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PROTEASE PRODUCTION FROM *Bacillus safensis* IN SUBMERGED FERMENTATION USING RESPONSE SURFACE METHODOLOGY

PRODUCCIÓN DE PROTEASAS A PARTIR DE *Bacillus safensis* EN FERMENTACIÓN SUMERGIDA USANDO METODOLOGÍA DE SUPERFICIES DE RESPUESTA

H.A. Shakir[1], R. Mahmood[1], M. Irfan[2], F. Deeba[1], I. Javed[1], J.I. Qazi[1]*

[1]*Department of Zoology, University of the Punjab new campus Lahore, Pakistan.*
[2]*Department of Biotechnology, University of Sargodha, Sargodha Pakistan.*

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Abstract

In present study, various culture parameters for *Bacillus safensis* were optimized in submerged fermentation for alkaline protease production using response surface methodology. Nutritional components were screened through Placket-Burman design. The optimized culture parameters were, 1% inoculum size, initial medium of pH 9.0, incubation temperature of 30 °C and fermentation time of 72 h. Among nutritional parameters, casein, KH_2PO_4 and peptone were found significant which was further optimized through Box-Bhenken Design with three levels. The experimental results showed that 4.0% casein, 0.05% KH_2PO_4 and 0.575% peptone yielded highest protease production. The proposed model was significant which clearly explained the response. Partial characterization revealed that crude protease produced by *Bacillus safensis* showed optimum activity at pH 8.0 and at 40 ºC. Kinetics revealed the K_m and V_{max} values of 1.027 mg/ml and 67.57 mg/ml/min using casein as substrate, respectively.

Keywords: alkaline protease, *Bacillus* sp, RSM, characterization, submerged fermentation.

Resumen

En este trabajo se optimizaron varios parámetros para *Bacillus safensis* cultivado en fermentación sumergida para la producción de proteasa alcalina usando metodología de superficies de respuesta. Los componentes nutricionales se examinaron usando diseño de Placket-Burman. Los parámetros de cultivo optimizados fueron, 1% tamaño de inóculo, pH inicial del medio de 9.0, temperatura de incubación de 30 °C y tiempo de fermentación de 72 h. Entre los parámetros nutricionales, la caseína, el KH_2PO_4 y la peptona fueron significativos y fueron sujetos a una opimización adicional usnado un Diseño de Box-Bhenken con tres niveles. Los resultados experimentales mostraron que 4% de caseína, 0.05% de KH_2PO_4 y 0.575% de peptona produjeron el mayor rendimiento de peptona. El modelo propuesto fue significativo y claramente describió la respuesta. Una caracterización parcial reveló que la proteasa cruda producida por *Bacillus safensis* mostró actividad óptima a un Ph de 8.0 y temperatura de 40 °C. Los parámetros cinéticos K_m y V_{max} mostraron valores de 1.027 mg/ml y 67.57 mg/ml/min cua, respectivamente, cuando se usó caseína como sustrato.

Palabras clave: proteasa alcalina, *Bacillus* sp, MSR, caracterización, fermentación sumergida.

1 Introduction

Proteolysis is the hydrolysis of peptide bonds by proteolytic enzymes. Amino acids and peptide fragments are formed as a result of proteolysis. Proteases are found almost everywhere in natural habitats and are important for differentiation and cell growth. These enzymes are not only essential in metabolic processes of the cells but are also of great importance in industrial field (Gupta *et al*., 2002). Proteases that are produced on commercial scale have widely been used in photographic, detergent, dairy and leather industries (Anwar and Saleemuddin, 1998). There is a long history of proteases' applications in detergent and food industries. The application of proteases in leather industry for bating of hides and dehairing as an alternative for the use of toxic chemicals is relatively new technique and has gathered attention from biotechnological point of view (Rao *et al*., 1998). These enzymes are substrate specific, have specific catalytic mechanism, temperature activity, active site, pH and stability profiles.

* *Corresponding author. E-mail*: qazi.zool@pu.edu.pk
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These enzymes take an advantage in industries of their distinctive catalytic and physical properties (Ward, 1991). The vast variety of proteases, in comparison to their action specificity has got attention around the world for their biotechnological and physiological applications (Rao *et al*., 1998).

