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Research Article

Statistical Optimization of Alkaline Protease Production by Newly Isolated *Bacillus* Strain using Industrial Skin Waste as a Novel Substrate

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MN and RN designed the study and analyzed data. RN, NM and MMR contributed new methods or models. YA helped in writing the manuscript.

Keywords
Protease, *Bacillus*, Molasses, Industrial skin waste, Optimization, RSM

Abstract | Protease is one among other many enzymes which possess the qualities of the vitally important hydrolytic enzymes. The present experimental work of the protease production was conducted by using skin waste from leather industry as a substrate. Different sources of nitrogen and carbon were screened in order to enhance the production of protease by a locally isolated *Bacillus* sp. In order to ensure the best results Response surface methodology (RSM) was employed to optimize five different independent variables including substrate, Molasses, temperature, pH and incubation period. Maximum protease activity was observed by using molasses (540.62 ± 26.77 IU/ml) as carbon source and $NaNO_3$ (606.95 ± 7.61 IU/ml) as nitrogen source. The optimum conditions predicted by RSM were substrate 2%, molasses 1.5%, temperature 35 ˚C, pH 9.0 and induction period of 96 hour. Almost 2.9 fold increase in protease production was observed after optimization at these optimal conditions. All these results indicated that skin waste from leather industry can be exploited as a potential substrate for alkaline protease production at optimized conditions by *Bacillus* sp.

Novelty Statement | In present research work industrial skin waste obtained from the local leather industry was exploited as substrate for alkaline protease production that make the process economically feasible and environment friendly by utilizing the industry waste.

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Introduction

Enzymes have become the center of attention for researchers due to various types of industrial and pharmaceutical uses with their eco-friendly nature (Corrêa *et al.*, 2014). There are countless benefits of the enzymes specifically proteases and its various characteristics such as pH profiles and thermo-stabilities conducive to various types of industrial demands. Proteases are the vitally important commercial enzymes because of their special needs in silver recovery, food processing, diagnostics, pharmaceutical companies and detergent producing industries (Kumar *et al.*, 2012). These catalysts can be roughly calculated for 60 to 65% of the world enzymes market which easily made them to be the most efficient and widely used in term of their industrial usages (Laxman *et al.*, 2005). Proteases are enzymes which hydrolyses peptide bonds among amino acids groups of proteins. The hydrolytic enzymes are accounted for almost 75% of the global sales for industrial usages which are used in different areas along with proteolytic enzymes that are almost 60% in them (Ningthoujam *et al.*, 2009; Chu, 2007).

Alkaline proteases are among the most important groups of commercially used enzymes which are

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produced by a myriad variety of microorganisms such as fungi and bacteria (Kaminishi *et al.*, 1994). Currently microorganisms are the best possible protease producing resources because of their compatibility to genetic manipulations as well as bioengineering capabilities (Nirmal *et al.*, 2011). Furthermore, most of the microbial proteases are extracellular enzymes secreted directly into the fermentation channel unlike plants and animals that have complex process (Lageiro *et al.*, 2007). Bacterial proteases are the best among other types of proteases such as fungal and animals (Ward, 1985). The *Bacillus* sp. is one of the mostly exploited groups of microorganisms for industrial production of diverse enzymes, vitamins, probiotics and insecticides. Moreover, these species are mostly used in industrial metabolites production (Dave *et al.*, 2015). These are the industrially potential microbes due to their extensive growth productivity and the shortest fermentation cycle. Generally, most of the *Bacillus* sp. are considered to be the safest status with the drug and food administration (Alcaraz *et al.*, 2010; Rai *et al.*, 2010).

Now a days numerous efforts have been made in the exploration of new resources of enzymes specifically protease that possesses the most compatible criteria for various types of biotechnological applications. The process of fermentation especially submerged and solid state trigger various aspects of microorganism's growth and production of enzymes (Hamzah *et al.*, 2009). However, researchers believe that the submerged fermentation is more effective rather than the solid state fermentation due to handy sterilization procedures that makes the system easier to tackle (Vidyalakshmi *et al.*, 2009).

