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Enhanced Production of Extracellular Alkaline Protease by *Bacillus cereus* GVK21 by Optimized formulations

Keshavamurthy. M[1], Vishwanatha. T[2], Suresh Kumar M[1], Subhaschandra M Gaddad[1*]

[1]Department of Post Graduate Studies and Research in Microbiology, Gulbarga University, Jnana Ganga, Kalaburagi – 585 106, Karnataka, INDIA.

[2]Department of Microbiology, Maharanis's Science College for Women, Palace Road, Bengaluru – 560001, Karnataka, INDIA

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***Corresponding Author Email:** smgaddad@gmail.com

Abstract

Bacillus cereus GVK21, isolated in our laboratory, is a potent producer of alkaline protease. In the present study optimization of the process and nutritional parameters is reported. Response Surface Methodology (RSM) based Box-Behnken design (BBD) was employed for the optimization of various nutritional factors such as carbon and nitrogen sources and physical parameters like incubation time, pH, temperature, inoculum size and age, agitation rate were optimized for the maximum yield of protease under submerged fermentation. The Maximum of 912 U/mL protease production was observed at 48 h of incubation, pH 9.0, 140 rpm shaking and a temperature of 40°C. Thus, with above selected process parameters the best carbon and nitrogen sources for *B.cereus* was found to be molasses 0.75% and Peptone 1.00%. 3D plots were generated to evaluate the damages in the response surface and to understand the relationship between the culture conditions and protease yield. The statistical optimization by RSM resulted in significant increase by about two-fold in the protease yield. The present study shows that protease production by *B.cereus* GVK21 is greatly influenced by cultural conditions and media constituents. This study indicates that the strain is capable to producing maximum levels of alkaline protease in a low-cost medium containing molasses and peptone as major carbon and nitrogen sources and can be exploited for industrial production of alkaline protease.

Keywords

Optimization, alkaline Protease, *Bacillus cereus,* Response surface methodology, culture conditions, submerged fermentation

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INTRODUCTION

Proteases are an important class of industrial enzymes and account for 60% of total global enzyme sales and represent one of the three largest groups of industrial enzymes (Sharma *et al.,* 2017). Proteases are extensively exploited commercially in food, detergent, leather and pharmaceutical industries (Ningthoujam *et al.,* 2009). Microorganisms are extensively used in the industrial production of proteases. *Bacillus* was the first to be used in the commercial production of protease in 1952 (Binod *et al.,* 2013). Several proteases have

been produced from many *Bacillus* species (Rao *et. al.,* 1998). *Bacillus* species like *B. subtilis* (Babe and Schmidt, 1998), *B. licheniformis* (Mabrouk *et al.*, 1999), *B. sphaericus* (Singh *et al.*, 1999), *B. proteolyticus* (Bhaskar *et al.*, 2007), *B. cereus* (Doddapaneni *et al.,* 2009; Bajaj *et al*., 2013) *and B. megaterium* (Rajkumar *et al.*, 2010) have been reported for protease production. Other bacteria such as *Pseudomonas fluorescens* (Kalaiarasi and Sunitha, 2009), *P. aeruginosa* (Raj *et al.*, 2012), *Vibrio etschnikovii* (Jellouli *et al.*, 2009), *V. alginolyticus* (Shanthakumari *et al.*, 2010) are also reported for protease production.

The production of proteases by microorganisms is influenced by several factors, such as ionic strength, temperature and mechanical handling. Media composition plays a significant role in enzyme production by microorganisms (Dorcas and Pindi, 2016). In order to obtain high and commercially viable yields of protease, it is essential to optimize fermentation conditions (Sharma *et al.,* 2015). No defined medium has been established for the best production of alkaline proteases from different microbial sources. Each organism has its own special conditions for maximum enzyme production (Schmidt, 2005; Sharma *et al.,* 2017).

Media rich in nitrogen sources, such as casein, gelatin, soybean meal, corn steep liquor, distiller's solubles, brewer's yeast, and carbohydrate sources such as starch or lactose are generally used for protease production (Blieva *et al.,* 2003). However, higher carbohydrate concentrations repress enzyme production (Mehta *et al.,* 2006).

