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Statistical Optimization of Milk Clotting Enzyme Biosynthesis by *Mucor mucedo* KP736529 and its Further Application in Cheese Production

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ABSTRACT

Microbial sources of bioagents are coinciding with the attempts to find cheap alternative substrates for high production. Rennet is the golden element in the dairy industry, particularly cheese making. Commonly, the use of statistical models to optimize culture medium components and conditions has increased in present-day biotechnology, due to its ready applicability and aptness. The present study adopted statistical approach for Milk Clotting Enzyme (MCE) production by *Mucor mucedo* KP736529 which was isolated, selected and molecularly identified, as the most potent active strain in MCE biosynthesis. Screening of various medium constituents using the statistical Plackett-Burman design showed glucose and KH_2PO_4 as the significant factors affecting MCE biosynthesis. Optimizing MCE production by the central composite design led to 6.12 fold increase in MCE production, compared with initial activity. Domiati cheese was manufactured using the *M. mucedo* KP736529 enzyme (E-cheese) or using commercial calf rennet (C-cheese) as control. During two months under pickling conditions, the yield and chemical properties of E-cheese were better than those of C-cheese. The fungal enzyme (MCE) showed proteolytic activity higher than calf rennet with the absence of taste defects such as bitter taste. Organoleptically, E-cheese scored the highest points.

Key words: Rennet, milk-clotting enzyme, domiati cheese, central composite design, medium components, *M. mucedo* KP736529

INTRODUCTION

Rennet is a complex of enzymes produced in the abomasums of milk-fed calves and lambs and acts the corner stone of milk coagulation in cheese making industry (Hutkins, 2006). Rennet contains many enzymes, chymosin or rennin (EC 3.4.23.4) is the major milk-clotting component of natural rennet preparations as its activity amounts to 90% of the total potency, there is also protease that coagulates the milk, causing it to separate into solids (curd) and liquid (whey), in addition to other important enzymes, e.g., pepsin and lipase (Kumar *et al*., 2010; El-Hersh *et al*., 2014).

According to the projections of OECD/FAO (2011), by 2020 milk production will reach 852898×10^3 t, indicating the continual increment of the world production. Approximately a third

of the world's milk production is used for cheese manufacturing (Farkye, 2004). Unfortunately, animal-derived milk coagulants are not sufficient to cover the cheese production demand, since only 20-30% of the world demand for milk-clotting preparation is covered by calf rennet (Jacob *et al*., 2011). Moreover, calves slaughtering is a problem threatening the production of meat (Kumar *et al*., 2010).

Because of the limited availability of mammalian rennet and most rennet of plants have proved to be unsuitable because they impart a bitter taste to cheese. Microbial sources can substitute for animal rennet. Therefore, the demand has prompted increased research efforts in the manufacture of recombinant and microbial milk-clotting enzyme (Poza *et al*., 2004). This alternative amazing source appears to be more promising because its production is cheaper, biochemical diversity is greater and genetic modification is easier (El-Tanboly *et al*., 2013). Many fungi are used for this purpose; nevertheless, milk-clotting enzyme of *Mucor* spp. e.g., *M. bacilliformis* (Machalinski *et al*., 2006) and *M. mucedo* DSM 809 (Yegin *et al*., 2010) have good characteristics. Another advantage of using microbial rennet source is that none of the available reports contains any indication that the use of microbial rennet, especially from *Mucor* sp., in cheese manufacture is unsafe (Tubesha and Al-Delaimy, 2003).

The aim of this work was to obtain fungal isolates capable of production milk-clotting enzyme, to be used as an economic alternative cheese coagulant; Domiati cheese was manufactured with the produced milk-clotting enzyme as a cheese example. For evaluating, the manufactured cheese with fungal milk-clotting enzyme and cheese made with commercial calf rennet were chemically and organoleptically, analyzed during two months of pickled storage.

MATERIALS AND METHODS

Fungal isolation and molecular identification: Among several fungi isolated from spoiled milk on skim milk agar, *Mucor* strain was selected based on the diameter of clear zone on skim milk agar as well as Milk-Clotting Activity test (MCA). The selected fungus was identification upon the morphological and microscopic investigations as *Mucor mucedo*, with the aid of Domsch *et al*. (1980).

The present fungal strain was molecularly identified in a previous study, the isolation of genomic DNA, amplification and 18S rRNA sequencing was performed by Macrogen Korea Company Gasan-dong, Geumchen-gu, Seoul, Korea (http://www.macrogen.com). The sequencing product was deposited in the GenBank database under accession numbers KP736529. The 18S rRNA gene sequence (667 bp) of the strain was aligned with the corresponding 18S rRNA sequences of the type strains of representative members of the fungi retrieved from the GenBank, databases using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul *et al*., 1997).

Submerged Liquid State Fermentation (LSF) medium and time course profile: Stock culture of the fungus was maintained on Sabouraud agar slants. To recover the spores, plates of Sabouraud agar were inoculated and incubated at 35°C for 72 h, the inoculum was obtained by scraping the agar surface in the presence of sterile water. The spore suspension was adjusted to account 10^6 spore mL^{-1}, by Neubauer Chamber. The composition of the proposed fungal rennet production medium contained (g L^{-1}), glucose (18), peptone (8), casein (4), KH_2PO_4 (2), olive cake (4) and corn steep liquor (CSL) (4). The initial pH was adjusted to 5.0. The medium was sterilized at 121°C for 15 min. The 10% (v/v) of inoculum was transferred to 500 mL Erlenmeyer flasks containing 90 mL of sterilized liquid fermentation medium. Incubation was carried out at 35°C under shaking at 150 rpm. For determination of the optimum incubation period, time course of 72 h was carried out, the milk-clotting activity was determined after every 12 h.