Leather production has become another contributing factor in the global climate crisis because of organic wastes, bad smell and heavy water consumptions. Usually various types of industrial wastes are left out during the process of hides transformation into leather products in both modern and traditional factories which badly influence our natural diversity in various forms across the world. During leather manufacturing process there are many different types of residuals parts are disposed to the environment without realizing that could also be used for the productive purposes and that's accounted for almost 60% which can be otherwise used for industrially productive purposes. The most obvious example is alkaline protease production process in which microorganisms utilizes different biological tannery wastes. Solid wastes come from various sources of leather production such as flesh wastes, chrome shaving, skin trimming keratin wastes and buffing wastes while its basic components are proteins. If these proteins and other chemicals which have exist in the chemically treated proteins are not properly utilized it can pose unsafe and dangerous contamination problem to the ecosystem (Ahmad and Ansari, 2015). These proteins can be easily utilized instead of dumping them into the environment.

For models biological processes and optimization, the researchers have been applied the response surface methodology (RSM) with a specific number of factors of variables to increase the yields of commercially significant products to many fold. This prompts to improve the yields and in turns decline in the production budget for their various commercial usages in pharmaceuticals, foods and detergent industries (Ferreira *et al.*, 2009). For optimization the researchers commonly employ on factor at a time technique but this method is hectic and tedious, therefore alternative use of statistical tools is being explored which makes the procedures easier. RSM is an example, being widely applied for modeling, optimization and analysis of problems related to the production of industrial enzymes (Priji *et al.*, 2015).

The alkaline proteases specifically extracellular proteases from the member of *Bacillus* genus are generally the serine proteases have been used from long time in many fields (Pathak and Rathod, 2018). The *Bacillus* sp. are an inexpensive source of enzymes since its wide dispersal, safety handling, easy cultivation and liability to genetic manipulations. The scientists are primarily interested in proteolytic enzymes that found one of the greatest diverse classes of microbial proteins in terms of characteristics. In spite the extended history of their studies this class of enzymes remains to exhibit great prospective for practical applications (Danilova and Sharipova, 2020).

In the present study we explore a *Bacillus* strain, which produces alkaline protease extracellular in a wide range of pH and temperature. The alkaline protease was produced from *Bacillus* sp. by using beef skin waste (defatted) from leather industry as substrate because it was not much exploited before for protease enzyme production in the literature cited. The effects of various nitrogen and carbon sources on alkaline protease production have been studied. RSM was used to obtain the maximum yield of the protease enzyme. Protease production process parameters including initial pH of the medium, temperature and substrate, molasses and incubation period were optimized by central composite experimental design (CCD). The obtained data was then processed by RSM to obtain the optimal levels and predictions.

Materials and Methods

Microorganism

Bacterial strain was isolated from soil sample. The soil samples were collected from various areas of Lahore such as fish market, poultry market and slaughter house. Soil samples were prepared as one percent solution with sterile water. This stock solution then serially diluted, allow to heat shock for 15 minutes at 80°C to kill all other vegetative cells and the inoculated on the skim milk agar medium and incubate at 37 °C for 24 h. The single colonies of

Bacillus sp then streaked on nutrient agar (Oxide medium) slant having pH 8.5. After 24 hours of incubation at 37 °C, the strains were preserved at 4 °C for experimental work.

Industrial skin wastes collection and pre-treatment

The industrial skin wastes were collected from leather industry Kasur, Lahore Pakistan. The skin wastes were cut into small pieces and treated with 1.25% ammonium chloride solution to remove absorbed salts calcium during processing (Ahmad and Ansari, 2015). Then washed with tap water and dried.

Production of alkaline protease

Alkaline protease production was carried out in medium comprised of (w/v %) K_2HPO_4 0.5%, NaCl 0.05%, $MgSO_4.7H_2O$ 0.05%, glucose 0.5% and 1% of the industrial skin waste as a substrate in the 250 Erlenmeyer flask for screening and for optimization same medium was used except glucose that was replaced with molasses (0.5-2.5%) and 0.5 % $NaNO_3$. pH of the medium was adjusted to 8.5 and autoclaved at 121°C for 15 minutes. Inoculums of 24 hours old culture in nutrient broth (Oxide medium) was added 2% (v/v) in the sterilized medium and incubate at 37 °C for 48 hours in a shaking incubator at 100 rpm. The sample was collected after fermentation and centrifuged at 5000 rpm at 4 °C for 15 minutes. The pellets containing cells were discarded and the supernatant was used to measure the enzyme activity.