Though a variety of microorganisms are used for protease production, *Bacillus* sp. are the most predominant and an ideal source for enzyme production as they grow rapidly and survive under harsh environmental conditions (Azlina and Norazila, 2013). Earlier researchers often used to perform one-factor-at-a time experiments, which vary only one factor at a time while keeping other variables fixed. However, statistically designed experiments that vary several factors simultaneously are more efficient when studying two or more factors. The conventional way of optimization has been done by monitoring the influence of one-factor-at-a-time approach keeping the other parameters constant. The major drawback of one-factor-at-a-time is that it is time consuming and production cost becomes expensive. To overcome these difficulties a statistically designed optimization study is helpful in determining the effects and interactions of fermentation variables (Govarthanan *et al.,* 2014). The application of statistically designed experiment will helpful in the reduction of production cost, improvement of product yield etc. Thus, a statistically designed optimization is the need of the hour. In recent years Response Surface Methodology (RSM) is widely being used for optimization studies of various Biotechnological and industrial processes. The present investigation reports the enhanced production of alkaline protease by *B. cereus* GVK21 (KY659318) by optimizing process parameters.

2. MATERIALS AND METHODS:

2.1 Microorganism:

Bacillus cereus GVK 21 was isolated in our laboratory from soil samples collected in from different locations within the Hutti gold mines, Raichur district (16° 11′ 45″ North Latitude and 76° 38′ 31″ East Longitude), Karnataka, India (Keshavamurthy *et al.,* 2018). The culture was maintained on nutrient agar slants.

2.2 Fermentation Medium and enzyme production:

Protease production was carried out using production medium [g/L: Glucose – 5.0; casein- 5.0; Yeast extract – 5.0; $MgSO_4 \cdot 7H_2O$ - 0.02 and KH_2PO_4 - 0.05 pH 9](Pant *et al.,* 2015).One mL of fresh bacterial inoculum was added in to 250 mL Erlenmeyer flask containing 100 mL of production medium. The flasks were placed in a rotary shaker incubator at 140 rpm at 37° C for 3-4 days. An aliquot of the culture supernatant was collected at regular intervals of 12 hand assayed for protease activity (Josephine *et al.,* 2012).

2.3 Optimization of medium components and culture conditions for Protease Production:

2.3.1 One Variable at a Time Approach (OVAT)

Production of protease by *Bacillus cereus* GVK21 was carried out in basal medium with different combinations of carbon and nitrogen sources (1% w/v) and 1% inoculum size at 37 °C for 72 h in a rotary shaker (120 rpm). The initial pH of the medium was adjusted to 7.0. Parametric optimization was performed with respect to pH (7 to 10), incubation period (24 to 120 h), temperature (25 to 50°C), agitation rate (100 to 200 rpm), inoculum size (0.5 to 3 mL) and inoculum age (6 to 30 h) were studied by using various levels of the test parameter and keeping the other parameters constant. The bacterium was grown in the production medium supplemented with different carbon sources (1% w/v) such as dextrose, fructose, glucose, lactose, mannose, maltose and molasses, and nitrogen sources (1% w/v) like peptone, yeast extract, beef extract, ammonium chloride and ammonium sulphate. At the end of the fermentation duration, the fermentation broth was centrifuged at 10,000

rpm for 5 min. The supernatant obtained by the centrifugation was used for measuring the protease activity. The un-inoculated flasks served as controls. All the experiments were done in triplicates and the average of enzyme activity was taken for statistical analysis to know the significance of each factor.