Alkaline proteases (EC.3.4.21-24, 99) are the proteases which are dynamic in a neutral to alkaline pH range (Gupta *et al*., 2002). At present, a large fraction of alkaline proteases available on the commercial scale are obtained from Bacillus strains (Mabrouk *et al*., 1999, Mehrotra *et al*., 1999). Optimization is an undeniable component of any commercial goal (Greasham, 1983). There is a major drawback of this technique that it does not comprise the interacting effects among variables and therefore it does not illustrate the inclusive effects of the process parameters (Baåÿ and Boyacä, 2007).

Therefore, in order to overwhelm the drawbacks of one factor at a time (OFAT), optimization can be carried out using response surface methodology (RSM). RSM is an assortment of mathematical and statistical techniques which are valuable for improving, developing and optimizing processes in which a response of interest is affected by several variables and the basic aim is to optimize the response. RSM is of great importance in development, design and formation of new products, in addition to it, improves the present product designs. RSM has removed all the demerits of conventional methods and has demonstrated to be helpful for the optimization of the metabolites' productions (Liu and Wang, 2007; Sayyad *et al*., 2007; Deepak *et al*., 2008).

Different second order models like Box-Behnken, Plackett-Burman and Central Composite designs are extensively used in RSM, as these designs can fit into various functional forms (Srinivas *et al*., 1994; Carvalho *et al*., 1997;Adinaryana and Ellaiah, 2002; Li *et al*., 2007; Xiao *et al*., 2007). The implementation of RSM in process of fermentation process can develop in increased product yields, decreased process variability and reduction in overall cost. Although RSM has some restrictions, studies are carried out without considering these boundaries (Baåÿ and Boyacä, 2007). The aim of the current study was to screen different species of bacteria for their protease producing potential, optimization of cultural and nutritional conditions by employing RSM and partial characterization of crude enzyme

2 Materials and methods

2.1 Selection of microorganism

The bacterial species *Aeromonas allosaccharophila* (KF625182), *Bacillus safensis* (KF551977), *Bacillus pumilus* (KF625178), *Aeromonas bestiarum* (KF625168), *Aeromonas salmonicida* (KF551975), *Exiguobacterium mexicanum* (KF625174), *Aeromonas hydrophila* (KF551976), *Aeromonas media* (KF551978) and *Obesumbacterium proteus* (KF625187) previously isolated from gut of *Labeorohita* were revived from the stock culture of Microbial biotechnology lab. Department of Zoology, University of the Punjab and screened for their protease producing potential.

2.2 Preparation of plate medium

The selected medium (2% casein, 1% gelatin, 1.8% agar, pH 7) was prepared following Naik *et al*. (2013) and the revived bacterial culture were streaked following standard microbiological practices. These plates were incubated at 37 ºC for 2 days. Formation of the clear zones indicated positive results for protease production. The strains which showed protease producing potential were carried out for enzyme assay to select the bacterium with highest protease producing potential.

2.3 Protease production

For protease production, 100 ml of the medium comprising of dextrose 1.0%, peptone 1.0%, KH_2PO_4 0.05%, $MgSO_4$ 0.02%, NaCl 0.2%, $CaCl_2$ 0.002% and casein 2.0% (Naik *et al*., 2013) was taken in 250ml capacity Erlenmeyer flask and sterilized. Each fermentation flask was inoculated (1%) using 24 h old cell culture and incubated at 37°C for 3-4 days. After incubation, the fermented medium was centrifuged at 10,000 rpm and 4 ºC for 10 min. The clear cell free extract was then used as a crude enzyme source.

2.4 Enzyme assay

Protease activity was determined by method as described Naik *et al*. (2013). Crude enzyme (0.5 ml) with substrate solution i.e 0.65% casein in Glycine-NaOH buffer of pH 9, (2.5 ml) was incubated at 37 ºC for 10 min. After incubation, 2.5 ml of trichloroacetic acid (0.11 M) was added in it and incubated at 37 ºC

for 30 min. Both the control and experimental reaction solutions were centrifuged at 10,000 rpm for 10 min at 4 ºC and supernatant (0.4 ml) was taken in another test tube to which 1.0 ml of sodium carbonate (0.05M) and 0.2 ml of Folin-Ciocalteu reagent were added. The absorbance was then recorded at 660 nm against enzyme blank after incubation at 37 ºC for 30 min. One enzyme unit (U) was defined as, the amount of enzyme that produce 1 μg of tyrosine per minute under the determined assay conditions. After the enzyme assay of five isolates, the better one was carried out for optimization.

