°C and

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CCCGAAGGGGACGTCCTATCTCTAGGATTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTT
GCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTT
GCGACCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCC
TAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCT
TTCGCTCCTCAGCGTCAGTTACAGACCAGAGAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTAC
GCATTTCACCGCTACACGTGGAATTCCACTCTCCTCTTCTGCACTCAAGTTCCCCAGTTTCCAATGAC
CCTCCCCGGTTGAGCCGGGGGCTTTCACATCAGACTTAAGGAACCGCCTGCGAGCCCTTTACGCCCA
ATAATTCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTT
TCTGGTTAGGTACCGTCAAGGTACCGCCCTATTCGAACGGTACTTGTTCTTCCCTAACAACAGAGCTT
TACGATCCGAAAACCTTCATCACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATT
CCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCA
GGTCGGCTACGCATCGTTGCCTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCGCCGCGGGTCCA
TCTGTAAGTGGTAGCCGAAGCCACCTTTTATGTTTGAACCATGCGGTTCAAACAAGCATCCGGTATTA
GCCCCGGTTTCCCGGAGTTATCCCAGTCTTACAGGCAGGTTACCCACGTGTTACTCACCCGTCCGCC
GCTAACATCAGGGAGCAAGCTCCCATCTGTCCGCTCGACTTGCATGTATTAGGCAC

Length of Query sequence is: 195 A - 254 T - 321 C - 229 G
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Figure 3. Sequence of the 16S rDNA of strain I33M (*B. mojavensis*).

counts performed on plates having between 20 and 300 colonies. Other fermentation parameters, such as pH, were continuously monitored using microprocessor-controlled probes.

RESULTS AND DISCUSSION

Screening of strains producing bacterial

Milk-clotting enzyme

In this study, some strain with different milk-clotting activity (MCA) were isolated from soil and identified as bacillus Gram positive by Biochemical tests (data not shown). Among these strains, four *Bacillus* sp (A, B, C and I33M) had a good milk-clotting activity and caseolytic effect on casein agar plates as shown in Figure 2 and were selected to be studied further. In the present work and in a first step, we report the results of extracellular protease activity and molecular identification and phylogenetic analysis of strain I33M which also has the best enzyme activity (Up to 3.3 IMCU/ml) followed by high proteolytic effect. It is well known that protease production is an inherent capacity of all microorganisms, however only those microbes that produce substantial amounts of extracellular enzyme are of industrial importance (Gupta et al., 2002; Kole et al., 1988). Thus, the most common and widely used bacteria for industrial proteases belong to the genus *Bacillus* (Ara et al., 2007). Ghaffour and Hasnain (2010) reported that the strains of *B. subtilis* was highly active on milk agar plates and caseinase activity was confirmed by growing the organism on casein agar plates concentration ranging from 0.3 to 1%. In light of the examined literature, no work, in our opinion, has reported on milk clotting proteases produced by *B. mojavensis*. So, According to the results, *B. mojavensis* could be a good producer of extracellular milk clotting protease. As Fungal milk-clotting enzymes, the *Bacillus* species could find applications in cheese industry.

Identification of the strain

The 16SrDNA gene sequence analysis has been described to be necessary mainly to detect some misidentification of *Bacillus* and related strains (Ben et al., 2011). Also, this technique is one of the most commonly used today for the identification of species, as it is a quick, reliable, and reproducible method compared to conventional technique using morphological and physiological criteria. For further characterization, the PCR product of strain I33M 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 I33M (Figure 3). This sequence consists of 999 bp and the length of Query sequence is: 195 A, 254 T, 321 C and 229 G. The BLAST search of 16S rDNA gene sequence against sequences in nucleotide database has shown 100% homology with *B. mojavensis* strain. Therefore, our strain was named *B. mojavensis* I33M and the data bases allowed us to elaborate a phylogenetic dendrogram for I33M and related strains (Figure 4). The phylogenetic analysis showed that the tested strain was closest to *B. subtilis.* Similar results were reported by Thuy and Bose (2011). Also, Roberts et al. (1994) report that isolate was subsequently found to belong to the closely related *B. subtilis*-like phenotype that has been recently described as *B. mojavensis. B. mojavensis* can be distinguished only by differences in whole-cell fatty acid composition, divergence in DNA sequence analysis, and resistance to genetic transformation between taxa within the *B. subtilis* group. Moreover, Bacon and Hinton (2001) revealed