Screening of carbon source

Different carbon sources like (Galactose, soluble starch, glucose, lactose, sucrose, maltose, fructose and cellulose) and the agro-industrial waste (molasses) were screened in the fermentation medium to observe the appropriate carbon source for the production of alkaline protease by *Bacillus* sp. (Table 1) The initial concentrations of all the above carbon sources were employed at 0.5% (w/v).

Table 1: Screening of different carbon source for alkaline protease production.

Sr. No.	Carbon source	Mean ± SD* (IU/ml)
1	Control	373.32 ± 10.03^{cd}
2	Glucose	397.57 ± 16.34^{de}
3	Fructose	417.72 ± 16.47^{e}
4	Sucrose	307.28 ± 7.58^{b}
5	Maltose	288.93 ± 16.52^{b}
6	Starch	356.54 ± 15.21^{c}
7	Galactose	294.90 ± 10.56^{b}
8	Glycerol	199.41 ± 16.64^{a}
9	Manitol	411.81 ± 22.56^{e}
10	Xylose	357.36± 30.96^{c}
11	Molasses	540.62 ± 26.77^{f}

*SD is standard deviation followed by different letters represent the significant difference among yield with different carbon sources.

Screening of nitrogen sources

Different inorganic and organic sources of nitrogen were supplemented to the medium to identify the suitable nitrogen source. The organic sources such as beef extract, peptone, yeast extract, urea, casein hydro lysates, and tryptone were initially employed to the production medium at 0.5% w/v. On the other hand, the inorganic sources like $(NH_4)_2SO_4$, $NaNO_3$, NH_4NO_3, and at the above same concentration of 0.5% (w/v) were employed for the hyper production of alkaline protease from *Bacillus* sp. (Table 2).

Table 2: Screening of different nitrogen source for alkaline protease production.

S. No	Nitrogen source	Mean ± SD (IU/ml)
1	Control	379.38 ± 39.53^{c}
2	Beef extract	446.19 ± 34.33^{d}
3	Yeast extract	420.03 ± 10.84^{cd}
4	Peptone	315.08 ± 27.06^{b}
5	$NaNO_3$	606.95 ± 7.61^{e}
6	Tryptone	453.26 ± 29.27^{d}
7	Casein hydrolysates	275.83 ± 28.26^{ab}
8	Ammonium sulphate	440.17 ± 17.81^{d}
9	Ammonium nitrate	389.25 ± 15.25^{c}
10	Urea	263.21 ± 12.76^{a}

*SD is standard deviation followed by different letters represent the significant difference among yield with different nitrogen sources.

Optimization by response surface methodology (RSM)

Optimization to enhance the production of protease from the isolated bacterial strain was conducted by RSM method. Important variables including substrate (%) X1, molasses (%) X2, temperature (°C) X3, pH- X4, and incubation period (h) X5, each with five different levels (Table 3) were used to construct the central composite experimental design (CCD) with 32 experimental runs (Table 4). RSM model was used to predict the expected outcomes from the observed data as shown in the Equation 1.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5 + \beta_{11} X^2_1 + \beta_{22} X^2_2 + \beta_{33} X^2_3 + \beta_{44} X^2_4 + \beta_{55} X^2_5 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{15} X_1 X_5 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{25} X_2 X_5 + \beta_{34} X_3 X_4 + \beta_{35} X_3 X_5 + \beta_{45} X_4 X_5 \quad \ldots(\text{Eq. 1})$$

Where response variables are represented by Y and the coefficient of regression is represented by β_0. Linear effect of coefficient represented by β_1, β_2, β_3 and β_4, and effect of coefficient of quadrant represented by β_{11}, β_{22}, β_{33}, β_{44} and β_{55}, and the effect of coefficient of interaction represent by β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, β_{34} and β_{45} for five different indecent variables (X_1= substrate, X_2= molasses, X_3= temperature and X_4= pH, X_5= incubation period).