2.5 Optimization of temperatura on enzyme production

Effect of temperature on protease production was noted with 1% inoculum of 24 h culture of *Bacillus safensis* the production medium. The fermentation cultures was incubated at three different temperatures i.e. 30 ºC, 37 ºC and 45 ºC. After 72 h of incubation, crude enzyme was obtained by centrifugation at 10,000 rpm at 4 ºC for 10 min and enzyme assay was performed according to above mentioned procedure.

2.6 Optimization of pH on enzyme production

In order to optimize pH for alkaline protease production, the medium was prepared with initial pH of 5, 7 and 9. These production media were then inoculated with 1% 24 h culture of the strain and incubated at the optimized temperature. After incubating the medium for 72 h, crude enzyme was obtained by centrifugation at 10,000 rpm at 4 ºC for 10 min and protease assay was followed according to above mentioned protocol.

2.7 Optimization of inoculum size on enzyme production

Effect of different inocula for alkaline protease production was studied on the fermentation medium of selected pH. Inoculation was done with 1%, 2% and 3% of 24 h old culture followed by incubation at selected temperature for 72 h. The cell free culture fluids were then processed for crude enzyme as mentioned earlier.

2.8 Optimization of incubation time on enzyme production

Fermentation medium of select pH with suitable inoculum size was incubated for 24, 48, 72 and 96 h at pre optimized temperature. After obtaining the crude enzyme at the prescribed different time intervals, enzyme assay was carried as mentioned before.

2.9 Experimental design

Plackett-Burman design was used, in order to find significant impact of different variables. Seven components of the medium were screened in twelve runs of experiments for alkaline protease production in submerged fermentation. The low level (-1) and high level (+1) values were selected (Table 1). The twelve experiments were performed to select the appropriate medium ingredients for higher alkaline protease production under optimum physical conditions. Minitab software was used to select the suitable medium components through Pareto chart Response surface methodology (RSM). Box-Behnken design was employed to optimize the three significant factors viz., casein, KH_2PO_4 and peptone which showed significant impact on protease production. The three independent variables were studied at three different levels (Table 2) and a set of 15 experiments was carried out. All experiments were carried out in triplicates.

Table 1. Range of parameters used for Plackett-Burman design.

Nutrient	Code	Low level (-1)	High level (+1)
Casein	A	1.0	4.0
KH_2PO_4	B	0.05	0.1
K2HPO4	C	0.1	1.0
$MgSO_4$	D	0.01	0.2
NaCl	E	0.2	1.0
Glucose	F	0.1	1.0
Peptone	G	0.15	1.0

Table 2. Ranges of the independent variables used in RSM.

Variable	Code	Levels		
		-1	0	+1
Casein (%)	A	1.0	2.50	4.0
KH_2PO_4 (%)	B	0.05	0.075	0.10
Peptone (%)	F	0.15	0.575	1.0

Analysis of variance (ANOVA) was used for statistical evaluation of the data. All the responses were calculated through the following second order polynomial equation:

$$y = \beta_o + \sum_{i=1}^{k} + \sum_{i=1}^{k} \beta_i X_i^2 + \sum_{i} \sum_{j} \beta_{1j} X_i X_j \qquad (1)$$

where Y_i is the predicted response, $X_i X_j$ are independent variables, β_o is the offset term, β_i is the ith linear coefficient, β_{ii} is the ith quadratic coefficient and β_{ij} is the ijth interaction coefficient. STATISTICA software (99th edition) was used for data analysis.

2.10 *Effect of pH on enzyme activity*

To evaluate the effect of pH on enzyme activity and stability, different buffers such as 0.1M acetate (pH 4,5), 0.1M phosphate (pH 6,7), 0.1M tris-HCl (pH 8,9) and 0.1M glycine-NaOH (pH 10,11) 0.1M substrate buffer solutions varying pH made using these buffers. The reaction mixtures containing the enzyme and the substrate solutions were used for enzyme assay as described before.