2.3.2 Statistical Optimization

Response surface methodology is a well-accepted statistical technique able to design and optimize the experimental process that involves choosing the optimal experimental design and estimate the effect of the several factors independently and also the interactions simultaneously. Response surface methodology combined with BBD was established using Design Expert software (Version 11.0, Stat-Ease Inc., Minneapolis, USA) to analyze and plot the response surface graphs. Four factors, namely, pH, incubation period, molasses and peptone, were optimized for enhanced protease production using the isolate *B.cereus* GVK21. Based on Box-Behnken design (BBD), the factors were analysed at two levels: -1, for low level, and +1, for high level. A total of twenty-nine (29) runs were performed to optimize the process parameters, and experiments were performed according to the experimental design matrix. The results were evaluated by applying the coefficient of determination (R^2), analysis of variance (ANOVA) and response plots. Employing RSM, the most widely used second-order polynomial equation was developed to fit the experimental results and identify the relevant model terms:

$Y=\beta_0 \sum\beta_i X_i +\sum\beta_i X_i \beta i_j+\sum X_i X_j$

where Y is the predicted response; b_0, b_i, and b_{ij} are constant regression coefficients of the model; and X_i and X_j represent independent variables. The experimental design helps in investigating linear, quadratic and cross product effects of these factors and also centre points for replication (Govarthanan *et al.,* 2015). ANOVA (Analysis of Variance) – a statistical analysis was performed where the data obtained in the present study were expressed as Mean ± SD and was further analyzed using one-way ANOVA test at 5% level of significance using IMB SPSS (20) version software.

2.4 Protease assay:

The protease activity of the crude enzyme was determined by the modified method of Joo *et al.,* (2002) briefly; 0.5 mL of Culture filter Supernatant (CFS) was added to 0.5 mL of 1% casein (as a substrate) in 0.1 M Tris-HCl (pH 9.0) and incubated at room temperature for 10 min. The reaction was terminated by the addition of 3 mL of 10% (w/v) trichloroacetic acid (TCA). The solution was centrifuged at 5000 rpm for 10 min. To the 3 mL of the clear supernatant, 5 mL 0.4 M sodium bicarbonate solution and 0.5 mL of Folin Ciocalteau reagent (FCR) were added, mixed thoroughly and incubated for 30 min at room temperature, in dark. The optical density was measured using a UV-VIS spectrophotometer (ELICO SL - 159) at 660 nm against the enzyme blank. The amount of the released aromatic amino acids was calculated using tyrosine standard.

One unit of protease is defined as the amount of the enzyme required to release 1µg of tyrosine per mL per min and was calculated according to the formula of Pant *et al.*, (2015).

RESULTS AND DISCUSSION:

Protease production is an inherent capacity of all microorganisms; and a large number of bacterial species are known to produce alkaline proteases (Lakshmi *et al.,* 2014). Among various bacteria, the *Bacillus* species are most significant and specific-producers of alkaline proteases (Rajkumar *et al.*, 2010; Bajaj *et al*., 2013).

A potent protease producing bacterium GVK21 was isolated in our laboratory and was identified as *B. cereus* GVK21 (Keshavamurthy *et al.,* 2018). In the preliminary studies, the bacterium produced 136 U/mL of protease activity in 24 hours.

With respect to the effect of pH on protease production, increase in pH of the medium up to pH 9.0 increased the protease production to a maximum of 286.5 U/mL, then after the enzyme activity steadily decreased (Fig.1). The pH of culture affects enzymatic processes and transportation of various components across the cell membrane (Sharma *et al.,* 2017). However, the molecular basis of pH affecting bacterial metabolism in culture broth is not well understood. Since proton motive force in chemiosmosis is affected by the medium pH value, it is possible that under optimum pH range, the relative metabolic efficiency is high (Singh *et al.,* 2010). Dodia *et al.,* (2006) reported that the optimum pH for growth of bacteria was 9.0 for the majority of the isolates, while the optimum pH with regard to enzyme secretion varied between pH 8.0 - 10.0. A pH of 9 has been reported as optimal for protease production by *Bacillus* sp. (Prakasham *et al.,* 2006) and *Bacillus* sp. strain APP1 (Chu, 2007), which is similar our observation.

