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Statistical optimization of *B. subtilis* MK775302 milk clotting enzyme production using agro-industrial residues, enzyme characterization and application in cheese manufacture

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ABSTRACT

The agricultural and industrial wastes are environmental pollutants. So, there is an increasing demand for more efficient exploitation of these wastes and their conversion into useful products. Recently, the limited availability of calf rennet led to a search for rennet substitutes for cheese manufacture. In this study, *Bacillus subtilis* subsp. *subtilis* strain 168 (*B. subtilis* MK775302) was isolated from the marine sponge *Pseudoceratina Arabica* and it was used for milk clotting enzyme (MCE) production using some agro-industrial wastes. The production medium containing agro-industrial residues was optimized by using Plackett-Burman (PB) and central composite designs (CCD). After optimization, the MCE productivity was increased by 2.3-fold compared to the non-optimized medium. The enzyme showed maximum activity at 70 °C, pH 5.0. Ultrafilterated (UF) white soft cheese production was studied by using the crude extract of *B. subtilis* MK775302 in a comparison with commercial rennet. The cheese produced by the crude extract of *B. subtilis* MK775302 had a higher ripening index, higher acidity, higher flavor intensity, and acceptable organoleptic score. Therefore, this study offered the production of a promising rennet substitute that is eco-friendly with low production costs and competes with the commercial coagulants for the production of acceptable UF white soft cheese.

1. Introduction

Agricultural and industrial wastes are among the common causes of environmental pollution. Recently, there has been an increasing demand for more efficient utilization of agro-industrial residues and their conversion into useful products (Ismail et al., 1995; Pandey and Soccol, 1998). Many new opportunities have been opened for the applications of agro-industrial residues in bioprocesses (mainly in enzyme and fermentation technology field) and this can help in providing alternative substrates and solving the pollution problems (Akinyele et al., 2011).

Proteases account for about 60% of global markets enzymes, which have a wide range of applications (Sharma et al., 2017; Souza et al., 2017). They are used in several industrial processes including food, detergents, baking, pharmaceuticals, and photography (Ben Elhoul et al., 2015; Abdel-Naby et al., 2017). They have been used in the dairy industry for cheese manufacturing, where they destabilize casein micelles and stimulate milk-clotting, as the main step for cheese production. Cheese is the most important product of milk in highly concentrated form. It has been considered as a valuable dairy product of high nutritional value which can be kept fresh for a long time (Osman, 2009). Chymosin (EC 3.4.23.4) is the main enzyme in calf rennet with a relatively low proteolytic activity. It is known as the most suitable coagulant which has a high milk clotting/proteolytic activity ratio (Afsharnezhad et al., 2019). The limited availability of calf rennet leads to a search for alternate rennet substitutes for cheese making industry (Shellomith and Preetha, 2018). Most plant coagulants are unsuitable because they give a bitter taste to the cheese (Imdakim et al., 2015). Attention has been focused on milk clotting enzyme (MCE) from microorganisms due to many reasons including its stability, availability, rapid growth, low costs and the ease of genetic modification (Wehaidy et al., 2018). Many nonpathogenic

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strains of Bacillus species have been used for MCE production (Ahmed et al., 2016).

Statistical designs are preferred for fermentation optimization because they reduce the total number of experiments and provide a better understanding of the interactions between several factors (Revankar and Lele, 2006).

The main purpose of the present study is the exploitation of some agro-industrial residues to produce microbial MCE (rennet substitute), with the use of the statistical factorial designs for maximization of the enzyme yield. The produced enzyme was characterized and evaluated in the manufacture of white soft cheese in a comparison with a commercial microbial coagulant.

2. Materials and methods

2.1. Materials

Orange peels and rice straw were air-dried and cut into small pieces (particle size: 1.0 mm), wheat bran and whey were used in the same form as they were supplied. Casein (Hammerstein grade) was purchased from Sigma-Aldrich. Microbial rennet powder Chy-Max (from Chr. Hansen, Denmark) was used for the control white soft cheese production. The crude MCE extract produced by the bacterial isolate *Bacillus subtilis* subsp. *subtilis strain 168* (*B. subtilis* MK775302) was used for the tested white soft cheese production. The pre-cheese buffalo's milk retentate was obtained from the Dairy Products Unit at the Animals Production Research Institute, Ministry of Agriculture, Giza, Egypt.

2.2. Isolation and identification of microorganism

Bacillus subtilis subsp. *subtilis strain* 168 (*B. subtilis* MK775302) was isolated from the marine sponge *Pseudoceratina arabica* collected from the Red Sea, Sharm El Sheikh, Egypt. The molecular identification of the isolate was performed using 16s rRNA gene sequencing technique by Sigma Company for Scientific Services, Egypt.

2.3. Media used for MCE production by B. subtilis MK775302

B. subtilis MK775302 was screened for MCE production using 3 basal media. The most potent medium was selected with other media components for the optimization studies.

2.3.1. Medium 1 (g/L)

Lactose, 10; peptone, 1.5; yeast extract, 1; $MgSO_4.7H2O$, 1.0; KH_2PO_4, 1.0; $(NH_4)_2HPO_4$, 7 and $CaCl_2$, 0.3 (Fiedurek and Ilczuk, 1990).

2.3.2. Medium 2 (g/L)

Wheat bran, 50; yeast extract, 3.0; $MgSO_4.7H2O$, 0.2; K_2HPO_4, 3.0; and glucose, 4.0. pH was 6.0 before sterilization (Wehaidy et al., 2016).

2.3.3. Medium 3

Whey protein containing 2% lactose (Osman et al., 1969).

2.4. Production of MCE from B. subtilis MK775302 by submerged fermentation

One cultured slant was scratched with 10 ml distilled water and 2 ml of cell suspension was added to 250 ml Erlenmeyer flasks containing 50 ml of the sterile production media. The flasks were incubated for 48 h at 37 °C on a rotary shaker at 150 rpm. The cells were centrifuged at 3000 rpm for 15 min and the supernatant was collected and assayed for milk clotting activity (MCA).

2.5. Assay of milk clotting activity (MCA)

0.5 ml of the enzyme solution was incubated with 2 ml of skimmed milk (12 g skimmed milk/100 ml of 0.01 M calcium chloride) at 40 °C and pH 5 (0.1 M acetate buffer). MCA was expressed in Soxhlet units and calculated using the following equation:

Soxhlet units = 2400/T × S/E; where S is the volume of milk (ml), E is the volume of the enzyme (ml) and T is the time necessary for coagulation (Arima et al., 1967).