2.11 *Effect of temperature on enzyme activity*

Temperature stability of the enzymewas tested at 30, 40, 50 and 60 ºC. Maximum stability of the enzyme was considered as the temperature corresponding to the best the enzyme.

2.12 *Enzyme Kinetics*

To find the effect of substrate concentration on the enzyme characterization, after extracting the crude enzyme, different substrate concentrations i.e. 0.50%, 0.55%, 0.60%, 0.65%, 0.70%, 0.75% and 0.80% were used in the selected pH buffer. The concentration corresponding to the best enzyme value was considered as stable for the enzyme.

3 Results and discussion

3.1 *Isolation and screening of protease producing bacteria*

In this study, five out of nine isolates showed positive results in the form of hydrolytic zones on casein-agar plates and the enzymes activity was quantified. *Bacillus safensis* showed highest amount of protease (155.22 ± 0.62 U/ml) in comparison with other strains and was used further study (Table 3).

3.2 *Optimization of temperatura on enzyme production*

The effect of temperature on alkaline protease production is shown in figure 1. Maximum amount of alkaline protease production was observed in medium incubated at 30 ºC temperature (150.30 ± 15.17 U/ml). Similar results have been reported for optimum temperatures at 30 ºC for *Bacillus pumilus* (Purohit *et al*., 2016), 35 ºC for *Bacillus licheniformis* (Sen and Satyanarayana, 1993) and Bacillus cereus FT 1 (Asha and Palaniswamy, 2018) and 37 ºC for *Bacillus subtilis* (Hameed *et al*., 1996; Chang *et al*., 1998; Ali *et al*., 2016).

Table 3. Selection of bacterial strains for protease production in submerged fermentation.

Sr. No.	Strain	Protease activity (U/ml)
1	*Aeromonas allosaccharophila*	89.19^{c} ± 0.72
2	*Bacillus safensis*	155.22^{a} ± 0.62
3	*Aeromonas bestiarum*	101.19^{b} ± 0.53
4	*Aeromonas salmonicida*	66.89^{d} ± 0.50
5	*Aeromonas media*	84.046^{e} ± 0.49

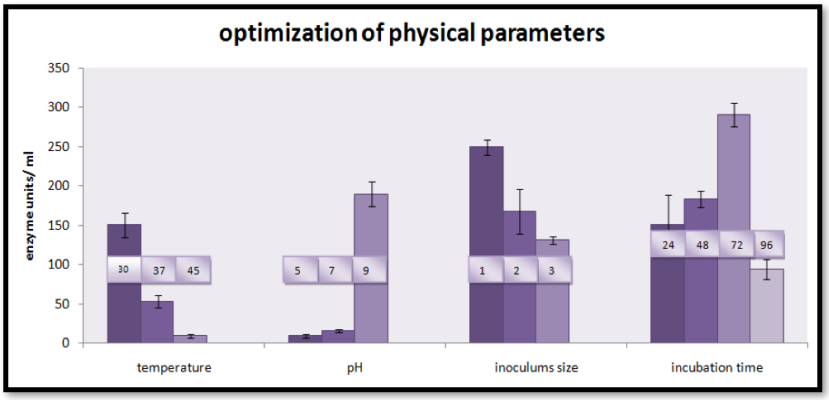

Fig. 1. Effect of temperature, pH, inoculum size and incubation period on protease production.

Optimum temperature in the range of 30 ℃-40 ℃ has also been reported for some other *Bacillus* species (Purva *et al*., 1998; Anandharaj *et al*., 2016).

3.3 Optimization of pH on enzyme production

The pH of the culture medium plays a critical role for the optimal physiological performance of the cells and transport of various nutrient components across the cell membrane ultimately affecting cells' productive potential including alkaline protease yields (Kumar and Bhalla, 2004). The impact of pH on alkaline protease production was shown in the figure 1. Maximum production of protease has been observed in medium with pH 9 (190.18 ± 15.63 U/ml). Kothari *et al*., (2013) has reported pH 8 for *B. safensis*. Likewise, pH ranges from 7-11 have been reported for alkaline protease production by different *Bacillus* species (Purva *et al*., 1998; Johnvesly and Naik, 2001; Genckal and Tari, 2006; Nascimento and Martins, 2006; Ahmad *et al*., 2010; Anadharaj *et al*., 2016; Purohit *et al*., 2016; Asha and Palaniswamy, 2018).