Table 3: Coded values of variables used for RSM Optimization.

Coded values	Actual values				
	X1	X2	X3	X4	X5
	Substrate %	Molasses %	Temperature °C	PH	Incub. period (h)
-2	1	0.5	25	7.5	24
-1	2	1	30	8	48
0	3	1.5	35	8.5	72
1	4	2	40	9	96
2	5	2.5	45	9.5	120

Table 4: CCD Design used for RSM optimization of alkaline protease production by *Bacillus*.

S. no.	Sub-strate	Mo-lasses	Temp	PH	Incu. Prd	Observed values	Predicted by RSM
1	1	1	1	1	-1	1289.363	1302.188
2	1	1	1	-1	1	1350.191	1348.313
3	1	1	-1	1	-1	1261.273	1271.997
4	1	1	-1	-1	1	1393.85	1388.341
5	1	-1	1	1	-1	1326.078	1320.445
6	1	-1	1	-1	1	1280.615	1295.573
7	1	-1	-1	1	-1	1276.092	1264.711
8	1	-1	-1	-1	1	1294.98	1310.058
9	-1	1	1	1	-1	1302.357	1286.672
10	-1	1	1	-1	1	1358.888	1370.877
11	-1	1	-1	1	-1	1295.995	1281.645
12	-1	1	-1	-1	1	1431.043	1436.069
13	-1	-1	1	1	-1	1501.842	1507.958
14	-1	-1	1	-1	1	1532.497	1521.166
15	-1	-1	-1	1	-1	1476.117	1477.388
16	-1	-1	-1	-1	1	1573.032	1560.815
17	2	0	0	0	0	1303.652	1289.061
18	-2	0	0	0	0	1509.711	1524.302
19	0	2	0	0	0	1348.944	1347.373
20	0	-2	0	0	0	1488.806	1490.376
21	0	0	2	0	0	1496.312	1490.632
22	0	0	-2	0	0	1494.411	1500.091
23	0	0	0	2	0	1434.179	1442.236
24	0	0	0	-2	0	1403.134	1395.077
25	0	0	0	0	2	1551.302	1543.245
26	0	0	0	0	-2	1358.477	1366.534
27	0	0	0	0	0	1505.162	1497.162
28	0	0	0	0	0	1502.162	1497.162
29	0	0	0	0	0	1499.162	1497.162
30	0	0	0	0	0	1498.162	1497.162
31	-1	2	0	-2	1	1295.55	1290.55
32	-1	-1	0	-2	1	1456.193	1449.193

Statistical analysis

For statistical analysis the version 7 software of STATISTICA were used to construct data and plots. To conclude the significance of parameters in RSM the analysis of variance (ANOVA) was used. To analyze the regression model performance, the adjusted R^2 and R^2 were calculated. From the desirability charts optimized levels of particular variables were obtained. The SPSS software was used for comparison of means by Post Hoc test.

Analytical procedure

The activity of enzyme was measured according to the slightly modified method of Yang and Huang (Yang and Huang, 1994). Activity was calculated in IU and One International Unit (IU) of protease enzyme is equal to one micromole of amino acid released by the per ml/min of enzyme source. Crude enzyme extract of 1 ml mixed with 2 ml of 1% casein solution in a glycine-NaOH buffer with pH adjusted at 10 and incubated for 30 minutes at 40 °C. 3 ml10% TCA of solution was added to stop the reaction. The solutions were centrifuged at 9000 rpm for 10 minutes. The optical density (OD) was Measured at 280 nm against the blank.

Results and Discussion

Screening of carbon source

Different source of carbon exhibited different effect on protease yield (Table 1). Among this molasses showed supreme level of yield (540.62 ± 26.77 IU/ml) with significant difference from other sources. Followed by fructose (417.72 ± 16.47 IU/ml) with a minimum yield was obtained using glycerol (199.41 ± 16.64) as compared with control (373.32 ± 10.03).