2.6. Optimization of B. subtilis MK775302 MCE production using statistical factorial designs

2.6.1. Plackett-Burman design (PB)

Eleven components (Wheat bran, Rice straw, orange peel, Whey, Peptone, Yeast, Lactose, Glucose, KH_2PO_4, $MgSO_4$, and $CaCl_2$) were selected for the study, each variable represented at two levels, high value (+1) and low value (−1). A 12 trial experiment was generated based on the rule R = n+1 where R is the run numbers and n is the number of variables. As illustrated in Table 1, each row represents an experiment, and each column represents an independent variable. The PB experimental design based on the following first-order model:

$$Y = \beta_0 + \Sigma^{k}_{i=0}\, \beta i Xi$$

Where Y represents the response (enzymes activity), B_0 is the model intercept, B_i is the linear coefficient, X_i is the level of independent variable and k is the number of involved variables.

Each trial was carried out in triplicates and the average enzyme activity was used as the response variable. Power calculations were accomplished using response type "Continuous" and parameters, Delta = 2, Sigma = 1. Power is evaluated over the −1 to +1 coded factor space.

In a balanced design, the standard errors should be similar to each other. Lower standard errors are better. The ideal VIF value is 1.0. VIF values above 10 are cause for concern. VIF values above 100 are cause for alarm, indicating coefficients are poorly estimated due to multicollinearity. Ideal R_i^2 is 0.0. High R_i^2 means terms are correlated with each other, possibly leading to poor models. Based on regression analysis, the variables that exhibited significant effect (95% confidence level, Prob > F ≤ 0.05) on enzyme activity were evaluated in further optimization experiments.

2.6.2. Central composite design (CCD)

After the identification of components that affect MCE production by Plackett–Burman design, three variables (Orange peel, Rice straw, and KH_2PO_4) were selected for response surface methodology of central composite design (CCD). The three significant variables were studied at five coded levels (−2, −1, 0, +1, +2). The CCD for MCE resulted in a total of 20 experimental trials including 8 trials for factorial design, 6 trials for axial points and 6 trials for replications of the central points (Table 3). Other media components were selected at the significant level concentrations from the PB design. The results of the CCD were expressed by the following second-order polynomial using a multiple regression technique according to the following equation:

$$Y = \beta_0 + \Sigma\, \beta_i X_i + \Sigma\, \beta_{ii} X^2_i + \Sigma\, \beta_{ij} X_i X_j$$

Where Y is the predicted response, β_0 the intercept term, β_i the linear coefficients, β_{ii} the quadratic coefficients, β_{ij} the interactive coefficients, X_i and X_j are the coded independent variables.

2.7. Statistical analysis of models

Statistical analysis of the models was performed according to the analysis of variance (ANOVA).

2.8. Characterization of crude B. subtilis MK775302 MCE

Some properties of the produced *B. subtilis* MK775302 MCE were studied. These include the optimum temperature, optimum pH, proteolytic activity and effect of sodium chloride concentration.

2.8.1. Optimum temperature

The optimum temperature of MCE was determined by assaying the enzyme activity at a temperature range from 40 to 80 °C.

2.8.2. Optimum pH

The optimum pH for the activity of MCE was determined by assaying the enzyme activity in the pH range from 5.0 to 7.0 using 0.1 M acetate buffer.

2.8.3. Proteolytic activity (PA)

One milliliter of the enzyme was added to 1 ml of 1% casein (Hammerstein grade) solution in phosphate buffer (0.1 M, pH 6.0). The reaction mixture was incubated in a water bath at 40 °C for 30 min. Then 2 ml of 20% trichloroacetic acid was added and the mixture was centrifuged at 4000 rpm for 10 min (Kunitz, 1947). The solubilized proteins in the supernatant were measured using the Lowry method at 750 nm (Lowry et al., 1951). One unit of the proteolytic activity (U) was defined as the amount of enzyme that liberates 1 μmol of amino acid equivalent to tyrosine per min under the assay conditions.

2.8.4. Effect of NaCl on MCA

The effect of NaCl on MCA was investigated by incubating MCE with an equal volume of NaCl with different concentrations (from 0 to 4%) at room temperature for 1 h. After the incubation, the MCA was determined (Ahmed et al., 2016).

2.9. Cheese manufacture

Two UF soft cheese treatments were conducted using control microbial rennet and the crude *B. subtilis* MK775302 extract as given by El-Shibiny et al. (2005) with some modifications. Fresh milk was heated at 72 °C for 15 s and ultra-filtrated. The ultrafiltration was carried out to a concentration factor of ~4.5 using tubular concentration module DC2, supplied by Amicon Corporation, USA, at 4 bar pressure and 50 °C. The average composition of the used retentate was: 35.85% total solids, 15.44 total protein, 14.58% fat, 4.63% lactose, and 1.20% ash and pH value 6.56. Sodium chloride was added to retentate at a level of 3% then heat-treated at 72 °C for ~15 s then cooled to 37 °C. Both treatments were inoculated with 1% active cheese culture (*Lactococcus lactis* spp *lactis* and *Lactococcus lactis* spp *cremoris*, 1:1). To both treatments 0.02% of calcium chloride was added, left at 37 °C for 1 h, coagulants were then added (with the level which completes coagulation within 40 min). The UF soft cheese samples were immediately distributed into plastic containers and incubated at 37 °C to complete the suitable curd formation. The top of the curd in each container was covered with a volume of pasteurized salted permeate (3%) height 1 cm then tightly closed with its lids and stored at 5 ± 2 °C for four weeks. Three replicates were carried out for each treatment. The produced cheese samples were examined when fresh and after 4 weeks storage.

2.10. Physico-chemical properties

The moisture content was determined in both milk and cheese samples and the total nitrogen (TN) of milk and cheese samples was determined by semi-micro Kjeldahl distillation as the method described in the Association of Official Analysis Chemists (AOAC, 1995). The titratable acidity (TA) for milk and cheese samples was expressed as lactic acid percentages. The fat content was determined by using a Gerber tube for both milk and cheese. The soluble nitrogen (SN) for cheese samples was determined by the semi-micro Kjeldahl distillation method (Ling, 1963). The total volatile fatty acids (TVFA) were determined in cheese samples according to the method described by Kosikowski (1982), the value was expressed as ml of 0.1 N NaOH/100 g cheese.