3.4 Optimization of inoculum size on enzyme production

Maximum amount of the enzyme was recorded with 1% inoculum size (250.33 ± 9.74 U/ml). Effect of inocula sizes is shown in the figure 1. Similar results have been discussed by Tiwari *et al*. (2015) and Suganthi *et al*. (2013). Borah *et al*. (2012) found that 5% v/v inoculum size gave higher proteases production. Similar results have also been discussed by other researchers (Shafee *et al*., 2005; Josephine *et al*., 2012).

Table 4. Plackett-Burman design matrix for screening of important variables for protease production.

Run #	Casein (A)	KH_2PO_4 (B)	K_2HPO_4 (C)	$MgSO_4$ (D)	NaCl (E)	Glucose (F)	Peptone (G)	Response
1	0.2	0.01	0.02	0.04	0.2	0.2	0.03	25.72
2	0.2	0.01	0.02	0.002	0.04	0.02	0.03	96.91
3	0.8	0.01	0.02	0.002	0.2	0.2	0.2	107.2
4	0.2	0.02	0.02	0.002	0.04	0.2	0.2	84.9
5	0.2	0.02	0.2	0.002	0.2	0.02	0.03	92.62
6	0.2	0.01	0.2	0.04	0.2	0.02	0.2	30.87
7	0.2	0.02	0.2	0.04	0.04	0.2	0.2	104.62
8	0.8	0.02	0.2	0.002	0.2	0.2	0.03	165.52
9	0.8	0.02	0.02	0.04	0.2	0.02	0.2	215.26
10	0.8	0.01	0.2	0.04	0.04	0.2	0.03	125.21
11	0.8	0.02	0.02	0.04	0.04	0.02	0.2	124.35
12	0.8	0.01	0.2	0.002	0.04	0.02	0.2	96.91

3.5 Optimization of incubation time on enzyme production

The results (Fig. 1) obtained at different time intervals depicted that 72 h of incubation was the optimum time for maximum production of alkaline protease (290.69 ± 14.76 U/ml). Effect of incubation time is also shown in the figure. Similar results were obtained by Naik *et al*. (2013) in their studies. Tiwari *et al*. (2015) reported maximum enzyme production after 48 h of incubation.

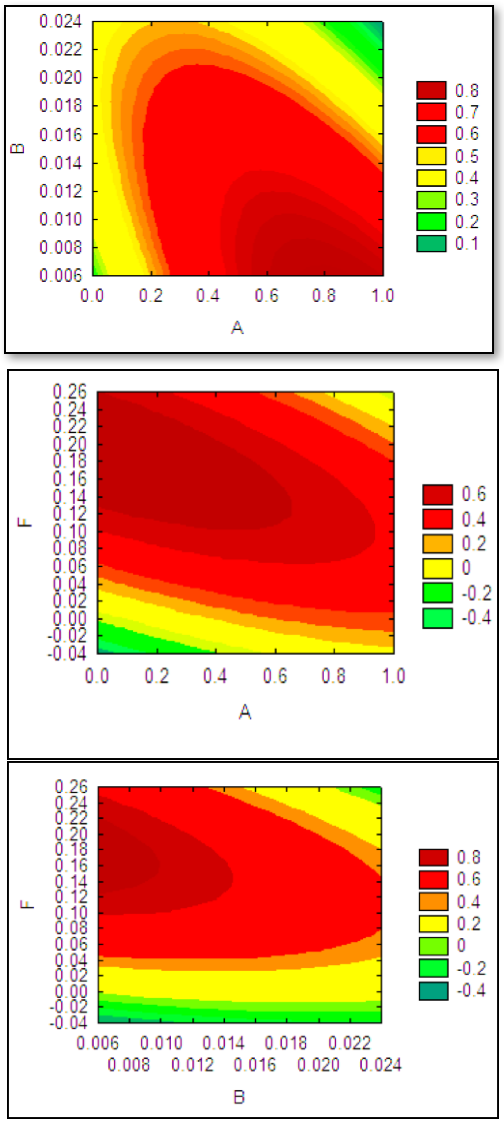

Fig. 2. Contour plots of the effects of KH_2PO_4 and peptone on protease production (where B is KH_2PO_4 and F is peptone).