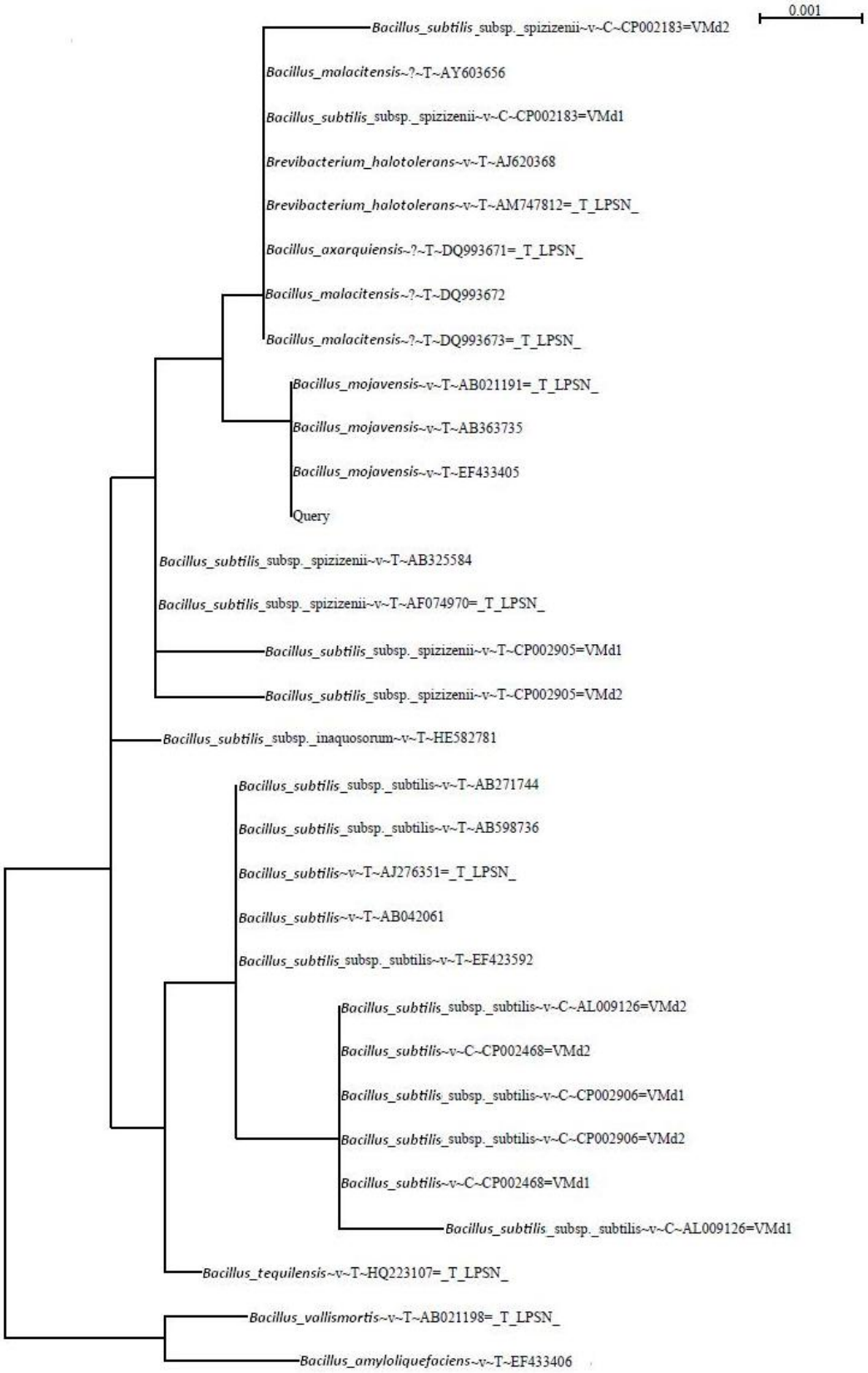

Figure 4. Phylogenetic tree derived from analysis of 16S rDNA sequence of strain I 33M and related sequences obtained from NCBI. Scale bar. 0.001 substitutions per nucleotide position. The GenBank accession number for each strain follow the species name.