2.11. Textural profile analysis

Textural profile analysis (TPA) was performed on cheese samples according to the method of Glibowski et al. (2008) by using the double compression test (TA-XT2i texture analyzer (Stable Microsystems, Godalming, UK). Experiments were carried out by compression tests that generated a plot of force (N) versus time (s). A 25 mm diameter perplex conical-shaped probe was used to measure TPA of the Uf soft cheese samples in their cups performing five repetitions. In the first stage, the samples were compressed by 30% of their original depth. The speed of the probe was 2 cm/min. during the pretest.

2.12. Sensory evaluation

Cheese samples were organoleptically scored for flavor (50 points), body and texture (40 points) and appearance (10 points) according to the scorecard suggested by Davis (1965). Samples were judged by the staff members of the Dairy Science Department, National Research Center.

3. Results and discussion

3.1. Identification of the bacterial isolate and production of MCE

The molecular identification of the bacterial isolate was performed by Sigma company for scientific services. The obtained nucleotide sequence (Fig. 1a) was submitted to the NCBI Gene Bank with the accession number MK775302. As indicated by the phylogenetic tree (Fig. 1b), the aligned sequence showed approx. 98% identity to *Bacillus subtilis* subsp. *subtilis* strain 168.

3.2. Production of MCE by B. subtilis MK775302

B. subtilis MK775302 was tested for MCE production on three different media using submerged fermentation technique. It was observed that no MCE productivity was obtained when media 1 and 3 were used. However, by using medium 2, *B. subtilis* MK775302 produced 269 SU/ml MCE. Therefore, medium 2 was used with other components for MCE production and optimization studies.

Many authors have produced MCE from the genus *Bacillus* (Zhang et al., 2013a; Lemes et al., 2016; Ahmed et al., 2016). Many authors also have used *B. subtilis* to produce MCE (Dutt et al., 2009 used *Bacillus subtilis*; Wu et al., 2013 used *Bacillus subtilis natto*; Narwal et al., 2016 used *Bacillus subtilis* MTCC 10422; Wehaidy et al., 2016 used *Bacillus subtilis* KU710517).

3.3. Optimization of B. subtilis MK775302 MCE production by statistical factorial designs

3.3.1. Plackett-Burman design (PB)

Plackett-Burman (PB) design is an efficient screening design when the main effects of the medium components are studied. It offers a fast screening procedure and mathematically computes the significance of many factors in one experiment. Eleven factors (A-L) included culture conditions and medium components were selected for the optimization process. Milk clotting enzyme average activity for the different trials was calculated as U/ml and represented in Table 1.

a. Nucleotide Sequence

>bac seq

GCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACAGCGATTC
CGCTTCACGCAGTCGAGTTGCAGACTGCGATCCGAACTGAGAACAGATTT
GTGGGATTGGCTTAACCTCGCGGTTTCGCTGCCCTTTGTTCTGTCCATTGT
AGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCC
CCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAA
TGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAAC
ATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTGCCC
CCGAAGGGGACGTCCTATCTCTAGGATTGTCAGAGGATGTCAAGACCTGG
TAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGC
GGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGG
CGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCCTAA
CACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGT
TCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTTACAGACCAGAGAGTCGC
CTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTG
GAATTCCACTCTCCTCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACCCT
CCCCGGTTGAGCCGGGGGCTTTCACATCAGACTTAAGAAACCGCCTGCGA
GCCCTTTACGCCCAATAATTCCGGACAACGCTTGCCACCTACGTATTACCG
CGGCTGCTGGCACGTA

b. Phylogenetic tree

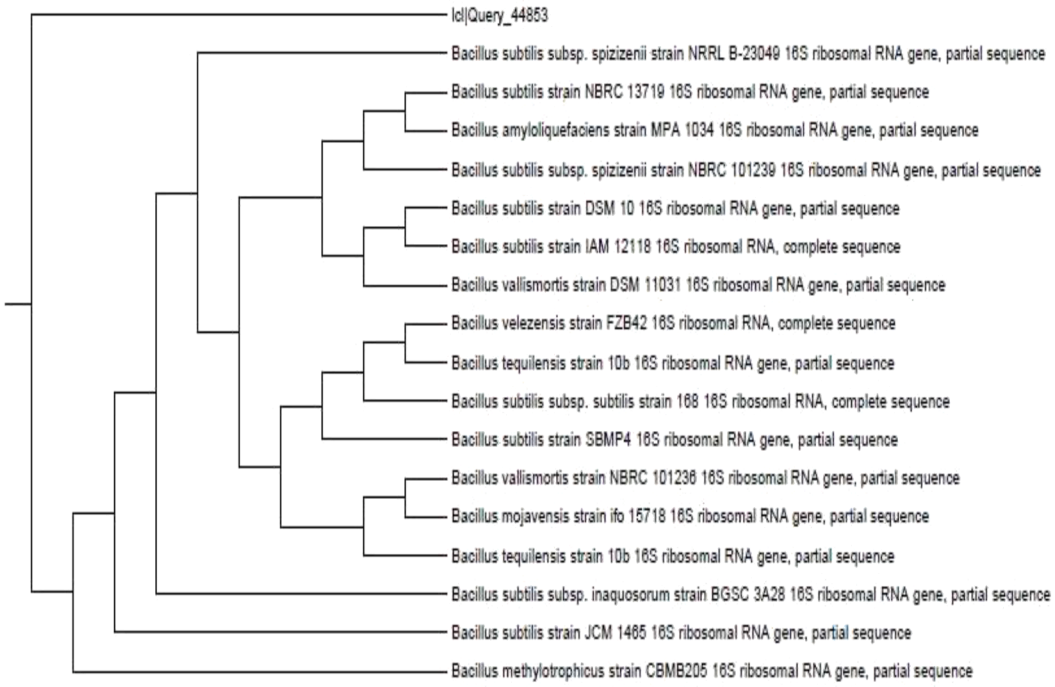

Fig. 1. Nucleotide sequence (a) and Phylogenetic tree (b) for marine sponge isolate.

The enzyme activity recorded a wide variation from (60.9–600.3 U/ml).

The ANOVA of the PB design for MCE activity is shown in Table 2, the model F-value of 14.30 implies that the model is significant. P-values less than 0.05 indicated the model terms are significant. In this case, Wheat bran, orange peel, peptone, and $MgSO_4$ are significant model terms.

Many previous reports described wheat bran as a better substrate for MCE production. It has been reported as an ideal medium component for microbial protease production (Dutt et al., 2009; Foda et al., 2012; Zhang et al., 2013b; Hang et al., 2016; Wehaidy et al., 2016). Foda et al. (2012) recorded that, wheat bran compared with other twelve industrial by-products used as substrates, resulted in the highest milk clotting activity by *Rhizomucor miehei*. Also, a medium containing 50 g/L wheat bran with xylose and yeast extract as carbon and

Table 1
Plackett–Burman experimental design for medium components and fermentation conditions for milk clotting enzyme.