The temperature affects growth rates of microorganisms, regulates the synthesis of the enzyme and also the enzyme production by changing the properties of the cell wall (Radhika Pilli and Siddalingeshwara, 2016). The highest protease activity was detected at 40°C (346 U/ml); however,

substantial activity was observed at 45°C (329.5 U/ml) and 50°C (313 U/ml) as well, as shown in Fig 2. The optimum temperature requirement reported for alkaline protease production by different microorganisms differs widely. The optimum temperature for protease production by *B. cereus* has been reported to be 30°C (Kebabci and Cihangir, 2011). A temperature of 37°C has been reported as optimal temperature for protease production by *B. aquimaris* VITP4 (Shivanand and Jayaraman, 2009). In contrast Darani *et al.,* (2008) and Seifzadeh *et al.,* (2008) reported a temperature of 40°Cas optimum for the production of protease by *Bacillus* sp. and*B. licheniformis* GUS1 respectively.

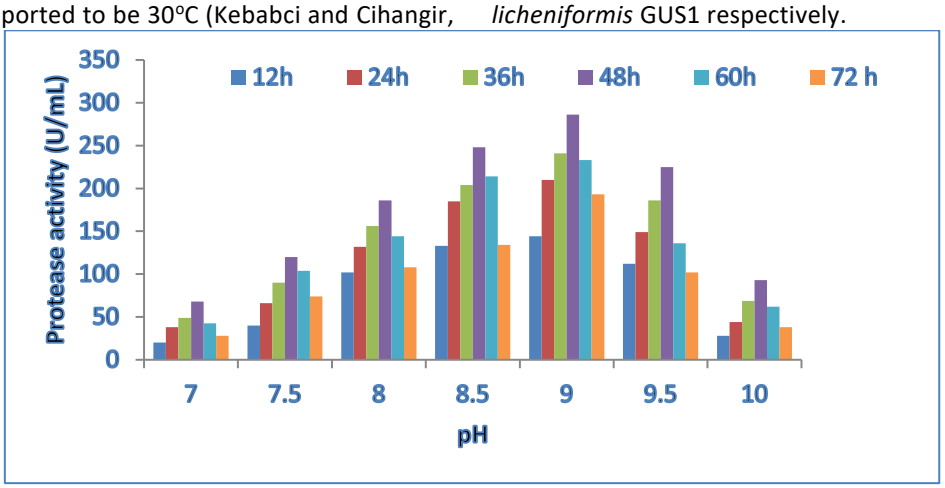


Fig. 1: Effect of different pH on Protease production.

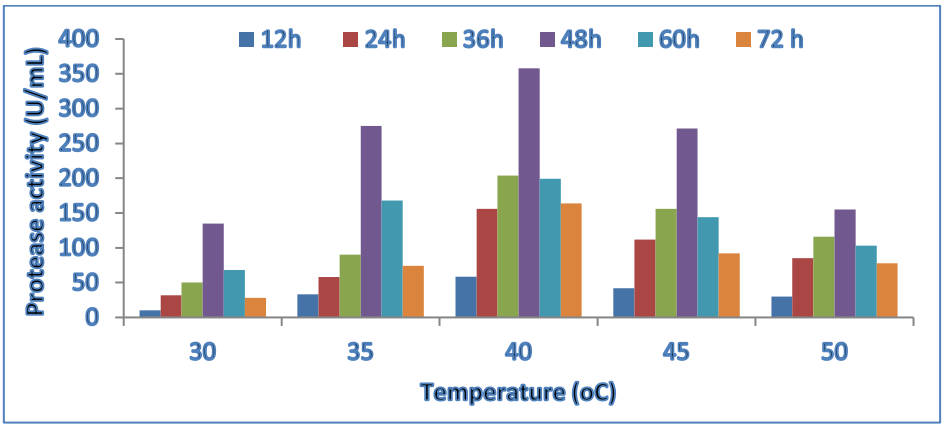


Fig. 2: Effect of different temperature on protease production.