Plackett-Burman design for selection of significant variables: The six independent nutritional variables (glucose, peptone, casein, KH_2PO_4, olive cake and corn steep liquor) of LSF medium were screened at high (+1) and low (-1) levels as well as the center point (0), using the two-level Plackett-Burman design in twelve combinations plus three center points (Table 1). The relation between the coded and actual values were described as in the following equation:

$$x_i = \frac{(X_i - X_0)}{\Delta X_i}$$

where, x_i is the coded value of an independent variable, X_i is the real value of an independent variable, X_0 is the real value of an independent variable at the center point and ΔX_i is the step change value. The design was performed in triplicates and the average of milk-clotting activity units were treated as responses. The main effect of each variable was simply calculated as the difference between the average of measurements made at the high level (+1) and the average of measurements observed at the low level (-1) of that factor, using the following first order model equation:

$$\text{Effect}(X_i) = \frac{2\left(\sum M_i^{+1} - M_i^{-1}\right)}{N}$$

where, X_i is the effect of the tested variable. M_i^{+1} and M_i^{-1} represent fungal rennet production from the trials where the variable (X_i) measured was present at high and low concentration, respectively and N is the total number of trials.

Table 1: Plackett-Burman design with actual (g L^{-1}) and codified values of medium components and corresponding rennet activity by *Mucor mucedo* KP736529

	Medium component						Fungal rennet (MCA)	
Run	Glucose (X_1)	Peptone (X_2)	Casein (X_3)	KH_2PO_4 (X_4)	Olive cake (X_5)	CSL (X_6)	Response	Fitted
1	25 (1)	4 (-1)	6 (1)	1 (-1)	2 (-1)	2 (-1)	4.17	4.01
2	25 (1)	12 (1)	2 (-1)	3 (1)	2 (-1)	2 (-1)	7.69	7.35
3	11 (-1)	12 (1)	6 (1)	1 (-1)	6 (1)	2 (-1)	0.00	-0.56
4	25 (1)	4 (-1)	6 (1)	3 (1)	2 (-1)	6 (1)	7.14	6.91
5	25 (1)	12 (1)	2 (-1)	3 (1)	6 (1)	2 (-1)	6.45	6.79
6	25 (1)	12 (1)	6 (1)	1 (-1)	6 (1)	6 (1)	3.25	3.32
7	11 (-1)	12 (1)	6 (1)	3 (1)	2 (-1)	6 (1)	2.51	2.90
8	11 (-1)	4 (-1)	6 (1)	3 (1)	6 (1)	2 (-1)	2.00	2.48
9	11 (-1)	4 (-1)	2 (-1)	3 (1)	6 (1)	6 (1)	3.23	2.58
10	25 (1)	4 (-1)	2 (-1)	1 (-1)	6 (1)	6 (1)	3.23	3.55
11	11 (-1)	12 (1)	2 (-1)	1 (-1)	2 (-1)	6 (1)	0.00	0.10
12	11 (-1)	4 (-1)	2 (-1)	1 (-1)	2 (-1)	2 (-1)	0.00	0.23
13	18 (0)	8 (0)	4 (0)	2 (0)	4 (0)	4 (0)	3.06	3.22
14	18 (0)	8 (0)	4 (0)	2 (0)	4 (0)	4 (0)	3.14	3.22
15	18 (0)	8 (0)	4 (0)	2 (0)	4 (0)	4 (0)	3.47	3.22

Number in parentheses are the corresponding coded values

Statistical design for fungal rennet production: Fermentation factors affecting fungal rennet production were optimized using the full Central Composite Design (CCD). The significant variables (Glucose, X_2 and KH_2PO_4, X_5) from screening experiment, in addition to the initial pH (X_7) of the culture medium were further investigated for studying the interaction among the three variables. The other medium components were kept at their minimal concentrations. Each of the three factors were examined at five different levels, at the center point and an axial point located at a specified distance (alpha, " = 1.682) from the design center in each direction on each axis. According to the applied design, 20 combinations were executed, the actual and code levels are shown in Table 2 and the observations of the three factors were fitted to the following second order polynomial quadratic model:

$$Y=\beta_0+\Sigma\beta_i X_i+\Sigma\beta_{ij}X_iX_j+\beta_{ii}X_i^2$$

where, Y is the predicted response, $\$_0$ is the model constant, X_i and X_j are independent variables, $\$_i$ is linear coefficients, $\$_{ij}$ is cross product coefficients and $\$_{ii}$ is the quadratic coefficients.

The statistical analysis of the results was performed with the aid of statistical software packages Design Expert (version 7, State-Ease, U.S.A.) and Minitab (version 16, Minitab Inc., USA).

Rennet extraction and assay: After specific time of cultivation, enzyme was separated from the mycelium through a Filtrak GmbH paper filter (Ø 90 mm). After centrifugation

Table 2: Coded and actual values of the independent variables used in central composite design matrix with experimental values of fungal rennet production by *Mucor mucedo* KP736529

	Coded value			Actual value			Fungal rennet (MCA)	
Run	Glucose (X_2)	KH_2PO_4 (X_5)	Initial pH (X_7)	Glucose (g LG^1)	KH_2PO_4 (g LG^1)	Initial pH	Response	Fitted
1	-1	-1	-1	20	2	4	5.67	5.90
2	1	-1	-1	30	2	4	14.29	14.06
3	-1	1	-1	20	4	4	7.07	7.02
4	1	1	-1	30	4	4	9.52	9.55
5	-1	-1	1	20	2	6	13.33	13.31
6	1	-1	1	30	2	6	14.29	14.34
7	-1	1	1	20	4	6	14.67	14.90
8	1	1	1	30	4	6	10.53	10.30
9	-1.682	0	0	16.591	3	5	11.76	11.53
10	1.682	0	0	33.409	3	5	14.29	14.52
11	0	-1.682	0	25	1.318	5	12.50	12.48
12	0	1.682	0	25	4.682	5	10.00	10.02
13	0	0	-1.682	25	3	3.318	5.61	5.62
14	0	0	1.682	25	3	6.682	12.50	12.48
15	0	0	0	25	3	5	10.30	10.05
16	0	0	0	25	3	5	10.10	10.05
17	0	0	0	25	3	5	9.90	10.05
18	0	0	0	25	3	5	10.00	10.05
19	0	0	0	25	3	5	9.89	10.05
20	0	0	0	25	3	5	10.11	10.05

(5000 rpm), the filtrate was used for enzyme assay. Milk-Clotting Activity (MCA) was calculated according to Otani *et al*. (1991), using the following equation:

$$\text{MCA units} = 2400/T \times S/E$$

where, T is the time necessary for the curd fragment formation (sec), S is volume of milk substrate (mL), E is the volume of enzyme extract (mL). At least three measurements were made for each experiment and the data given is an average of these results.