Run	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Factor 7	Factor 8	Factor 9	Factor 10	Factor 11	Activity (SU/ml)
	A: Wheat bran g/f	B: Rice straw g/f	C: orange peel g/f	D:Whey ml/f	E: Peptone g/L	F: Yeast g/L	G: Lactose g/L	H: Glucose g/L	J:KH2PO4 g/L	K: MgSO4 g/L	L: CaCl2 g/L	
1	2.5	0	3	5	3	3	5	4	3	1	0.5	507.50
2	2.5	0	0	10	1.5	3	10	2	3	1	1	348.00
3	5	3	0	10	3	3	5	2	1.5	1	0.5	107.30
4	5	0	0	5	3	1.5	10	4	1.5	1	1	154.57
5	5	3	3	5	1.5	1.5	10	2	3	1	0.5	600.30
6	2.5	3	0	10	3	1.5	10	4	3	0.5	0.5	116.00
7	2.5	3	3	5	3	3	10	2	1.5	0.5	1	380.48
8	5	0	3	10	1.5	3	10	4	1.5	0.5	0.5	246.50
9	5	3	0	5	1.5	3	5	4	3	0.5	1	60.90
10	5	0	3	10	3	1.5	5	2	3	0.5	1	153.70
11	2.5	3	3	10	1.5	1.5	5	4	1.5	1	1	591.60
12	2.5	0	0	5	1.5	1.5	5	2	1.5	0.5	0.5	260.13

R^2 = 0.9449, **Adjusted R^2** = 0.8788, **Predicted R^2** = 0.6827.

Table 2
Analysis of variance (ANOVA) for PB design for milk clotting enzyme production.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	45141.27	6	7523.55	14.30	0.0052	**significant**
A-Wheat bran	7681.08	1	7681.08	14.60	0.0124	
B-Rice straw	343.47	1	343.47	0.6527	0.4559	
C-orange peel	20352.80	1	20352.80	38.67	0.0016	
E-Peptone	4688.65	1	4688.65	8.91	0.0306	
G-Lactose	268.85	1	268.85	0.5109	0.5067	
K–MgSO4	11806.41	1	11806.41	22.43	0.0052	
Residual	2631.28	5	526.26			
Cor Total	47772.55	11				

nitrogen sources caused maximum MCE productivity by *Bacillus subtilis* KU710517 (Wehaidy et al., 2016). Magnesium is essential for bacterial growth and cell division. Thus, it is used in many media for enzyme production (Webb, 1951).

The coefficient R^2 for the design was 0.9449 and indicated that the data variability could be explained by the model very well. The predicted R^2 of 0.6827 is in reasonable agreement with the adjusted R^2 of 0.8788; i.e. the difference is less than 0.2. The multiple regression analysis on the experimental data resulted in the following first-order polynomial equation to explain the milk clotting production:

R1 = +236.97833–58.69600 Wheat bran + 10.34333 Rice straw + 79.62111orange peel-76.43111 Peptone + 5.49067 Lactose + 363.85333 $MgSO_4$

The Pareto chart for MCE (Fig. 2) showed that orange peel, MgSO4, rice straw were the most influencing factors in the medium followed by lactose and KH_2PO_4. Kembhavi et al. (1993) emphasized the requirement of phosphorus for protease production by a *Bacillus subtilis* NCIM no. 64. It is utilized to form various cellular components. In agreement with our results, a phosphate concentration of 3 g/L resulted in maximal MCE productivity (Wehaidy et al., 2016). On the other hand, this concentration was higher than that investigated by

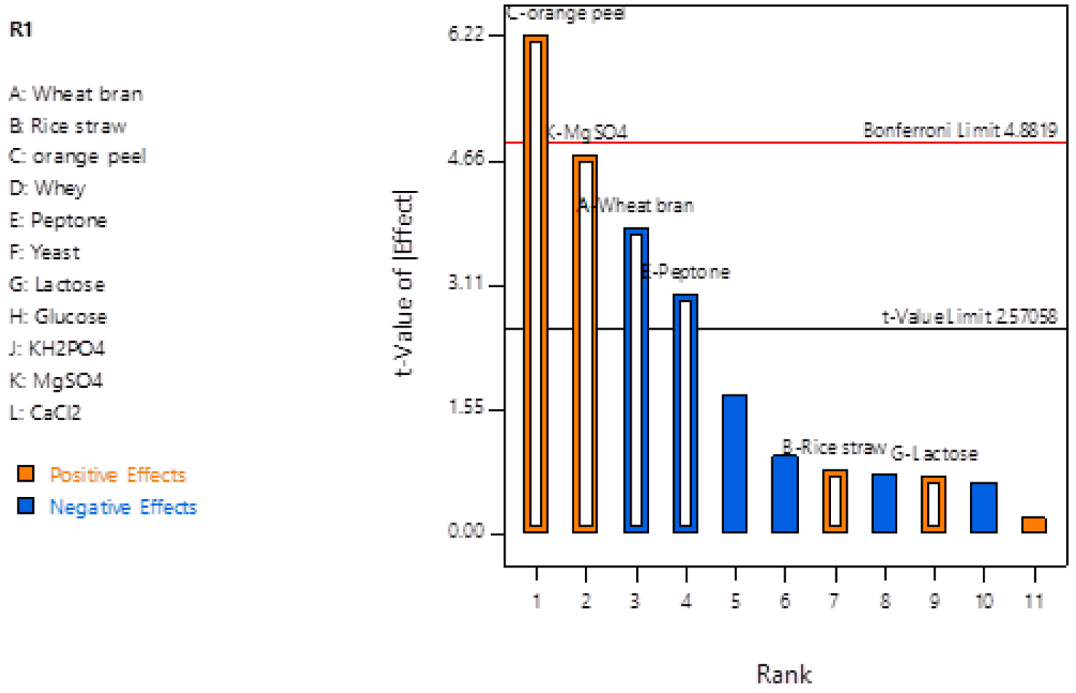

Fig. 2. Pareto chart showing the effect of each factor on milk clotting enzyme production.

other authors for maximum MCE production (Abou Ayana et al., 2015).

Orange peels and rice straw were reported to enhance the vegetative growth of the microorganism (Foda et al., 2012). Orange peels had a positive effect on MCE production and enhanced the growth of microorganisms due to their content from sugar, proteins, moisture, ash, and minerals (Al-Saadi et al., 2009).