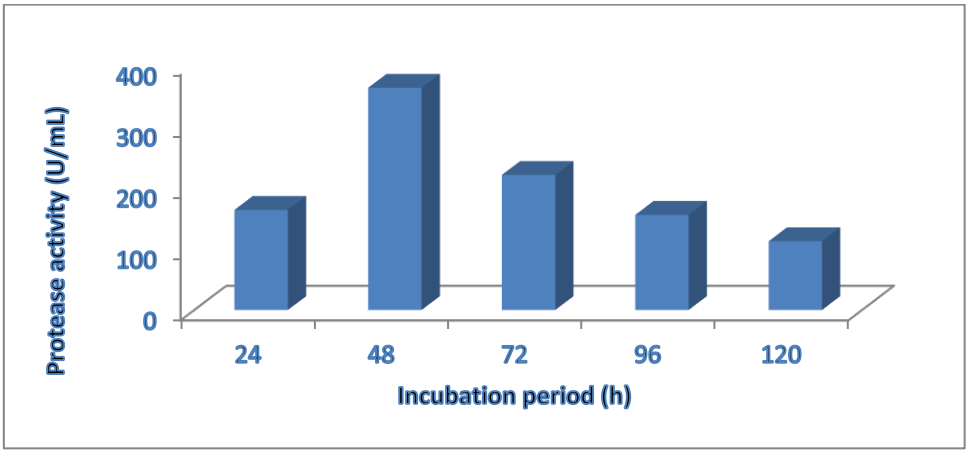


Fig. 3: Effect of incubation period on protease production.

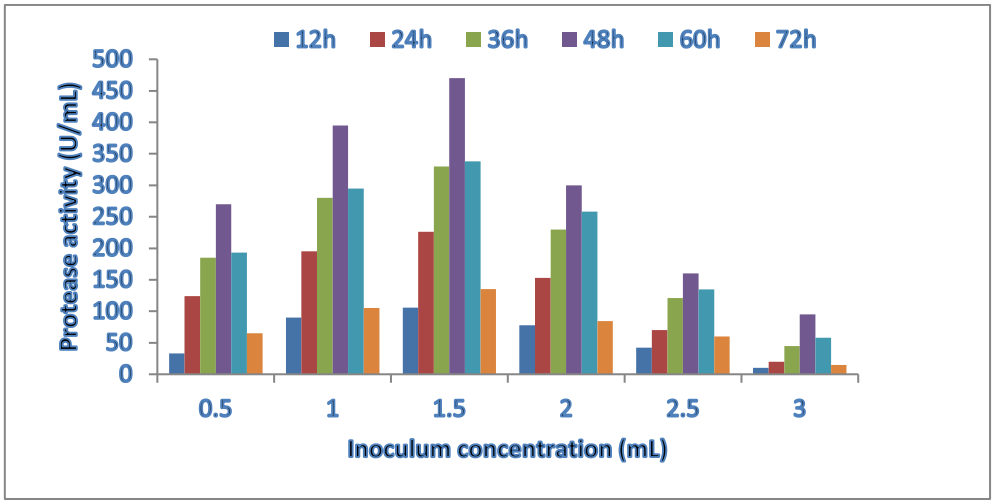


Fig. 4: Effect of inoculum concentration on protease production.

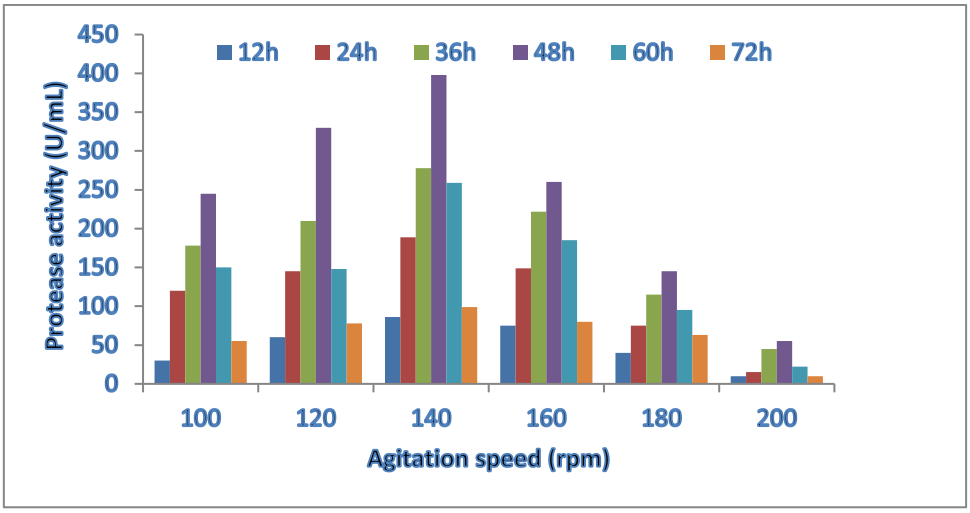


Fig. 5: Effect of agitation speed on protease production.