Nitrogen source

For high productivity of enzymes different sources of inorganic and organic nitrogen were screened to find out the suitable source of nitrogen for high yield of alkaline protease from newly isolated *Bacillus* (Table 2). Among these $NaNO_3$ showed significantly higher yield (606.95 ± 7.61 IU/ml) as compared to the other nitrogen sources (Table 2) and is followed by tryptone (453.26 ± 29.27), Beef extract (446.19 ± 34.33 IU/ml), ammonium sulphate (440.17 ± 17.81), yeast extract (420.03 ± 10.84), ammonium nitrate (389.25 ± 15.25), peptone (315.08 ± 27.06), casein hydrolysates (275.83 ± 28.26), urea (263.21 ± 12.76) respectively as compared with control (379.38 ± 39.53).

Optimization using RSM

The second-order quadratic model used for the experimental data analyzed by multiple regression was represented in Equation 2. Alkaline protease activity was predicted by using the model.

$$Y= 480.817 - 504.242X_1 - 580.774X_2 - 58.84_2X_3 + 608.134X_4 - 78.505X_5 - 22.620X_1^2 - 78.287X_2^2 - 0.018X_3^2 - 78.505X_4^2 - 0.018X_5^2 + 101.515X_1X_2 + 1.258X_1X_3 + 41.340X_1X_4 + 0.465\ X_1X_5 - 2.554\ X_2X_3 + 42.249\ X_2X_4 + 2.359\ X_2X_5 + 7.022\ X_3X_4 + 0.000X_3X_5 + 4.401X_4X_5 \ldots$$ (Eq. 2)

Where; Y is presenting the enzyme production and X1, X2, X3, X4 and X5 are indicating the substrate, molasses, temperature, pH and incubation period respectively. This model is highly significant as indicated by the calculated statistical parameters (Table 5). The obtained F calculated value was 54.54 and a small value for p (0.000008). On the other hand, R^2 value (0.991) very close to 1 is indicating the accuracy of model. Only a small percentage of the data (0,009%) was not expressed by the calculated model which may be observed from R^2 value. the adjusted R^2 value (0.97) is also supporting the model accuracy and significance. Main effects of incubation time and temperature were found significant as indicated in the Table 5. Quadratic effects of all the five variables used, including Substrate, molasses, temperature, pH and incubation were found significant. Substrate effected the protease production most significantly. Molasses, incubation time, pH and temperature effects were following the substrate effect in descending order. The interaction effects of substrate-molasses and incubation period - pH were found significant. The significant interaction effects are represented by 3D surface plots (Figures 1 and 2). The predicted optimal levels by RSM were; substrate 2%, molasses 1.5%, temperature 35 °C, pH 9.0 and induction period of 96 hours (Figure 3). The graph between observed and predicted values is given in Figure 4. The predicted protease production was 1560.815 IU/ ml, at these optimum levels. The observed protease activity in the experiments at the optimum levels was 1573.032IU/ml.

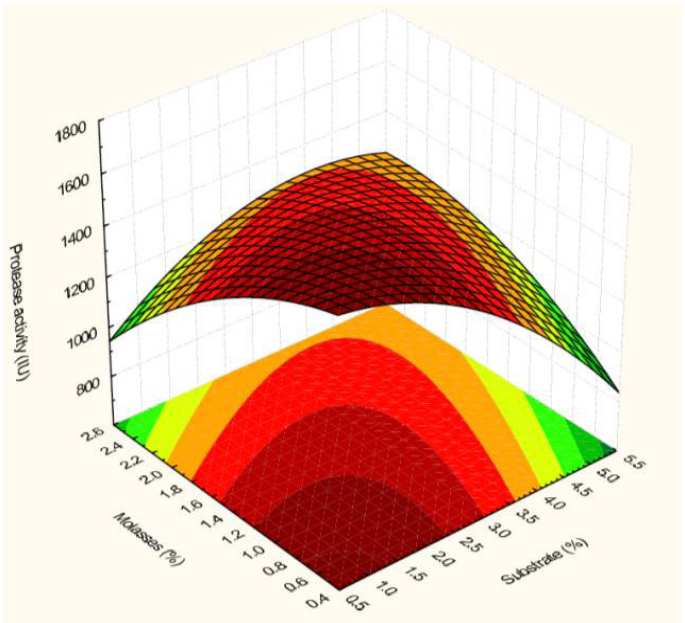

Figure 1: Surface plot representing the interaction effect of substrate and molasses on protease production.