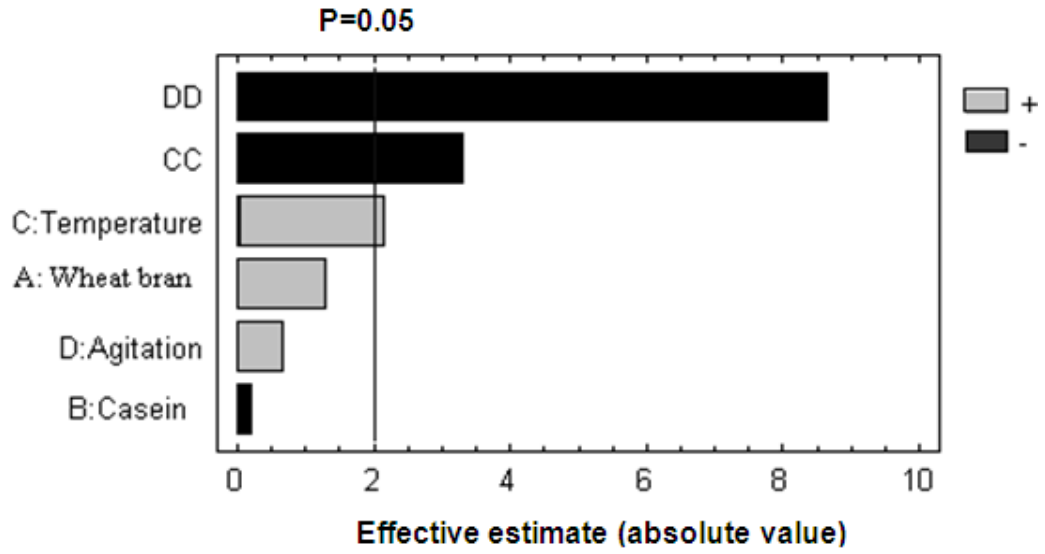

Figure 5. Bar graph of standardized estimated effects of the different variables tested in the prospective experiment on milk clotting protease production by strain I33M (*B. mojavensis).* The variables tested were wheat bran. temperature. agitation and casein. The point at which the effect estimates were statistically significant (at P = 0. 05) is indicated by the vertical line.

Table 3. Estimated effects for Milk clotting activity.

Factor	Value
Average	2.27234 ±0.103779
A:Wheat bran	0.212778 ± 0.161821
B:Casein	-0.0316667 ± 0.161821
C:Temperature	0.351667 ± 0.161821
D:Agitation	0.107778 ± 0.161821
CC	-1.20806 ± 0.369922
DD	-3.20306 ± 0.369922
Block	0.0466667 ± 0.125346

Standard errors are based on total error with 52 d.f.

in their works that the identity of a patented endophytic bacterium was established by 16S rRNA sequence analysis as a strain of *B. mojavensis*, a recently erected species within one of the *B. subtilis* subgroups.

Optimization by response surface methodology

The statistical design approach using response surface methodology was used to study the interactive effects of various chemicals and physical factors on protease (milk clotting) production by *B. mojavensis*. The primary aim is to maximize the milk clotting activity and to minimize the proteolytic activity. The experiment was conducted in 60 runs and the input variables that have the maximum influence on the final response of the system were identified from the interaction of four factors namely, wheat bran (carbon source) and casein (nitrogen source) (nutritional factors), temperature and agitation (physical factors), was examined through RSM following the CCD. We recall that the respective low and high levels (% w/v) with the coded levels in parentheses for the factors were defined as 3 (-1) and 7 (+1) for wheat bran, 0.02 (-1) and 2.0 (+1) for casein, 25°C (-1) and 50°C (+1) for temperature, 100 rpm (-1) and 200 (+1) for the agitation.