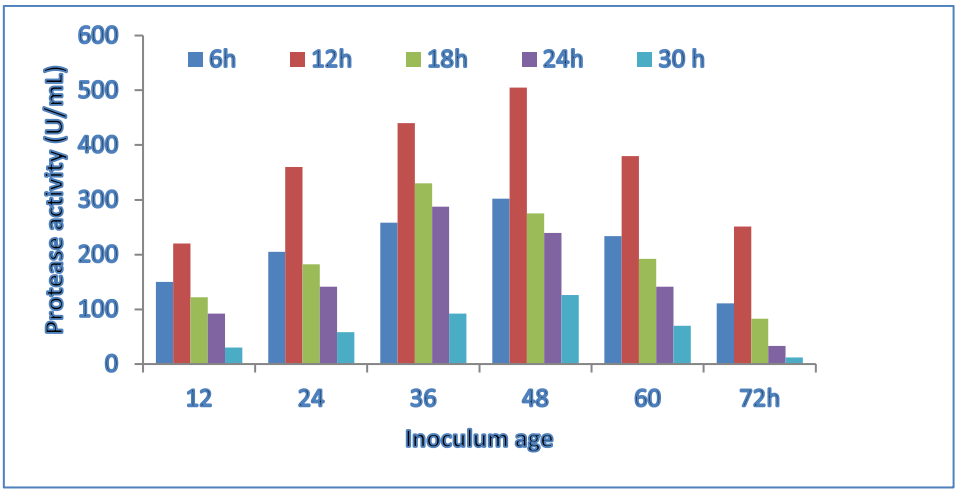


Fig. 6: Effect of inoculum age on protease production.

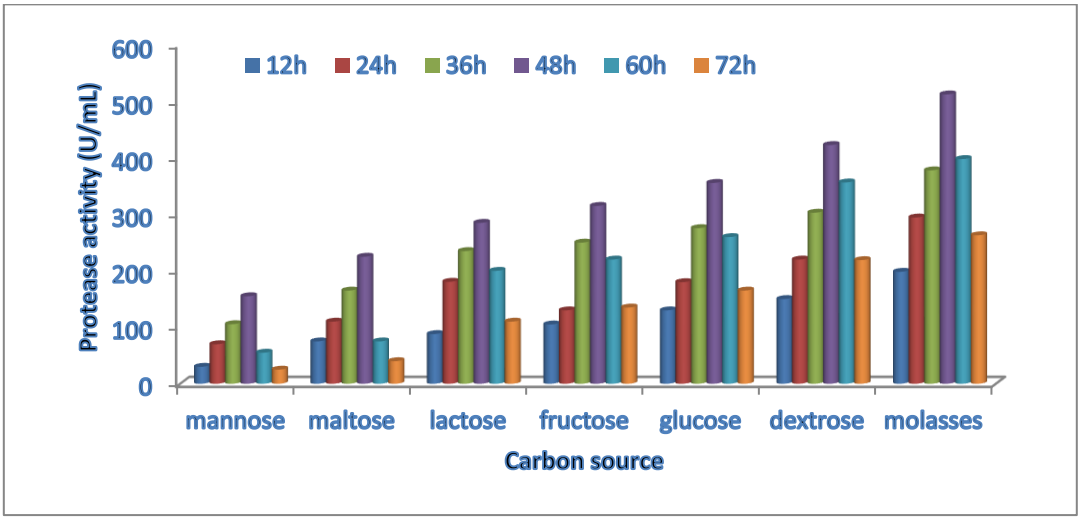


Fig.7: Effect of different carbon sources on protease production.