3.3.2. Central composite design (CCD)

The most significant parameters for enhancement production of MCE based on PB design results were used in the second design (CCD) to determine the optimal concentration of each variable. The design matrix and the corresponding experimental data of the three independent variables are shown in Table 3.

The Model F-value (Table 4) of 10.05 implies the model is significant. P-values less than 0.05 indicate model terms are significant. In this design, all the linear coefficients (A, B, C) and only the interactions (AB, AC) are significant model terms, whereas none of the quadratic coefficients had a significant effect. The fit of the model was checked by the value of R^2, which was 0.9004, indicating that the model can explain about 90.04% of the variability in the response. The Adeq Precision ratio of the design (11.182) indicates an adequate signal and that this model can be used to navigate the design space. It is well known that for Adeq Precision, a ratio greater than 4 is desirable.

Table 3
Central composite design for milk clotting enzyme production.

Run	Factor 1 A: Orange peel	Factor 2 B: Rice straw	Factor 3 C: MgSO4	Actual value	Predicted value
1	4.5	4.5	4.5	486.91	437.59
2	3	6	6	410.52	394.68
3	4.5	4.5	4.5	437.46	437.59
4	3	3	3	630.83	588.62
5	6	3	6	338.24	329.20
6	4.5	4.5	7.02	416.22	408.80
7	4.5	4.5	4.5	405.76	437.59
8	6	6	3	434.29	418.07
9	4.5	4.5	4.5	446.97	437.59
10	6	6	6	329.68	354.17
11	4.5	4.5	4.5	399.42	437.59
12	1.98	4.5	4.5	481.84	513.86
13	3	6	3	358.21	349.53
14	4.5	1.98	4.5	481.84	505.78
15	4.5	4.5	1.98	443.80	476.28
16	7.02	4.5	4.5	374.06	367.10
17	3	3	6	573.77	572.27
18	6	3	3	456.48	454.60
19	4.5	4.5	4.5	453.31	437.59
20	4.5	7.02269	4.5	324.61	325.73

R^2 = 0.9004, **Adjusted R^2** = 0.8108, **Predicted R^2** = 0.5184.

Table 4
Analysis of variance (ANOVA) for CCD design for milk clotting enzyme production.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	9948.48	9	1105.39	10.05	0.0006	**significant**
A-Orange peel	2587.48	1	2587.48	23.52	0.0007	
B-Rice straw	3894.32	1	3894.32	35.40	0.0001	
C–KH2PO4	547.01	1	547.01	4.97	0.0498	
AB	2041.61	1	2041.61	18.56	0.0015	
AC	591.68	1	591.68	5.38	0.0428	
BC	188.18	1	188.18	1.71	0.2202	
A^2	1.50	1	1.50	0.0136	0.9094	
B^2	85.47	1	85.47	0.7769	0.3988	
C^2	4.40	1	4.40	0.0400	0.8456	
Residual	1100.06	10	110.01			

Table 4 shows ANOVA analysis for CCD experimental design of MCE. The closure between the actual and predicted values of MCE production using CCD confirms the high significance of the design as shown in Table 3. Three-dimensional (3D) response surface graphs Fig. 3 were plotted to illustrate the relationships between response and experimental levels of each variable for milk clotting enzyme. Each figure explains the effect of two variables while the other factor was held at zero level. The significant interaction between every two variables is clear from the figures. The multiple regression analysis of the experimental data of milk clotting enzyme resulted in the following second-order polynomial equation:

R1 = +1093.52456–79.93535 Orange peel-136.83822 Rice straw + 3.39745 $MgSO_4$ + 22.50700 Orange peel * Rice straw-12.11644 Orange peel * $MgSO_4$ + 6.83311 Rice straw * $MgSO_4$ + 0.454299 Orange $peel^2$-3.43102 Rice $straw^2$ + 0.778075 $MgSO_4^2$

The final optimized medium components for MCE production (after excluding the negative factors that had no significant effect on MCA) were as following (g/L):

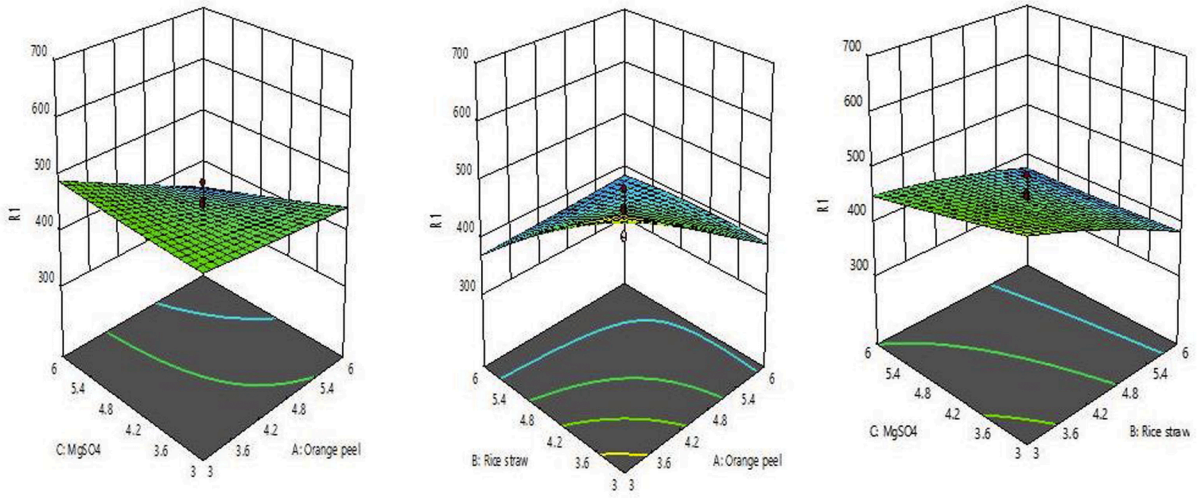

Fig. 3. Response surface 3D contour plots for milk clotting production; showing the interaction between the variables, (A) orange peel, (B) rice straw and (C) $MgSO_4$ Other variables were kept constant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

wheat bran 100; rice straw 60; orange peel 60; lactose 10; KH_2PO_4 3.0; $MgSO_4$ 3.0 and $CaCl_2$ 0.5

The results indicated that MCE experimental yield (630.83 SU/ml) and MCE statistical predicted yield (588.62 SU/ml) are closely related which confirm the validation of the model. The optimization process using the factorial designs showed a great effect on MCE productivity. After the optimization process, the MCE activity (630.8 SU/ml) was increased by 2.3-fold compared with the non-optimized medium (269 SU/ml). This result is better than those obtained by other authors when the statistical optimization methods were used. For example, a 1.94-fold increase in MCE production by *Bacillus subtilis* was achieved (Dutt et al., 2009), a 1.76-fold increase in MCE production by *Bacillus amyloliquefaciens* D4 was obtained by using Plackett–Burman design and Box–Behnken response surface methods (Zhang et al., 2013b) and a 1.7-fold increase in MCE production by *Bacillus subtilis natto* using 2^4 factorial design and CCD (Wu et al., 2013). The main medium components, orange peels, and rice straw are considered waste materials that are hardly removed. So, the use of these substrates in the production medium offered the advantages of lowering the production costs and solving environmental pollution problems.