The results of CCD experiments for studying the effects of these variables on protease production in milk clotting activity (IMCU/ml) are presented in Table 2 along with the observed responses.

Statistical analysis of results showed that in the range studied, quadratic effect of temperature and agitation has a strong effect on protease production followed by the quadratic effect of agitation. The temperature have a significant effect but slightly higher than the limit of significance for 95% of significance (Figure 5). Table 3 shows the estimate effects and interactions. The results

Table 4. Analysis of variance for Milk clotting activity.

Source	Sum of squares	Df	Mean square	F-Ratio	P-Value
A:Wheat bran	0.407469	1	0.407469	1.73	0.1943
B:Casein	0.009025	1	0.009025	0.04	0.8456
C:Temperature	1.11303	1	1.11303	4.72	0.0343
D:Agitation	0.104544	1	0.104544	0.44	0.5083
CC	2.51345	1	2.51345	10.66	0.0019
DD	17.6693	1	17.6693	74.97	0.0000
blocks	0.0326667	1	0.0326667	0.14	0.7112
Total error	12.255	52	0.235673		
Total (corr.)	76.2317	59			

R-squared = 83.924%; R-squared (adjusted for d.f.) = 82.1041%; Standard error of est. = 0.485462; Mean absolute error = 0.330268; Durbin-Watson statistic = 2. 11832 (P=0. 2560); Lag 1 residual autocorrelation = -0.0634366.

Table 5. Regression coefficients for milk clotting activity.

Constant	-18.5167
A:Wheat bran	0.0531944
B:Casein	-0.0159933
C:Temperature	0.304002
D:Agitation	0.193262
CC	-0.00386581
DD	-0.000640613

of the second-order response surface model in the form of analysis of variance (ANOVA) are given in Table 4. Fischer F-test demonstrates significance for the regression model where the coefficient of determination (R^2) was 0.8392. The R-Squared statistic indicates that the model as fitted explains 83.924% of the variability in milk clotting activity and this value indicates the aptness of the model. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 82.1041%. The standard error of the estimate shows the standard deviation of the residuals to be 0.485462. The mean absolute error (MAE) of 0.330268 is the average value of the residuals. The following regression equation (in terms of coded factors) of the levels of protease produced expressed in IMCU /ml (Y) as a function of wheat bran (A), casein (B), temperature (C) an agitation (D).

$$Y= -18.5167 + 0.0531944* A – 0.0159933*B + 0.304002*C + 0.193262*D – 0.00386581*C^2 - 0.000640613*D^2.$$

The regression coefficients are listed in Table 5. The magnitude of the effects indicates the level of the significance of the variable on protease production and among the variable screened; it seems that only the physical factors (temperature and agitation) were identified as most significant variables influencing milk clotting protease production as shown in Figure 5 than the substrate, wheat bran and casein. In fact, the results of observed responses for the experiments performed using CCD (Figure 2) showed that temperature and agitation are limiting factors for enzyme production of whatever substrate concentration. The combination of factors levels which maximize milk clotting activity has given a medium composed of wheat bran: 7.0; casein: 0.00941462; temperature: 39.2587 and agitation: 150.799) and the optimum value of milk clotting activity is 2.40661 (IMCU/ml).

After this, the three-dimensional response surface is plotted and represented as shown in Figure 6, which shows the relative effects of two factors, viz. temperature and agitation in combinations when all the factors (wheat bran and casein concentration) were kept at their central levels. The response surface obtained is convex and the convexity seems high enough. This suggests that there were well-defined optimum operating conditions. It was observed that there was an enhancement in protease production at relatively high temperatures and the maximum protease production was obtained at 38 to 39°C (Figure 6). On the other hand, above this temperature the protease production is very low. The same results are obtained with agitation. So, our model indicates that temperature has a major effect on protease