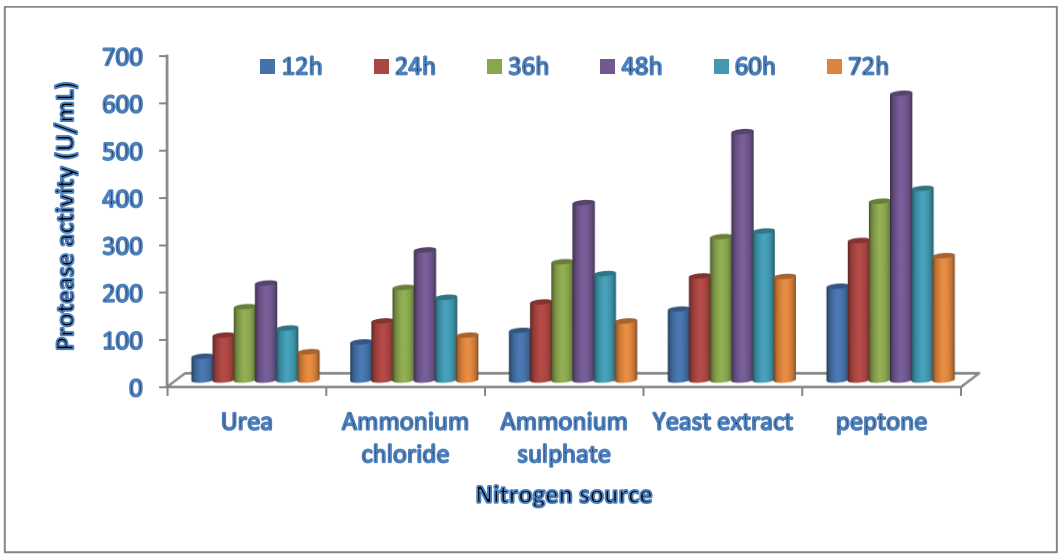


Fig. 8: Effect of different nitrogen sources on protease production.

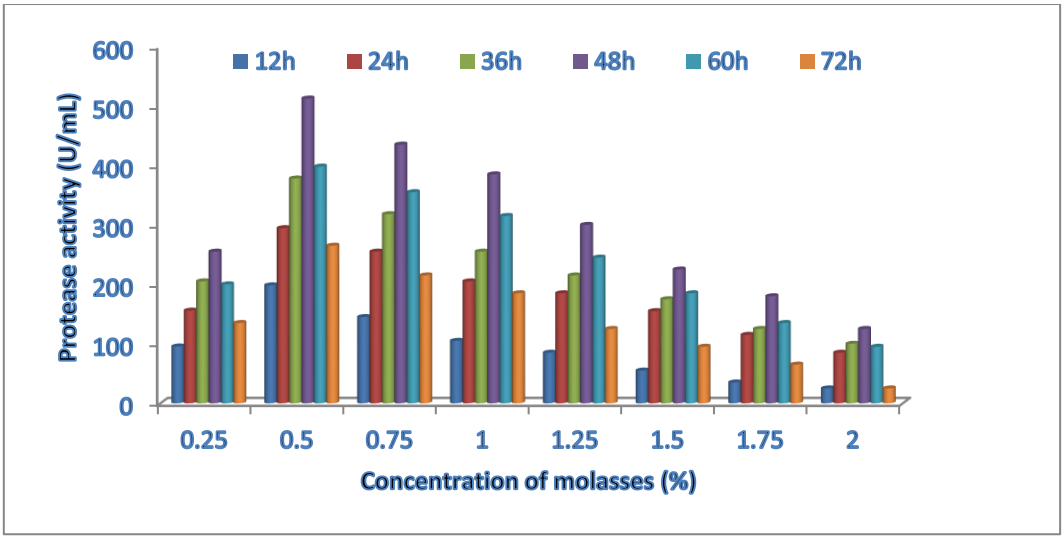


Fig. 9: Influence of different concentration of molasses on protease production.