3.4. Characterization of B. subtilis MK775302 crude MCE

3.4.1. Optimum temperature

As shown in Fig. 4, the temperature profile of MCE showed that the activity was increased by increasing the reaction temperature up to 70 ˚C. Further increase in reaction temperature resulted in a decrease in MCA. This might be due to the denaturation of the enzyme at higher temperatures. This optimum temperature is higher than those reported by some authors (55 ˚C for MCE from *Bacillus sphaericus* and 60 ˚C for MCE from *Penicillium oxalicum*) (Hashem, 1999; El-Bendary et al., 2007). On the other hand, other authors have reported higher optimum temperatures for MCE (75 ˚C from *Bacillus licheniformis* and 85 ˚C from *Aloe variegate* and *Bacillus subtilis*) (Ahmed and Helmy, 2012; Wehaidy et al., 2016).

3.4.2. Optimum pH

The enzyme proved to be an acidic protease. The maximum MCA (800 SU/ml) was obtained at pH 5.0 (data not shown). Above this pH, the activity decreased gradually, this might be due to the unsuitability of high pH for the enzyme activity and the denaturation of the enzyme protein at higher pH. A Similar optimum pH value was reported by other authors (MCE from *Rhizomucor miehei* by Foda et al., 2012; MCE from *Bacillus subtilis* by Wehaidy et al., 2016; MCE from *Cynara cardunculus* by Zikiou and Zidoune, 2019). The reduction in milk pH resulted in a decrease in the milk-clotting time and an increase in MCA (Elmazar et al., 2012). This higher activity of MCE in acidic pH is highly advantageous in industrial applications (El-Sayed et al., 2013). Other authors have also reported a decline in MCA at pH near neutrality (Chazarra et al., 2007; Wehaidy et al., 2016).

3.4.3. Effect of NaCl on MCA

Sodium chloride is usually used in cheese manufacture. As it plays important roles in cheese ripening, controlling microbial growth, consistency of curd, absorption of excess water and controlling cheese texture (Afsharnezhad et al., 2019).

As illustrated in Fig. 5, the enzyme retained 100% activity at concentrations from 0.1 to 1% NaCl. At higher NaCl concentrations, MCA was decreased gradually. Many authors also noticed a loss in MCA at high NaCl concentrations (El-Tanboly et al., 2000; El-Bendary et al., 2009; Afsharnezhad et al., 2019). It was suggested that the activity loss at high concentrations of NaCl might be due to enzyme denaturation caused by the high concentrations of salt (Afsharnezhad et al., 2019). So, to avoid the loss of MCA, it is recommended to use low NaCl concentration in the cheese manufacture.

3.4.4. Proteolytic activity (PA) and MCA/PA ratio

The proteolytic activity (PA) and MCA/PA ratio are important parameters to judge the efficiency of MCE in cheese production. MCE with low PA and high MCA/PA ratio is preferred for cheese manufacture, to avoid proteolysis of the produced cheese (Wehaidy et al., 2016; Ahmed et al., 2018). Also, He et al. (2012) reported that MCA/PA ratio is a very important criterion for evaluating milk clotting enzyme as a rennet substitute. Since MCE with high PA would extremely hydrolyze casein causing reduction of cheese yield, weaknesses of flavor and texture. In this study, MCA/PA ratio and PA were calculated as 2353 and 0.34 U/ml, respectively at the optimum reaction conditions. This MCA/PA ratio is much higher than those reported by Ismail (2019) for calf rennet extract (MCA/PA was 1470, MCA was 455.7, PA was 0.31 U/ml) and for commercial calf rennet (MCA/PA was 613.2, MCA was 288.2, PA was 0.47 U/ml). Much lower MCA/PA ratio (9.7) was obtained by Lizardi-Jiménez et al. (2019) for crude MCE from *Rhizopus microspores* var. *chinensis*. Therefore, the crude extract from *B. subtilis* MK775302 with its high milk clotting activity and low proteolytic activity is suitable for cheese manufacture and can be considered as an appropriate rennet substitute.

3.5. Physico-chemical properties of white soft cheese

3.5.1. Moisture content

The moisture contents of fresh cheese samples were 65.54 and 65.4% for the commercial rennet (control), and *B. subtilis* MK775302 crude extract cheese, respectively. The moisture contents of the two treatments are almost similar. It is well known that the high acidity

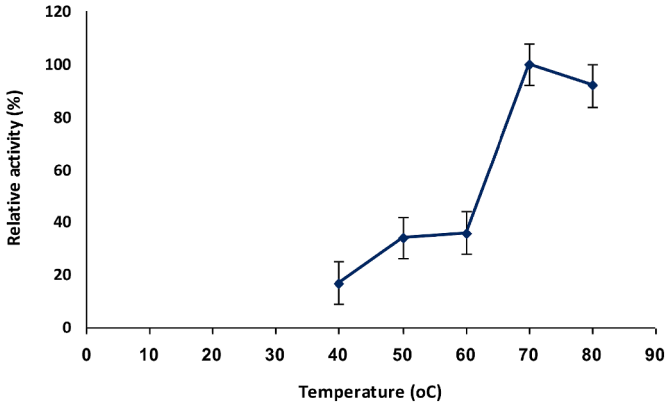

Fig. 4. Effect of reaction temperature on *Bacillus subtilis* MK775302 MCA.