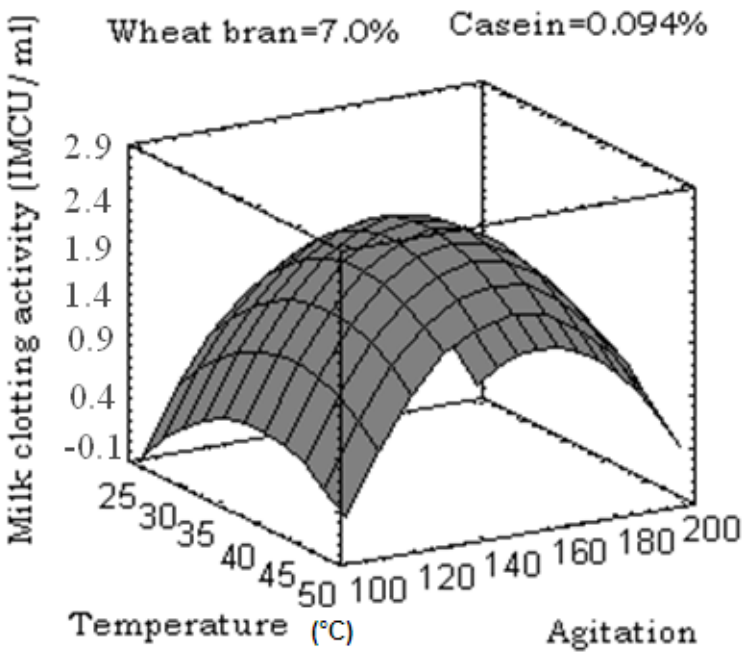

Figure 6. Response surface curve of milk clotting protease production from strain I33M (*B. mojavensis*) showing interaction between temperature and agitation.

production by the *B. mojavensis* strain. This result is reported by Thys et al. (2006) and other RSM studies also showed that temperature is a very relevant factor for microbial protease production (Wu and Hang, 2000). The RSM methodology was found to be very efficient to determine the optimal conditions for protease production by *Bacillus* sp. PE-11 (Thys et al., 2006). In general, it is clearly shown that no defined medium has been established for producing protease from different microbial sources (Gupta et al., 2002). Each organism or strain has its own requirement of special conditions for maximum enzyme production. So, in our case, the physical factors, temperature and agitation are more significant than nutritional factors.

Growth and protease production at optimal conditions in laboratory-scale bioreactor

During the period of growth, we notice that the lag phase is short and the exponential phase, after 10 h of fermentation, the stationary phase begins and continues for 48 h, the growth rate is 1.9228 while the generation time is 29 min 36 s (Figure 7). In terms of the stationary phase, there have been a number of studies conducted on *B. subtilis* fermentation. These studies show that it can last from 3 to 8 days. Thus, Dutt et al. (2008) report that clotting activity decreases after 45 h whereas maximum is reached after one day (Shieh et al., 2009). The pH was fairly stable throughout the fermentation period (Figure 7). This could be explained by the fact that the bacteria consuming sugars produced acids that were quickly neutralized by the basic products from the proteolytic activity of *Bacillus*. The milk clotting and proteolytic activities increase over time while the protein level remains more or less telling; the two activities are linked (Figures 8 and 9).

On the clotting enzyme produced by *B. mojavensis*, the results show that this protease is a secondary metabolite because its rate continues to increase during stationary phase when a primary metabolite is produced during the exponential phase.

Conclusion

From this study, it can be concluded that milk clotting protease from *B. mojavensis* can be considered as a suitable alternative to the conventional rennet but it is more important to increase in the milk clotting activity (MCA) per proteolytic activity (PA) index. On the other hand, the present work show that protease production in *B. mojavensis* can be improved by controlling various factors simultaneously viz. agitation and temperature and this interaction could only be well understood by suitable RSM selection. In fact, this statistical method which includes factorial design and regression analysis helps in evaluating the effective factors and in building models to study interaction and select optimum conditions of variables for a desirable response. In our case, maximum