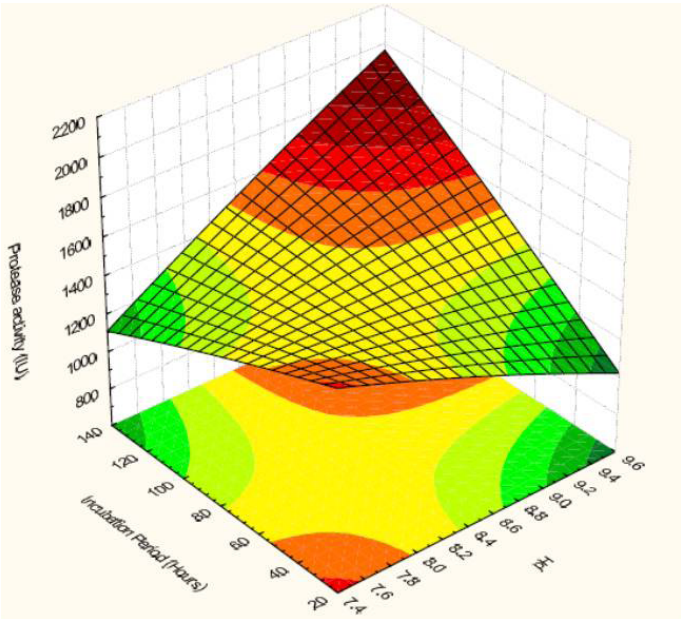

Figure 2: Surface plot representing the interaction effect o of incubation period and pH on protease production.

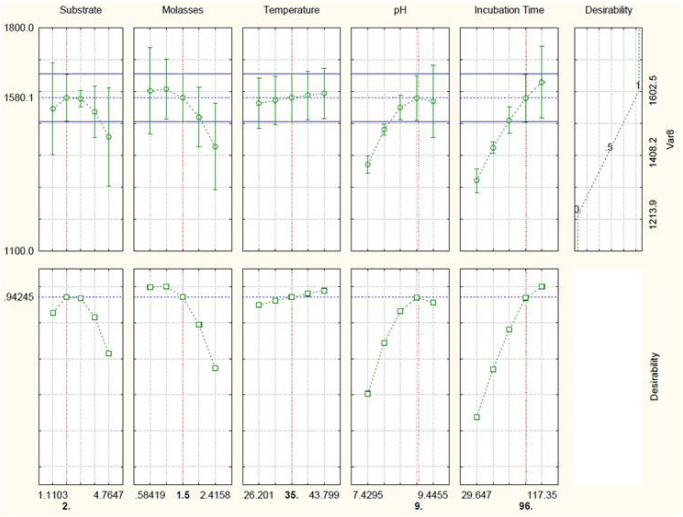

Figure 3: Desirability chart representing the optimum levels for independent variables and predicted protease activity on them.

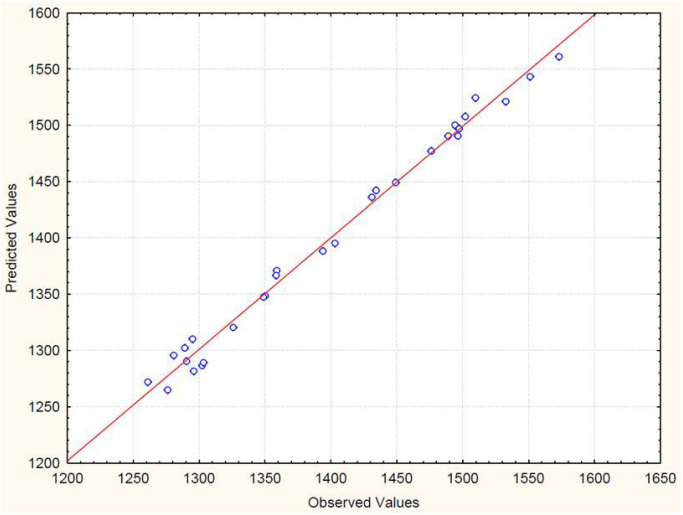

Figure 4: The graph between observed and predicted values for the production of protease enzyme.