3.6 Optimization of medium components

For medium optimization through response surface methodology, first seven nutritional parameters were screened through Placket-Burman design using twelve run experiments (table 4). From this experimentation, three parameters *i.e.*, casein, KH_2PO_4 and peptone were found significant was and were therefore further optimized through Box-Bhenken design of response surface methodology (Table 5). The response obtained was calculated by second order polynomial regression equation:

$$Y\ (protease\ \ activity) = 60 + 166.4A + 4348.8B + 1104.6F - 54.4A^2 - 67500.0B^2 - 2136.7F^2 - 3166.7A.B - 521.0A.F - 14800.0B.F \quad (2)$$

where A is casein, B is KH_2PO_4 and F is peptone.

Statistical analysis of the data was also performed by analysis of variance (ANOVA) to determine the importance of significant parameters that were already optimized using BBD. The F and P values essentially exhibit the individual and interactive effects of the independent variables.

This method of analysis was conducted for evaluation of effects of the variables and their probable interactions. Coefficients of the full model were analyzed for their significance and the insignificant ones were eliminated from the model by backward elimination. The ANOVA analysis of optimization is given in the table 6. The model was found highly significant and sufficient to represent the actual relationship between the response and significant variables as indicated by the small P-value (<0.001).

The regression equation obtained from ANOVA showed that the R^2 (multiple correlation coefficient) was 0.986718. This is an estimate of the fraction of overall variation in the data accounted by the model, indicating that the model is capable of explaining 98.67% of the variation in response (for a good statistical model, the R^2 value should approach the value of nearly 1.0). Figure 2 indicated the effect of different nutritional parameters on protease production which represented that each parameter had significant effect on protease production. Sobucki *et al*. (2017) optimized peptone and NH_4Cl as nitrogen sources for protease production using *Bacillus* sp. CL18.

Table 5. Box-Behnken Design for the optimization of medium components for protease production by *B. safensis* in submerged fermentation.

Run #	A*	B**	F***	Protease activity (U/ml)		Residue Value
				Observed	Predicted	
1	1	0	-1	180.370	182.362	-1.992
2	-1	0	-1	154.10	153.047	1.052
3	0	1	1	182.370	182.037	0.332
4	1	-1	0	205.820	203.495	2.325
5	1	0	1	176.960	178.012	-1.052
6	0	0	0	202.390	199.150	3.240
7	1	1	0	185.10	184.380	0.720
8	0	-1	1	202.960	204.232	-1.272
9	0	1	-1	173.670	172.397	1.272
10	0	0	0	197.810	199.150	-1.340
11	0	-1	-1	169.10	169.432	-0.332
12	-1	0	1	203.830	201.837	1.992
13	-1	1	0	188.810	191.135	-2.325
14	-1	-1	0	190.530	191.250	-0.720
15	0	0	0	197.250	199.150	-1.900

*casein, **KH_2PO_4, ***peptone

3.7 *Effect of pH on enzyme activity*

The results depicted that catalytic activity of protease was most active at pH 8 (167.80 ± 4.03 U/ml) indicating that the enzyme was an alkaline protease (figure 3). Further increase or decrease in pH from 8 resulted decline in protease activity. Activities of proteases produced from *Bacillus* species have been reported to perform best at varying pH levels for instance, at pH 7.5 (Asker *et al*., 2013), 8.5 (Ghafoor and Husnain, 2010; Tiwari *et al*., 2015), 9.0 (Rajkumar *et al*., 2011; Mazar *et al*., 2012; Marathe *et al*., 2018) 10.0 (Deng *et al*., 2010) and 11.0 (Nadeem *et al*., 2013).

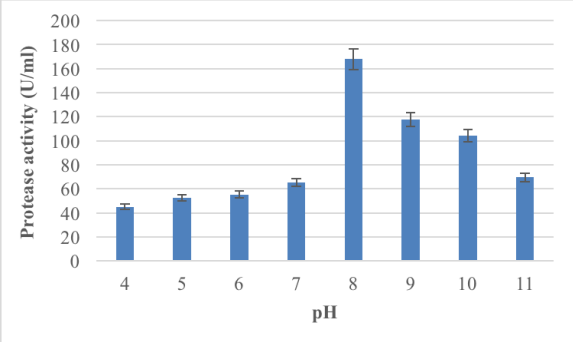

Fig. 3. Effect of pH on protease activity produced by *B. safensis* in submerged fermentation.