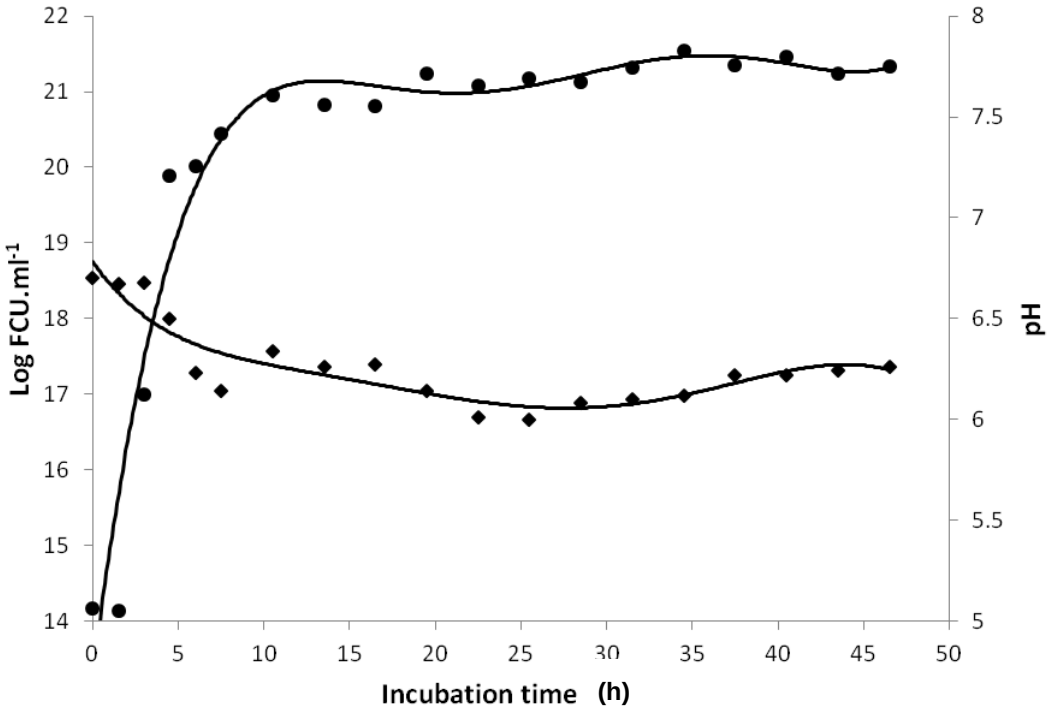

Figure 7. Evolution of the growth of the strainI33M (*B. mojavensis*) and pH of the medium in the fermenter [CFU (●). pH (♦)].