Preliminary cheese manufacturing for determination of fungal rennet concentration: The appropriate concentration of milk-clotting enzyme of *M. mucedo* KP736529 for curd production was determined, in which 500 mL fresh whole buffalo milk was salted with 4% NaCl, then heated at 75°C for 1 min and immediately cooled to 38°C. Milk was divided into 5 equal portions and the fungal rennet (MCE) was added at the rate of 70, 150, 320 and 650 MCA units in liquid form in comparison to 1 mg powder calf rennet (standard rennet, obtained from Ch-Hansen, Copenhagen, Denmark) per 100 mL milk. All treatments were incubated at 38°C until complete coagulation. The gelation or coagulation time was followed visually and determined as the time from the addition of rennet or MCE until complete curding (Van den Bijgaart, 1988). Curd tension (g cm^2) was estimated by Westphal Balance according to El-Shabrawy (1973). Curd syneresis was estimated after 24 h as volume of whey (mL/100 mL milk) which drained or expelled from curd through Whatman No.1 filter paper according to Van Dijk (1982). Weight of fresh curd after 24 h was taken as cheese yield. The body properties were also determined.

Ultra Filtration (UF) Domiati cheese manufacturing: UF-Domiati cheese was made from pasteurized buffalo milk according to the method described by Renner and Abd El-Salam (1991). The fungal rennet (MCE) was added based on the results obtained in preliminary manufacture experiment, another set of cheese made by calf rennet was used for comparison. All cheese samples were pickled in preheated 10% salty whey in polyethylene bags and stored at room temperature (22-25°C) and analyzed at 15 days interval beginning from zero time (fresh cheese) until 60 days of storage. All treatments were carried out in triplicates. Fat, Total Nitrogen (TN), Non-Protein Nitrogen (NPN), Soluble Nitrogen (SN), Titratable Acidity (TA) and Moisture Contents of cheese samples were determined according to AOAC (2007). Total Volatile Fatty Acids (TVFAs) was estimated as mL 0.1 N NaOH/10 g cheese, according to Koskowiski (1987). The spectrophotometric method of Vakaleris and Price (1959) was used for measuring tyrosine and tryptophan contents in cheese samples and expressed in mg %.

Organoleptic properties of the different treatments were evaluated by 10 panelists using scale of 0-10 points for visual aspects, 0-40 points for consistency characteristics and 0-50 points for flavor and taste.

RESULTS AND DISCUSSION

Fifteen fungal isolates, including the genus of *Aspergillus* (5), *Hirsutella* (1), *Mucor* (3) *Penicillium* (3) and *Trichoderma* (3) were isolated from spoiled milk. Based on screening of renneting profile of the fungal isolates on both skim milk agar and LSF medium, *Mucor* sp. was selected for MCE production as the potent strain, in which the fungus showed highest clear zone

on skim milk agar as well as high Milk-Clotting Activity (MCA) on LSF medium compared with the other isolates. The quantitative determination of fungal rennet production of the tested strain recorded 3.51 MCA, after 2 days of incubation.

The most active fungal isolate in MCE production showed the typical morphological and microscopic characteristics of *M. mucedo*, this was confirmed by the molecular identification. The sequencing product of the fungus was deposited in the GenBank database under accession numbers KP736529. Comparing the nucleotide sequence with other fungal species sequences using BLAST algorithm on NCBI GenBank with the present *M. mucedo* KP736529 (Table 3) revealed 100% similarity with *M. mucedo* strain CBS 987.68 (accession number JN939204.1). Query coverage (percent of the query sequence that overlaps the subject sequence) reached 98%. The nucleotide sequence indicated also that the present strain is very closely related to all *M. mucedo* strains with not less than 99% similarity to presently investigated *M. mucedo* strain. However, other *Mucor* species showed lower similarity (95%). Therefore, it is proposed that this strain should be included in the genus *Mucor* as *M. mucedo* KP736529, the taxonomic classification of the fungus is Eukaryota, Fungi, Dikarya, Zygomycota, Zygomycetes, Mucorales, Mucoraceae, *M. mucedo*.

The advantage of using natural microbial rennet producers over the genetically manipulated or ones that have been isolated from a different environmental set-up is the easier adaptation and succession when inoculated into the medium containing similar composition. In addition, the genetic manipulation to improve the enzymes production is not stable, therefore, the medium manipulation was found to be more stable and effective procedure for high productivity of milk-clotting enzymes. However, the fungal physiological state, during the process of fermentation, may not always be kept at its optimum for enzyme production, the physiological balance may be set back from productive to non-productive cell growth or forward to unwanted sporulation, leading in both cases to decreased enzyme productivity. For the above reasons, after the selection of high rennet producing fungi, the main task is the development and maintenance of conditions for maximal enzyme production.

Rennet biosynthesis as a function of time: The results of the periodic fungal rennet biosynthesis (Fig. 1) show that the biosynthesis was achieved earlier after 12 h of incubation on broth medium. Maximum MCE biosynthesis was observed after 48 h of incubation by the tested *M. mucedo* KP736529 with MCA being 3.51 SU. The renneting activity after 60 h was sharply reduced by about 47% from the maximum production, being 1.86 MCA, with continual reduction to the end of fermentation period. However, two days of incubation is reasonably accepted from the bioprocessing point of view, as compared with natural calf rennet extracted from the innermucosa of the fourth stomach chamber. So, this period was considered in the rest of the study.