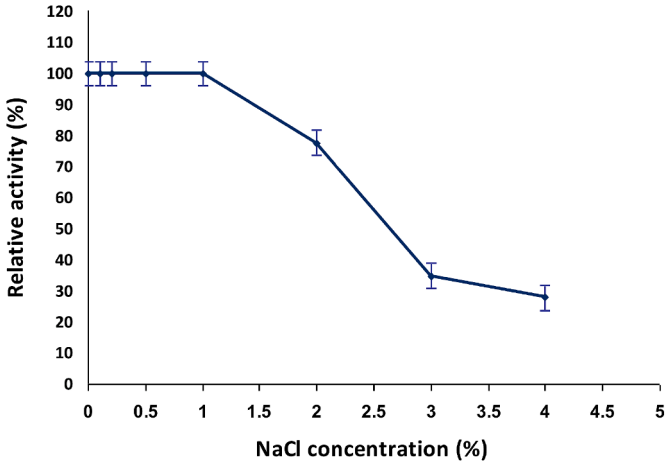

Fig. 5. Effect of NaCl concentration on *Bacillus subtilis* MK775302 MCA.

due to starter growth in the milk reduces the coagulation time, and decreases the moisture content (El-Abd et al., 2003).

3.5.2. Titratable acidity (TA %)

Changes in titratable acidity (TA) of white soft cheese made from commercial microbial rennet (control) and *B. subtilis* MK775302 MCE are presented in Fig. 6. The results showed that TA of fresh UF-white soft cheese samples were 0.17 and 0.20% for control and crude enzyme cheeses, respectively. The corresponding values of acidity after 4 weeks of storage were 2.14 and 2.44% for the same treatments. The obtained data indicated that white soft cheese made with crude MCE had higher TA than control cheese. A similar observation was obtained by Ahmed et al. (2016) when crude MCE from *Bacillus stearothermophilus* was compared with a commercial coagulant. This might be due to the accumulation of lactose degradation products such as lactic acid and other volatile acids (Merheb-Dini et al., 2010; Lemes et al., 2016). TA was increased in both treatments during the storage period (28 days), since it was increased from 0.17 to 2.14% and from 0.20 to 2.44% for the control and crude enzyme, respectively. Increasing the acidity during storage is due to lactose fermentation in the presence of starter cultures with the production of acidic compounds and also due to degradation of intermediates components of protein and fat (El-Din et al., 2010; El-Kholy, 2015; Khalifa and Wahdan, 2015; Ahmed et al., 2016).

3.5.3. pH values

The pH of protein-rich food is an important step in the production of cheese. The final quality of cheese can be affected by the type of coagulant used. The results in Fig. 7 showed that pH of fresh UF-white soft cheese samples was 6.11 and 5.90 for the control, and *B. subtilis* MK775302 crude extract cheese, respectively. There was a considerable decrease in pH of the control and crude enzyme cheese treatments throughout the storage period (28 days) since it was decreased from 6.11 to 4.2 and from 5.90 to 4.0, respectively. The decrease in pH values during maturation may be due to the presence of bacteria (mainly lactic acid bacteria) that resist pasteurization and the presence of starter bacteria after pasteurization (Guo et al., 2011; Ahmed et al., 2016). This decrease in pH is very important to prevent pathogenic bacterial growth during ripening (Ahmed et al., 2016).

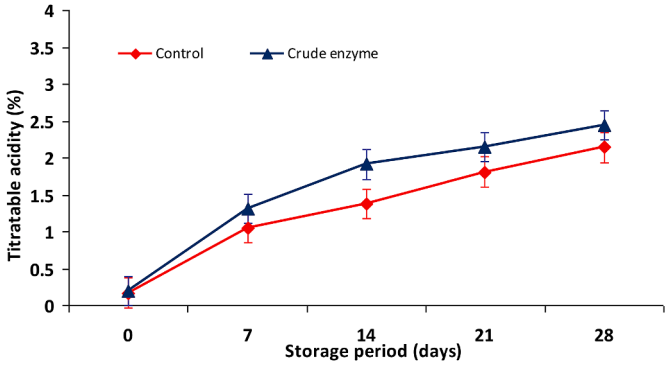

Fig. 6. Titratable acidity percentage of UF white soft cheese treated with different coagulants during the storage period.

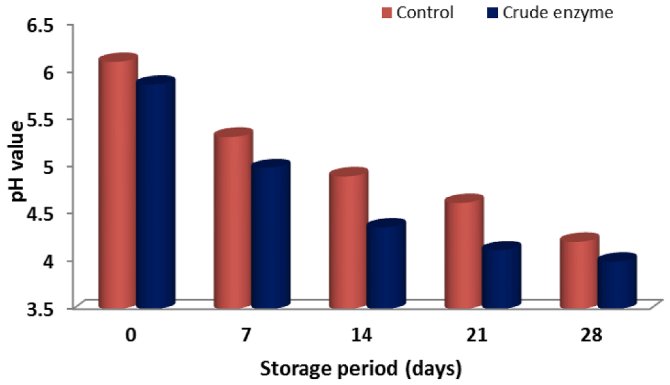

Fig. 7. pH value of UF white soft cheese treated with different coagulants during the storage period.

3.5.4. Total nitrogen (TN %)

The total nitrogen in UF-white soft cheeses with the two different coagulants was illustrated in Fig. 8. The results indicated that TN contents in the two experimented cheese samples were gradually increased with the advancement of the storage period and this might be due to the decrease in the moisture contents in cheese. Similar findings were observed by other authors (El-Kholy, 2015; Hattem and Hassabo, 2015).

3.6. Ripening indices of cheese

3.6.1. Soluble nitrogen (SN or WSN %)

The soluble nitrogen (SN) is regarded as a ripening index for cheese as it reflects the extent of proteolysis (Chen et al., 2003). Fig. 9 includes the SN in the experimented UF soft cheese samples during a period of 28 days at 5 °C. It was clear that in both cheese treatments the rate of accumulation of SN increased with the prolongation of the storage period and this might be attributed to increasing the rate of proteolysis. Similar observations were noticed by other authors (Khalifa and Wahdan, 2015; Meng et al., 2018). The fresh cheese of the crude enzyme extract had higher SN content (0.18%) compared to the control (0.17%). After 28 days, the crude enzyme cheese sample also had higher SN content (0.66%) than the control sample (0.58%). This could be due to the activity of proteinases and peptidases released from *B. subtilis* MK775302 crude MCE, which resulted in higher proteolysis in the cheese sample.

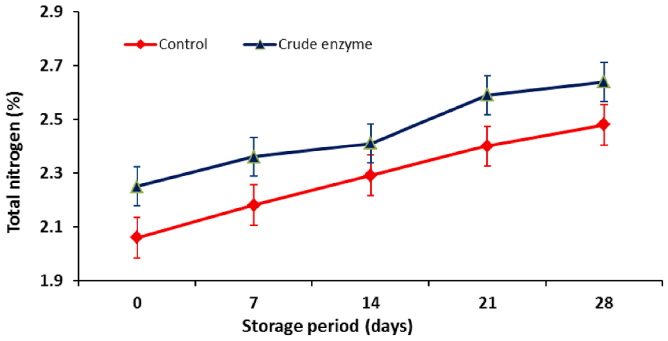

Fig. 8. Total nitrogen percentage of UF white soft cheese treated with different coagulants during the storage period.