3.8 *Effect of temperature on enzyme activity*

Different incubation temperatures were tested to check the optimum temperature of protease produced from *B. safensis* in submerged fermentation. Results (figure 4) revealed that maximum protease activity was found at 40 ºC (195.82 ± 9.68 U/ml). Further, increase in temperature lead to decline in enzyme activity. Some strains of *Bacillus* species produce protease enzyme having optimum activity at 50 ºC (Rajkumar *et al*., 2011; Asker *et al*., 2013). Marathe *et al*. (2018) reported optimum temperatura of 55 ºC for máximum alkaline protease activity from *Bacillus* sp.

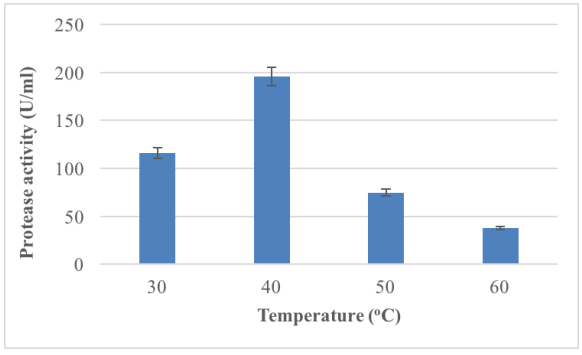

Fig. 4. Effect of temperature on protease activity produced by *B. safensis* in submerged fermentation.

Table 6. Analysis of Variance for protease production

Effect	SS*	DF**	MS***	F-value	P-value
Model	3073.16	9	341.463	41.272	0.000
A	426.599	1	426.599	51.563	0.000
A^2	88.562	1	88.562	10.704	0.022
B	42.877	1	42.877	5.182	0.071
B^2	10.514	1	10.514	1.270	0.310
F	1744.91	1	1744.91	210.908	2.8E-05
F^2	879.937	1	879.937	106.359	0.000
A.B	90.25	1	90.25	10.908	0.0214
A.F	705.965	1	705.965	85.330	0.000
B.F	158.256	1	158.256	19.128	0.007
Error	41.366	5	8.273		

Protease enzyme produced from different isolates of *Bacillus* species have also been reported as to be thermophilic having optimum temperature of 60 ºC (Deng *et al*., 2010; Mazar *et al*., 2012; Nadeem *et al*., 2013) whereas some strains exhibit optimum activity at 65 ºC (Ghafoor and Husnain, 2010).

3.9 Enzyme Kinetics

Enzyme kinetics (K_m and V_{max}) of the crude protease produced from *B. safensis* was determined using casein as substrate. The crude protease enzyme exhibited K_m and V_{max} of 1.027 mg/ml and 67.57 mg/ml/min, respectively. A recent study revealed that serine alkaline protease produced from *Aeribecillus pallidus* C10 had K_m and V_{max} of 0.197 mg/mL and 7.29l mol.ml^{-1}.min respectively (Yilodrim *et al*., 2017). Alkaline protease produced from *B. licheniformis* A10 had K_m and V_{max} of 0.033 mg/ml and 8.17 mmol.ml^{-1}.min^{-1}, respectively. Kamran and Bibi (2015) reported K_m and V_{max} value, of 2.3 mg/ml and 473 U/min from *Bacillus* sp., respectively. Thermostable alkaline serine protease produced from *Bacillus licheniformis* NMS-1 had V_{max} and K_m of 263mU/mg and 2.7 x 10^{-3} mg/ml, respectively (Mathew and Gunathilaka 2015).

Conclusions

Results of this study were successful to obtain an isolate of *B. safensis* that is capable of producing high yield of alkaline protease by employing the response surface methodology in sub merged fermentation. The culture parameters that were optimized included (%) casein 4.0, KH_2PO_4 0.05 and peptone 0.575, at pH 9 with 1% inoculation at 30 ºC incubation for 72 h. These results suggest potential utilization of this strain for enhancing nutritional levels of fish feed to promote growth of fishes for commercialization of aquaculture.

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