Table 3: Similarity of 18S rRNA between *Mucor mucedo* strain SEE1 and the most related representatives of the genus *Mucor* presented on NCBI BLAST web services homepage (blast.ncbi.nlm.nih.gov)

Mucor species	Accession	Query coverage (%)	Similarity to *M. mucedo* (%)
Mucor mucedo strain CBS 987.68	JN939204.1	98	100
Mucor mucedo NRRL3635	AF113470.1	96	99
Mucor mucedo strain CBS 640.67	JN939202.1	98	99
Mucor mucedo strain CBS 640.67	HM849687.1	98	99
Mucor mucedo strain KACC 46082	JN315044.1	97	99
Mucor mucedo strain KACC 46084	JN315045.1	97	99
Mucor aligarensis strain CBS 993.70	JN206461.1	98	95
Mucor flavus strain CBS 126.70	JN206469.1	98	95

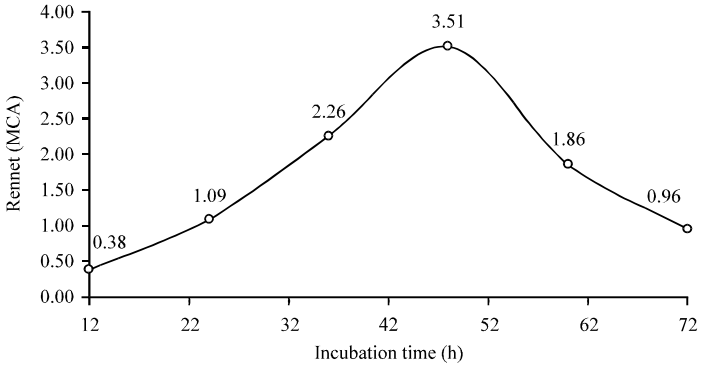

Fig. 1: Time course of the renneting profile by the isolated *Mucor mucedo* KP736529

Screening the components of fungal rennet production medium using Plackett-Burman: Plackett-Burman design has been adopted at two levels for studying the effect of six variables on the fungal rennet production. The design matrix for screening of the significant variables and the corresponding response are shown in Table 1, in which, the run number 2 recorded the highest fungal rennet production (7.69MCA). High degree of similarity was observed between the predicted (fitted) and experimental (response) values of rennet which reflects the accuracy of the results.

The adequacy of the model was calculated and the variables evidencing statistically significant effects were screened by ANOVA (Table 4). The model error separates the experimental error into its components; the lack of fit and the random (pure) error. However, the lack of fit error did reveal significant effect ($p = 0.133$) which means the high precision of the design. The main effect recorded significant p-value, this is an evidence of the significant effect of the different selected medium components. However, the main effect test is nonspecific and will not allow for a localization of specific mean pair wise comparisons. A main effect test will merely look at whether overall there is something about a particular factors that is making a difference. Therefore, ANOVA provides detailed data about every single factor, in which, glucose and KH_2PO_4 were determined to be significant factors; both of them recorded positive effect (4.032 and 3.062, in sequence). Their p-values being less than 0.05. The lower p-value (less than 0.05) indicate the more significant factor on the production process. To confirm these results, the magnitude and the importance of an effect were determined using Pareto chart of the effects (Fig. 2a). The chart displays the absolute value of the effects and draws a reference line on the chart, any effect that extends past this reference line is potentially important. In this respect, effects of glucose and KH_2PO_4 showed potential importance. Another confirmation was made by the points of normal probability plot of residuals (Fig. 2b), that form a straight line, indicating that the residuals are normally distributed. If the points on the plot depart from a straight line, the normality assumption may be invalid. Both glucose and $(NH_4)_2SO_4$ are simple carbon and nitrogen sources, respectively, that is required, nearly in all growth stages of microorganism, the small amount encourage the growth of the fungus at the initial growth stage. The other variables including peptone, casein, olive cake and CSL, recoded insignificant effect. Casein, olive cake and CSL exerted a negative effect, whereas the other variables exerted positive effects on fungal rennet production. Commonly, there is no general medium for fungal rennet production by different microbial strains. Anyhow, the optimum levels of the two significant variables (glucose and KH_2PO_4) which stimulated fungal rennet production were further discovered by the Central Composite Design (CCD) of response surface methodology.

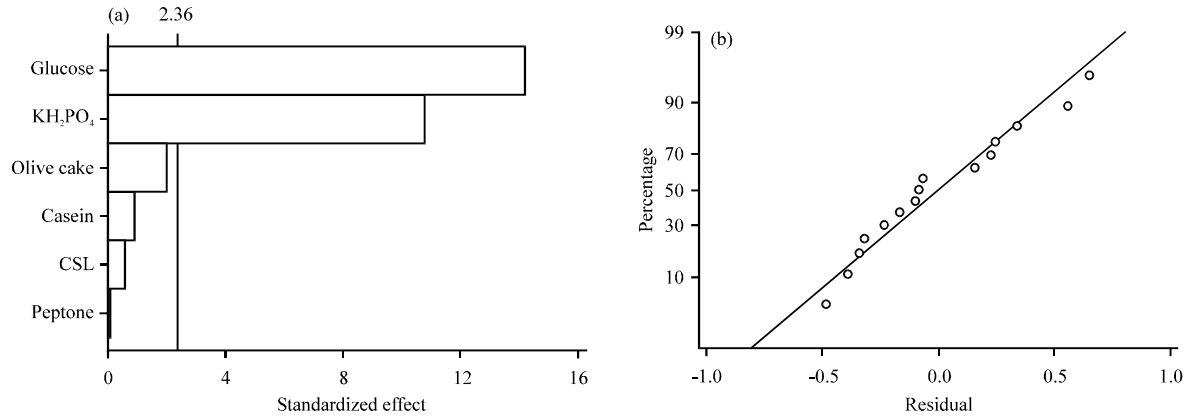

Fig. 2(a-b): Pareto chart of the (a) Standardized effects and (b) Normal probability plot of the different medium components used for fungal rennet production by *Mucor mucedo* KP736529