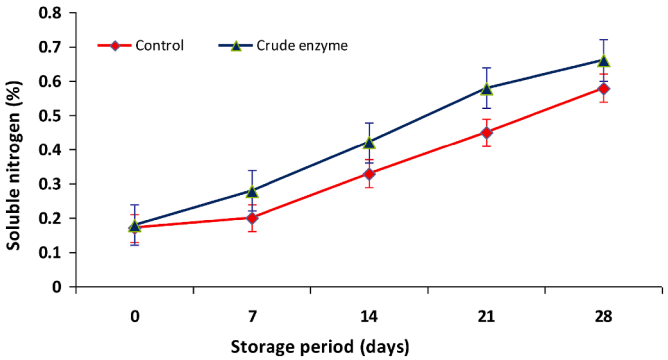

Fig. 9. Soluble nitrogen percentage of UF white soft cheese treated with different coagulants during the storage period.

3.6.2. Total volatile fatty acids content (TVFA)

The rate of accumulation of TVFA (ml of 0.1 N NaOH/100 g cheese) increased with increasing the storage period in both treatments. The values of TVFA in fresh samples were 17.34 and 18.81 for the control and crude enzyme cheese, respectively. The corresponding values after 4 weeks were 94.07 and 94.35 for the same treatments. Therefore, the crude enzyme cheese possessed a higher value of TVFA compared to the control. These variations could be due to the different lipolytic activity of the two coagulants. Other authors also observed a gradual increase in TVFA contents throughout the ripening period which might be attributed to increasing the rate of lipolysis during the ripening period (El-Hawary et al., 2015; Hattem and Hassabo, 2015).

3.7. Textural profile analysis (TPA)

The results of different TPA parameters are presented in Table 5. During the storage period, the cheese hardness was gradually increased in both treatments. However, the crude enzyme cheese exhibited lower hardness than the control treatment. Awad (2016) also reported an increase in the hardness of Karish cheese during the storage period which is in agreement with the present results. Changes in hardness can be attributed to the changes in the total solids content of cheese, as a closed relation was apparent between hardness and the total solids. However, this doesn't exclude the effect of changes in acidity on the observed hardness. The crude enzyme cheese treatment exhibited lower gumminess than the control cheese. The gumminess also was affected by two factors, the coagulant and the storage period. Generally, during the storage period (28 days) gumminess tended to increase in all treatments. Similar to gumminess, the chewiness of soft cheese samples from both treatments was increased up to 28 days of storage. The control cheese also exhibited higher chewiness than the crude enzyme treatment. This difference in textural properties between samples might be due to the type of coagulant and the difference in free amino acids and fatty acids contents. Changes in texture profile during the storage period can be generated by proteolysis, glycolysis, lipolysis and pH changes (Lemes et al., 2016).

3.8. Sensory evaluation

The acceptability of both treatments was increased with extending the storage period. After 28 days storage period, the general acceptability of cheese samples as total scores were 90 and 86.5 points for the control and crude extract cheese samples, respectively (Fig. 10).

Table 5
Textural profile analysis of Soft cheese treated with different coagulants during ripening.

Textural properties	Ripening Period (day)	Treatments	
		Control	Crude extract
Hardness N	**0**	3.60	2.00
	7	3.60	2.30
	28	4.00	2.60
Cohesiveness (B/A area)	**0**	0.719	0.640
	45	0.729	0.630
	90	0.747	0.677
Springiness Mm	**0**	0.777	0.642
	7	0.791	0.639
	28	0.797	0.751
Gumminess N	**0**	2.589	1.280
	7	2.624	1.449
	28	2.989	1.761
Chewiness N/mm	**0**	2.011	0.822
	7	2.074	0.926
	28	2.382	1.322

N = Newten, mm = Millimeters.

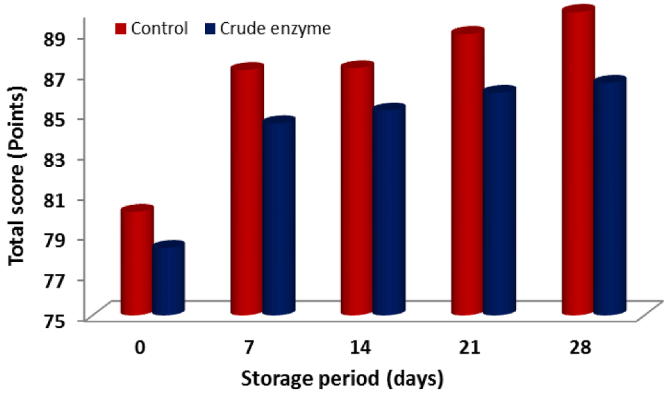

Fig. 10. Sensory evaluation during storage of UF white soft cheese treated with different coagulants during the storage period.

These results indicated that the cheese made by *B. subtilis* MK775302 crude extract is acceptable and had a slightly lower score compared to the control cheese sample (only 3.5 points difference). This variation in acceptability may be due to the differences in the free fatty acids and amino acids contents of each treatment which have a significant influence on the flavor of the produced cheese. Usually, cheese made by MCE derived from *Bacillus* has unpleasant taste due to the high bitter peptides contents (Singh et al., 2005; Meng et al., 2018). However, the cheese made by *B. subtilis* MK775302 crude extract in this study had no bitterness even after 28 days storage period.

4. Conclusions

It can be concluded that *B. subtilis* MK775302, isolated from the marine sponge *Pseudoceratina arabica* is an excellent producer for MCE. The optimization process using the PB and CCD factorial designs showed a great effect on MCE productivity. After optimization, productivity was increased by 2.3-fold when compared with the non-optimized medium. The crude extract from *B. subtilis* MK775302 was used for cheese manufacture and yielded acceptable UF white soft cheese with higher ripening index, higher flavor intensity, and lower pH compared to the commercial microbial rennet. So, this study recommended using the crude enzyme extract from *B. subtilis* MK775302 as a promising rennet substitute that can replace the commercial coagulants in the local markets. The exploitation of orange peel and rice straw in the production medium offered the advantages of lowering the production costs and solving environmental pollution problems.

Declaration of competing interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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