Table 5: ANOVA table calculated RSM representing the significance of model and variables.

Effect	SS	Deg. of freedom	MS	F	P
Intercept		0			
(X_1)	445.35	1	445.35	2.0142	0.1786
(X^2_1)	10915.63	1	10915.63	50.03	0.000013
(X_2)	311.26	1	311.26	1.4266	0.2554
(x^2_2)	8171.79	1	8171.79	37.4541	0.000052
(X_3)		0			
(x^2_3)	4.32	1	4.32	0.0198	0.89041
(X_4)		0			
(X^2_4)	8217.43	1	8217.43	37.6632	0.00005
(X_5)		0			
(X^2_5)	2382.57	1	2382.57	10.9201	0.00629
(X_1) * (X_2)	41220.79	1	41220.79	188.919	0
(X_1) * (X_3)	633.22	1	633.22	2.9023	0.11419
(X_2) * (X_3)	652.43	1	652.43	2.9903	0.10938
(X_1) * (X_4)	307.7	1	307.7	1.4103	0.25798
(X_2) * (X_4)	167.34	1	167.34	0.767	0.39834
(X_3) * (X4)		0			
(X1) * (X5)	78.89	1	78.89	0.3616	0.55883
(X2) * (X5)	938.4	1	938.4	4.301	0.60272
(X3) * (X5)		0			
(X4) * (X5)	11900	1	11900	54.5426	0.000008
Error	2616.18	12	2616.18		

A newly isolated *Bacillus* sp. (data will be published in our next research manuscript on the basis of molecular analysis) was used for protease production. It is generally known that both carbon and nitrogen sources availability help in the production of microbial protease, which play a significant role in synthesis of enzyme (Negi and Banerjee, 2010). For production of protease enzyme different carbon source were used to screen such as Molasses, sucrose, starch, glucose, fructose, maltose, galactose, glycerol, mannitol and xylose. Among all of these the best activity for enzyme production was shown by molasses. Abidi and Limam Nejib were also presented the maximum protease activity on 1.0% molasses by *Bacillus subtilis* in mineral medium (Abidi and Limam-Nejib, 2008).

Molasses have superior effect on the production of enzyme because of growth promoting substance in it which promotes the bacterial growth along with production of different concentration of enzyme. For large scale fermentation molasses is considered economical and cost effective main energy source. Similar effects were observed by Abidi and Limam Nejib among different carbon sources used in protease production with highest in the medium containing molasses (205.00 U/ml) (Abidi and Limam-Nejib, 2001).

The production of protease enzyme suppressed in the existence of organic nitrogen sources in the production medium while the alkaline protease production may be found to be best with inorganic nitrogen source such as $NaNO_3$. Different inorganic and organic source for protease production were secerned out. The maximum proteolytic activity observed by $NaNO_3$ was 606.95 IU/ml. Maximum protease activity was obtained by using $NaNO_3$ as nitrogen source. Sodium $NaNO_3$ ameliorated the production level up to 10.52% and 62.40 of neutral and alkaline protease, respectively (Gul *et al*., 2015).

The culture medium condition and nutrients was optimized through RSM by *Bacillus* sp. for production of alkaline protease enzyme. Now days in biotechnology the RSM a statistically model was used to optimize the superlative states for the production of enzymes owing to easy reliability, validity and applicability (Haaland, 1989). The RSM is best method for optimizing the enzyme activity. RSM is time saving, efficient, and highest yield method for batter understanding the variable interactions have been reported in different studies successfully optimizing of enzyme activity and subsequently achieved heights product formation. After optimization by RSM almost 2.9 fold increases in production was observed. A 2.5 fold increase in protease production by *Bacillus* sp. JER02 with statistical optimization by RSM was observed in another study (Badoei-Dalfard and Karami, 2013). Protease activity was achieved at highest level by *Bacillus subtitles* isolated from local tannery, up to 8.9 fold by a research group (Hussain *et al*., 2017). These values are in close agreement with the results obtained in the present study.