Table 4: Estimated effect, regression coefficient and ANOVA of Plackett-Burman matrix of medium components for rennet produced by *Mucor mucedo* KP736529 (coded units)

Source	Effect	Coefficient	Sum of squares	Mean square	F-value	p<0.05
Main effects	-	-	78.091	13.015	53.710	0.000[s]
Glucose	4.032	2.016	48.763	48.763	201.230	0.000[s]
Peptone	0.022	0.011	0.001	0.001	0.010	0.941[ns]
Casein	-0.255	-0.128	0.195	0.195	0.810	0.399[ns]
KH_2PO_4	3.062	1.531	28.121	28.121	116.050	0.000[s]
Olive cake	-0.558	-0.279	0.935	0.935	3.860	0.090[ns]
CSL	-0.158	-0.079	0.075	0.075	0.310	0.595[ns]
Residual error	-	-	1.696	0.242	-	-
Lack of fit	-	-	1.602	0.321	6.780	0.133[ns]
Pure error	-	-	0.095	0.047	-	-
R^2	0.979					
Adjusted R^2	0. 956					
Predicted R^2	0.882					

[s]Significant, [ns]Not significant at p<0.05

Optimization of fungal rennet production using CCD: The central composite design was adopted to explain the interaction among three independent variables, viz., glucose and KH_2PO_4 that previously chosen based on the screening of Plackett-Burman, in addition to the initial pH value of the fungal rennet production medium by *M. mucedo*. The independent variables were used at five different concentrations. The response and fitted fungal rennet production as a function of CCD experiments are presented in Table 2. Fitted data with a second order polynomial function indicate the accuracy of the model. The analysis of variance (Table 5) indicates that the overall model term is significant, where the p-value is less than 0.0001 and the F-value recorded 341.6. Additionally, the individual model terms of X_2, X_5, X_7, X_2X_5, X_2X_7, X_2^2, X_5^2 and X_7^2 were significant ($p<0.05$) but the interaction of X_5X_7 was the only exception, meaning that there was no significant interaction between the two independent variables; X_5 (KH_2PO_4) and X_7 (pH). Thus, the related term could be eliminated from the equation of prediction. The lack of fit F-value (0.1463) is not significantly relative to the pure error.

Table 5: Analysis of variance for response surface quadratic model for production of rennet by *Mucor mucedo* KP736529, based on CCD

Source	Sum of squares	Degree of freedom	Mean square	F-value	p<0.05
Model	137.125	9	15.236	341.6	<0.0001[s]
X_2	10.771	1	10.771	241.5	<0.0001[s]
X_5	7.300	1	7.300	163.7	<0.0001[s]
X_7	56.824	1	56.824	1274.2	<0.0001[s]
X_2X_5	15.832	1	15.832	355.0	<0.0001[s]
X_2X_7	25.404	1	25.404	569.6	<0.0001[s]
X_5X_7	0.111	1	0.111	2.5	0.1451[ns]
X_2^2	15.938	1	15.938	357.4	<0.0001[s]
X_5^2	2.583	1	2.583	57.9	<0.0001[s]
X_7^2	1.793	1	1.793	40.2	<0.0001[s]
Residual error	0.446	10	0.045		
Lack of fit	0.327	5	0.065	2.7	0.1463[ns]
Pure error	0.119	5	0.024		
R^2	0.997				
Adjusted R^2	0.994				
Predicted R^2	0.979				
Adequate precision	62.130				
Std. Dev	0.210				
C.V %	1.950				
PRESS	2.880				

[s]Significant, [ns]Not significant at p<0.05

The coefficient of determination (R^2), the adjusted R^2 and predicted R^2 values are 0.997, 0.994 and 0.979, respectively, the values of predicted R^2 and adjusted R^2 are in reasonable agreement. The value of R^2 indicates that the model can explain 99.68% of variation in the fungal rennet production that is explained by the factors. The adjusted R^2 and predicted R^2 are measures of how well the fitness of the data. These values can help selection the model with the best fit. Predicted R^2 can prevent overfitting the model and is more useful than adjusted R^2 for comparing models. Overfitting refers to models that appear to explain the relationship between the predictor and response variables for the data set used for model calculation but fail to provide valid predictions for new observations. Large value of predicted R^2 suggest models of greater predictive ability. However, the higher of their values, the more accuracy of the relationships between the variables (factors) and response (rennet).

The model has also, an adequate precision value of 62.13, this is an index of the signal to noise ratio or the adequate precision, this value indicates the validity of the data and its value is great enough, suggesting that the model can be used to navigate the design space. This is an essential prerequisite for a model to be a good fit. The fitting of the model was tested, it was confirmed to be fit by the insignificant lack of fit F-value (2.7). The model shows standard deviation (Std. Dev.) with low variation or dispersion from the average of rennet production being 0.21. The low Std. Dev. value indicates that the fungal rennet production points tend to be very close to the mean, also, indicates the low variation or dispersion from the average and the rennet points are not spread out over a large range of values. Coefficient of variation (C.V) which describes the variation of a test as a percentage of fungal rennet production mean recorded 1.95%. The accuracy of the experimentation procedure, presented by the C.V gives indication of good method performance since C.V of 5.0% or less is good indicator. Finally, the assessment of the model's predictive ability by the

Predicted Residual Sum of Squares (PRESS) recorded small value of 2.88, in general, the smaller the PRESS value, the better the model's predictive ability. The PRESS is used to calculate the predicted R^2 which is generally more intuitive to interpret. Together, these statistics can help prevent overfitting the model because it is calculated using observations not included in model estimation. All the previous values lead to the conclusion that, the model could be effectively used to measure the particular model fits at each point in the design, this in turn, confirms a satisfactory adjustment of the quadratic model to the experimental data. It should be considered that the polynomial model is a reasonable approximation of the true functional relationship on a relatively small region of the entire space of the independent variables.