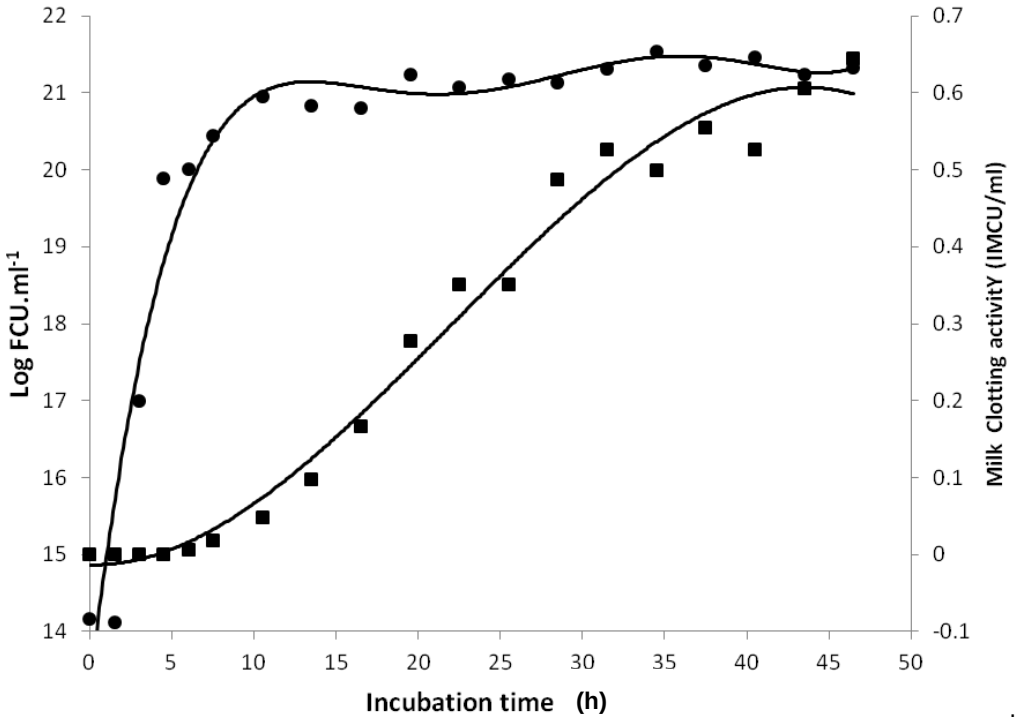

Figure 8. Production of milk clotting enzyme during growth of *Bacillus mojavensis* in optmized medium. CFU (●) and enzyme activity (■) were monitored.

milk clotting protease production was achieved at temperature 39°C and agitation 150 RPM.

However, additional studies about quality of the enzyme are needed in the future to confirm its usefulness

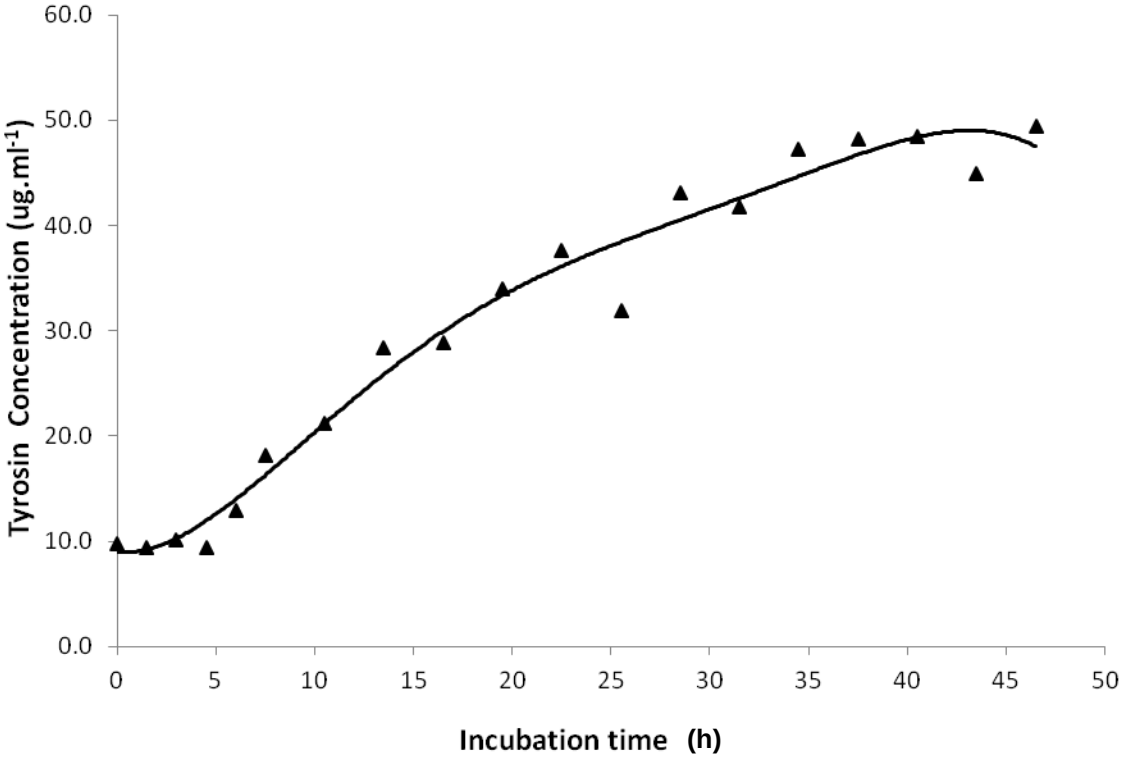

Figure 9. Curve of the proteolytic activity of strainI33 M (*B.mojavensis*) depending on the incubation time.

in the dairy processing.

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