In ANOVA the resulted F and P value are used to determine the model significance of variables. The model is considered significant with higher F value and p value than 0.05. The significance of variable effects was represented by their p values for the main effects, quadratic effects and interaction effects of variables. The effects of substrate level molasses and pH incubation time interactions were significant according to their p vales smaller than 0.05 p values. The ranking information of variables according to their effect on production was obtained from their F values. The effects of variables with larger F value have larger effect whereas smaller effect from the variables with smaller f values. Positive and negative sign indicated the positive and negative effect of variables as represented in Table 5.

According to RSM analysis, the maximum effect on protease was due to substrate level of 2%. The amount of protein in the substrate is high i.e. 78.81% of its total dry weight (Unpublished data). Due to high level of protein concentration bacteria digest the substrate and secret the required components in the fermentation medium. Due to high level of protein concentration bacteria digest

the substrate and secret the required components in the fermentation medium. For maximum enzyme production and cell growth, temperature is considered as most critical parameter that varies for different microorganism and has to be controlled. The protease produced by *Bacillus* with an optimum temperature at 30°C because the optimum growth temperature for bacteria is 30–35°C. The optimal temperature requirements reported for the production of alkaline protease by various microorganisms varies widely. Similar results of optimum temperature have been reported for various species of *Bacillus* (Asokan *et al*., 2011).

A bacterium isolated from soil and identified as *Bacillus cereus* KM 05 and a statistical method RSM was applied for optimization for better yield. It was explored that the predicted model has high value of R^2. 1.5 fold increased in protease production obtained (Jayakumar *et al*., 2021). Similarly, Shakir *et al*. (2019) reported the production of alkaline proteases from *Bacillus safensis* using RSM for optimization under submerged fermentation. The nutritional constituents were screened by Placket-Burman design. The optimized culture conditions were inoculum size 1%, medium pH 9.0, temperature 30 °C, and 72 h of incubation time.

An alkalophylic strain that was newly isolated grows at optimum pH 8. Sharma *et al* also stated that the maximum production of protease in a medium was observed having pH 8.5 as in the case of newly isolated bacterial strain AKS-4 and for AKS-6 bacterial case the pH. 10.5. The culture pH Effect all the component includes in enzymatic process and various cell membrane transposition material (Sharma *et al*., 2015). However, at molecular level the bacterial metabolism affected by pH in culture broth is ambiguous. Since in chemiosmosis the proton motive force in a medium is affected by the pH value, therefore it is possible to optimize the pH range, under optimum condition the metabolic efficiency is relative high (Singh *et al*., 2010). For highest protease production was achieved at optimum incubation periods of 96 hours. Whereas Ratnasri and his colleagues suggested that highest protease activity of bacterial isolates AKS-4 and AKS-6 were after 5 days (120 hours) and similarly they also reported that height activity of protease by *Bacillus cereus* strain S8 was achieved after 3 days (72 hours) (Ratnasri *et al*., 2014).

Kumar *et al*. (2008) used proteinaciuos solid wastes produced by leather processing industries as a substrate for the production of alkaline protease and found the maximum protease activity of 1160–1175 U/ml. These studies are in agreement with the results obtained in the present study.

Conclusions and Recommendations

The present study was conducted to optimize alkaline protease production from newly isolated bacillus using skin waste as a substrate. Form our study it was concluded that industrial skin waste is a good sources of nutrients fir protease production and in addition it can minimize the environmental pollution from leather industries. This preliminary attempt will hopefully be helpful to replace traditional hazardous chemicals used in leather processing. Furthermore, molasses and sodium nitrate were found the best carbon and nitrogen sources. RSM was observed as suitable statistical tool to optimize a bioprocess with accuracy in less time.

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Conflicts of interest

The authors have declared no conflict of interest.

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