Model diagnostic was evaluated to verify the aptness of the model using the graph of the actual response values versus the predicted response values of rennet, this helps detecting the value or the group of values that are not easily predicted by the model and this in turn, indicates how well the model satisfies the assumptions of the analysis of variance. As seen in Fig. 3a, the points of response values versus the predicted ones comprise a straight line, indicating a satisfactory correlation between the experimental and predictive values of fungal rennet production that indicates the good fit of the model. To test the influence of each point on the model fit, the leverage plot was depicted (Fig. 3b), if a point has a leverage of 1, then the model must go through that point which controls the model. Leverages near one should be reduced by adding or replicating points. These values provide measures of the influence, potential or actual of individual runs. Additionally, the plot can be used to spot near colinearity between terms. When the terms are collinear, the points will be compressed toward a vertical line.

In order to determine the optimal levels of each variable (KH_2PO_4, glucose and pH) for maximizing fungal rennet production, the three-dimensional response surface graphs were constructed by plotting the fungal rennet production on the z-axis against any two independent variables while maintaining the other variables at their central levels. As is shown in Fig. 4a, an increase in rennet secretion was observed when KH_2PO_4 and glucose concentration increased. Concomitant increase in fungal rennet production was observed with the high glucose concentration and pH (Fig. 4b). However, as is shown in Fig. 4c, fungal rennet production increases

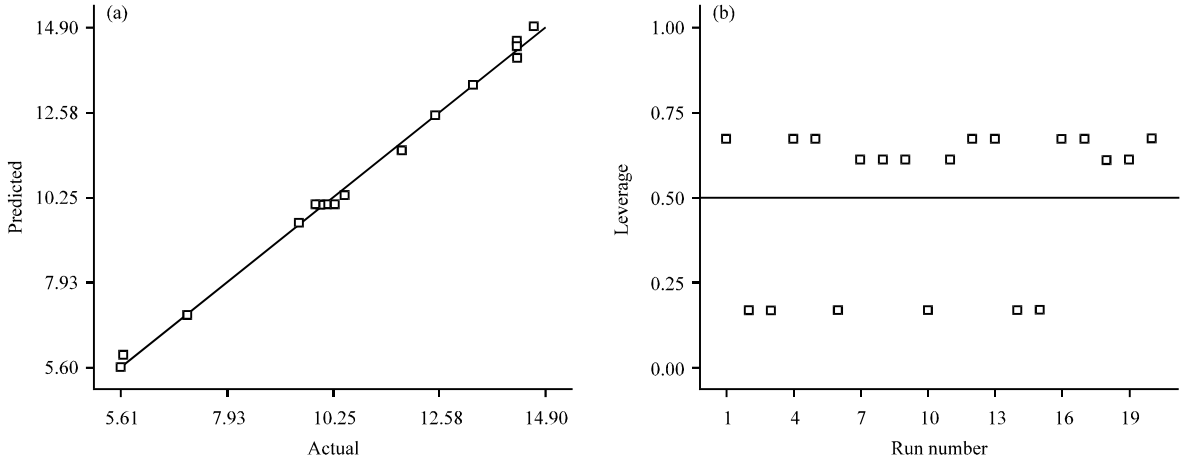

Fig. 3(a-b): Parity plot showing the distribution of (a) Experimental versus predicted values and (b) Leverage versus run plot of fungal rennet production

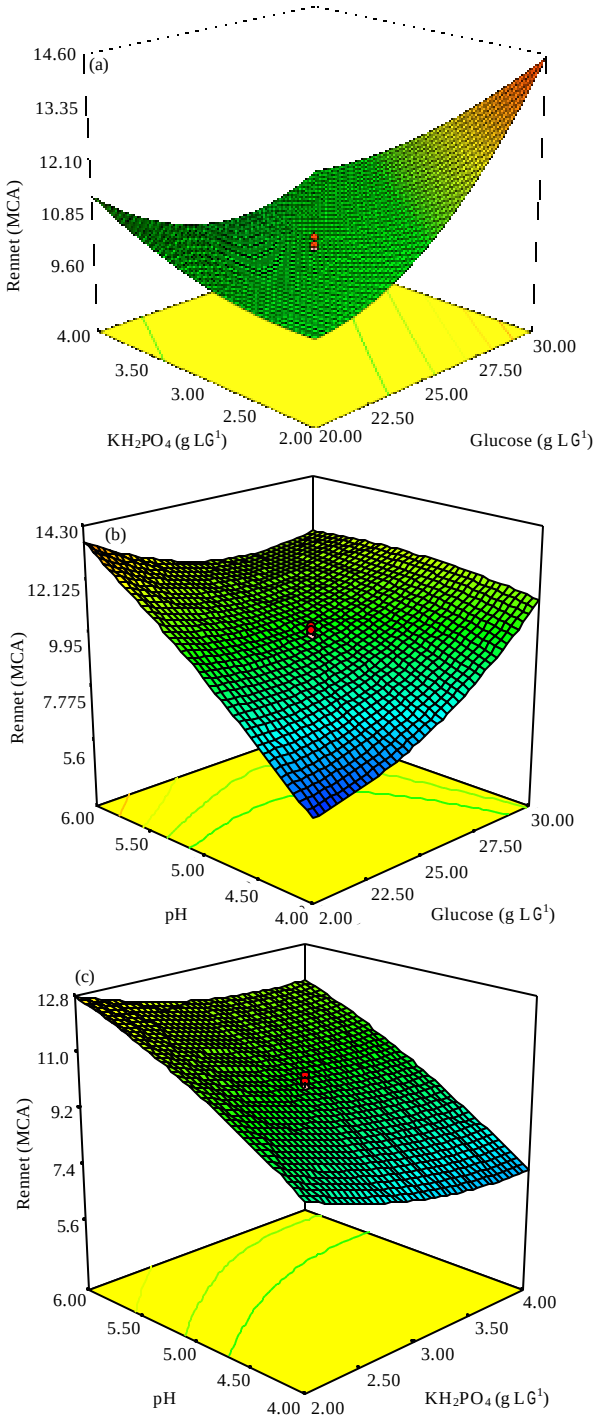

Fig. 4(a-c): Three-dimensional response surface plots for (a) Fungal rennet production showing the interactive effects of KH_2PO_4 and glucose, (b) Rennet showing the interactive effects of glucose and pH and (c) Fungal rennet production showing the interactive effects of KH_2PO_4 and pH

further with decrement of KH_2PO_4 but increased continuously with the higher pH values. Based on ANOVA, X_5X_7 is the only insignificant model, it is already known that, if there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve the model. In the present study, only one model is insignificant, therefore, there is no need to simplify the second-order polynomial equation for fungal rennet production. The fitted value was calculated from the final equation in terms of coded factors as follows:

$$\text{Rennet} = 10.05+0.89(X_2) -0.73(X_5) +2.04(X_7) -1.41(X_2X_5) -1.78(X_2X_7) +0.12(X_5X_7) +1.05(X_2^2) +0.42(X_5^2) -0.35(X_7^2).$$

The experimental data was fitted into the aforementioned equation and the optimum level of each variable was determined to be 33.38 g L^{-1} glucose, 1.32 g L^{-1} KH_2PO_4 and 4.22 for initial culture pH. According to the predicted equation, the fitted value of MCA of the resulted rennet was calculated to be 21.62 MCA. this value of rennet was experimentally validated and the activity reached to 21.50±0.53 MCA, recording 6.12 fold increase, in comparison with the initial activity (3.51 MCA).

Application of *M. mucedo* KP736529 enzyme in cheese manufacturing: The main target of any new product is its application in biotechnology field, so, the next experiment, is a practical application of the obtained fungal rennet in dairy products area. Domiati cheese was chosen as an example for this purpose.

Optimum concentration of fungal enzyme as milk clotting: This preliminary study was undertaken to estimate the appropriate fungal rennet concentration to be added during Domiati cheese manufacturing. The results given in Table 6 confirm that the addition of 320 or 650 MCA unit of the fungal enzyme as a rennet substitute were the most appropriate concentrations, since the properties of the resulted curds were very close to that of the control manufactured with commercial calf rennet. Economically, the concentration of 320 MCA unit/100 mL milk was selected and used in all next trials.

Evaluation of the adoptive UF-Domiati cheese manufactured with *M. mucedo* KP736529 enzyme: Data of Table 7 show the yield and some chemical characteristics of UF-Domiati

Table 6: Properties of fresh curd made with different concentration of milk-clotting rennet of *Mucor mucedo* KP736529 as well as calf rennet

Rennet type and concentration	Concentration (MCA)	Coagulation time (min)	Curd tension (g cm^{-2})	Curd syneresis (mL whey/100 mL milk) after 24 h of manufacturing	Cheese yield (g/100 mL milk) after 24 h of manufacturing	Properties of body
Fungal rennet (MCA unit/100 mL milk)	70	215	68	50	Difficult to calculate accurately because of watery body	Watery body
	150	140	75	54	41.2	Slightly watery
	320	50	145	62	37.3	Soft and acceptable
	650	32	155	65	38.1	Soft and acceptable
Standard (1 mg calf rennet/100 mL milk)		45	162	70	31.3	Firm and acceptable

Table 7: Chemical properties of Domiati cheese manufactured with *Mucor mucedo* KP736529 enzyme in comparison to commercial calf rennet under pickled storage for two months

	Cheese from commercial calf rennet during storage period (days)					Cheese from *M. mucedo* rennet during storage period (days)				
Criterion	0 (fresh)	15	30	45	60	0 (fresh)	15	30	45	60
Yield (%)	27.00	25.50	24.00	22.30	19.20	32.20	30.00	28.10	26.40	26.30
Moisture (%)	59.00	56.20	53.10	51.00	48.40	64.50	61.00	57.60	55.10	53.50
TA (%)	0.41	0.60	1.06	1.30	1.56	0.38	0.57	1.02	1.26	1.50
Fat (%)	22.40	23.00	23.20	23.50	23.80	20.80	21.00	21.60	22.00	22.30
TN (%)	2.01	2.04	2.08	2.11	2.15	1.75	1.77	1.83	1.87	1.89
SN (%)	0.22	0.32	0.52	0.61	0.72	0.23	0.45	0.73	0.96	1.12
NPN (%)	0.23	0.27	0.35	0.39	0.42	0.27	0.35	0.43	0.45	0.51
Tyrosine (mg %)	19.50	35.50	41.50	43.00	48.20	24.90	40.10	45.00	51.50	62.60
Tryptophan (mg %)	49.50	60.20	75.60	75.90	76.20	51.00	61.70	76.80	77.30	78.10
TVFA/10 g cheese (as mL 0.1 N NaOH)	11.20	20.50	23.30	25.30	27.80	12.50	23.70	25.30	26.80	28.50

cheese made with either commercial calf rennet (C-cheese) or *M. mucedo* milk clotting enzyme (E-cheese), during ripening. The yield of E-cheese was higher than C-cheese, this may be resulted from the high moisture content of cheese either in fresh or along the storage pickling period. On the contrary, the cheese made with calf rennet contained higher moisture than that made with fungal enzyme from *Thermomucor indicae-seudaticae* (Merheb-Dini *et al*., 2012).

For Total Acidity (TA), it could be noticed a gradual increases in TA during ripening in both C-cheese and E-cheese along the 60 days ripening period but the rate of increase was more pronounced in C-cheese, probably due to accumulation of lactose degradation products such as lactic acid and other volatile acids. These results are inconsistent with Merheb-Dini *et al*. (2012). Fat and TN content were higher in C-cheese than E-cheese, owing to the moisture variation but they lay in the normal range. Although, Merheb-Dini *et al*. (2012) and Abbas *et al*. (2013) did not find a significant difference in fat and TN between E-cheese and C-cheese when they have used fungal rennet from *Rhizomucor miehei* NRRL 2034.

For the development of texture, taste and aroma characteristics of ripened cheeses, a balanced degradation of proteins into peptides and amino acids is necessary (Singh *et al*., 2003) and the detection and quantification of these degradation products are used as parameters to express the ripening index of cheeses (McSweeney and Fox, 1997). Therefore, the formation of nitrogenous compounds during the ripening of cheese were studied to monitor and objectively evaluate cheese ripening. Gradual increment could be observed in Soluble Nitrogen (SN) contents in both cheese types as a result of proteolysis. There were obvious differences in the proteolytic action of the fungal enzyme and commercial calf rennin. E-cheese had more SN contents than C-cheese. Fungal preparation had more proteolytic activity and hydrolyzed more peptides bands and liberated more SN and free amino acids (Hynes *et al*., 2001), that is why Abbas *et al*. (2013) noticed that E-cheese contained obvious increases of SN and SN/TN than present in C-cheese.

Data also indicate that E-cheese recorded lowbrow increment in NPN compared to C-cheese, in this respect, Abd-Rabou and El-Senaity (2005) indicated that Edam cheese produced by *Rhizomucor miehei* milk-clotting enzyme was higher in SN and NPN content than C-cheese. It should be mentioned to the results of Reps *et al*. (2006), who indicated that the amount of milk N-compounds and fat in whey (obtained by Mucor proteinase preparation) was higher than that

obtained in coagulation with rennin during the manufacturing of camembert cheese. After 2 weeks-ripening, the content of N-compounds in cheese produced by *Rhizomucor* proteinase was higher than in rennet-cheese. Their obtained results explicitly indicate that the protein degradation process is more intense in cheese produced with fugal enzyme and thus ripens faster. There was changes in tyrosine and tryptophan contents with the progress of time as well as with kind of cheese, in which E-cheese had higher values through all intervals in comparison with C-cheese. At the end of storage period, ripened E-cheese had 62.6 and 78.1 against 48.2 and 76.2 mg% of tyrosine and tryptophan for C-cheese. These results are in accordance with those reported by Abd-Rabou and El-Senaity (2005) and Abbas *et al*. (2013).

Another important observation, the Total Volatile Fatty Acids Contents (TVFAs) showed great differences between both cheese kinds, in which E-cheese gained higher content of TVFAs either at fresh or during ripening. E-cheese possessed as 12.5, 23.7, 25.3, 26.8 and 28.5 mL 0.1 N NaOH per 10 g cheese against 11.2, 20.5, 23.3, 25.3 and 27.8 for C-cheese at fresh and after 15, 30, 45 and 60 days of ripening, respectively. These variations could be related to the higher lipolytic activity of fungal enzyme. Abd-Rabou and El-Senaity (2005) and Reps *et al*. (2006) confirmed that fungal protease had a positive effect on TVFAs of cheese as compared to commercial rennet. Lastly, it could be observed that E-cheese had more nitrogenous compounds than C-cheese which confirms that the milk-clotting enzyme from *M. mucedo* KP736529 has higher proteolytic action compared to the commercial calf rennet.

Organoleptic evaluation: The obtained enzyme has proteolytic action which may result in impaired cheese organoleptic characteristics. The rennin of microbial origin might be contaminated by other enzymes which might affect cheese ripening by causing bitterness during storage (Rogelj *et al*., 2001), this is one of many reasons that organoleptic evaluation was carried out. Data (Table 8) of the organoleptic scoring of E-cheese and C-cheese, generally, revealed the acceptability of both kinds of cheese. Fresh C-cheese seemed to be firm and acceptable body and texture with clean and plain flavor, while, E-cheese had a soft body, spreadable texture and pronounced desirable flavor with more acceptability. No clear differences was observed in the visual aspects of the two kinds of cheese. No bitterness or flavor defects could be observed in E-cheese and its

Table 8: Organoleptic evaluation of Domiati cheese manufactured with *Mucor mucedo* KP736529 rennet in comparison to commercial calf rennet during pickling storage period

Cheese type	Storage period (days)	Visual aspect (0-10)	Consistency (0-40)	Flavor and taste (0-50)	Total (100)	Criticism comment
C-cheese	Fresh	9.0	34 Firm	40	83.0	White color
	15	9.0	34 Hard	45	88.0	Firm body
	30	9.0	35	47	91.0	Clean and plain flavor
	45	9.0	35	44	88.0	
	60	9.0	37	42	88.0	
E-cheese	Fresh	9.5	37	43	89.5	Clear white
	15	9.5	37	46	92.5	Soft body
	30	9.5	38	48	95.2	Clean flavor
	45	9.5	38	48	95.5	More spreadable
	60	9.5	39	49	97.5	

ripening. Generally and owing to the proteolytic and lipolytic actions, ripening period improved all the organoleptic properties of both C-and E-cheese. For flavor and taste assessment, C-cheese possessed 40, 45, 47, 44 and 42 points when fresh and after 15, 30, 45 and 60 days against 43, 46, 48, 48 and 49 points for E-cheese, respectively. On the other hand, E-cheese has gained more point in visual aspect and consistency than C-cheese. Total acceptability of E-cheese was 97.5 points against 88 points for C-cheese at the end of storage period. Abbas *et al*. (2013) reported that E-cheese made with milk clotting enzyme from *Rhizomucor miehei* NRRL 2034 gained high scoring than C-cheese. However, the curd obtained with *Rhizomucor* clotting enzyme of Camembert, Edam and Cheddar cheeses was slightly less firm than that obtained with rennin preparation (Reps *et al*., 2006).

CONCLUSION

Microbial sources can substitute for animal rennet. This alternative amazing source appears to be more promising because its production is cheaper, biochemical diversity and safety. *Mucor mucedo* KP736529 can produce milk-clotting enzyme, this fungal rennet is low cost and has excellent characteristics for manufacturing of cheese whether fresh or during storage.

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