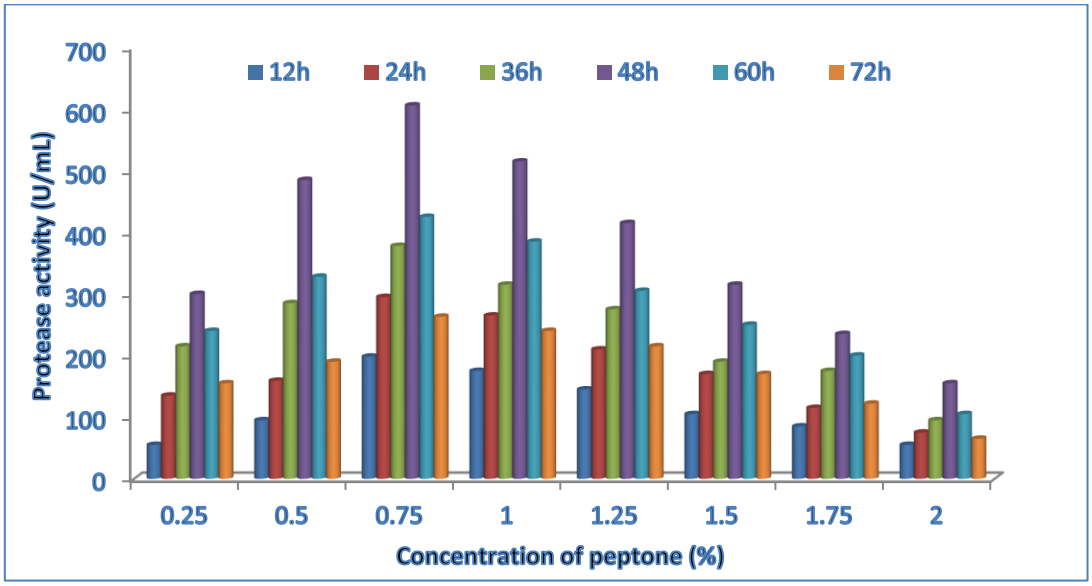


Fig.10: Influence of different concentration of peptone on protease production.

Incubation period affects the enzyme production significantly and it varies from 24 h to a week depending upon type of microorganism and other culture conditions such as inoculum size, metabolic state of cell, pH and temperature. The maximum protease production was observed at 48 h (403 U/mL), later on the activity decreased (Fig 3). Santong *et al.,* (2008) reported that *Bacillus* sp. BA40 produced 1.158 U/mL protease activities, *B. licheniformis* LBBL-11 showed 18.4 U/mL at 48 hours (Olajuyigbe and Ajele, 2008).

Parameters such as incubation period, agitation speed, inoculum concentration and inoculum age, play important role in the production of the enzyme. The importance of inoculum size on microbial fermentation process is widely accepted. Out of five inoculum size tested, 1.5 ml was found to be the most suitable for high production of protease by *B.cereus* GVK21 in submerged fermentation at 48 hrs of fermentation (Fig. 4), thereafter no appreciable change in production of protease with high inoculum size could be observed. An agitation 140 rpm (Fig: 5) and 12 hrs of inoculums age (Fig: 6) are found to be optimum for the maximum protease production by *B.cereus* GVK21.

The production of enzymes by microorganisms is strongly influenced by the composition of the medium. Carbon and nitrogen sources are also important factors for the production of protease. Molasses and peptone resulted in the highest protease activity (513 U/mL and 606 .5 U/mL per min, respectively, while the least enzyme activity was observed with maltose and mannose (Fig. 7) and ammonium chloride (Fig.8). Different concentrations of carbon (Molasses) and nitrogen (peptone) were examined for enzyme production, 0.5 g of Molasses (Fig: 9) as carbon source and 0.75 g of peptone (Fig:10) as nitrogen source have yielded maximum protease. Other studies on *B.cereus* reported that an incubation period of 72 h, pH 9.0 at 37° C and fructose and beef extract as the carbon and nitrogen sources, respectively, resulted in the highest production of protease (Sreedevi *et al.,* 2017). Dorcas and Pindi (2016) reported that the optimum pH, temperature and incubation period and agitation rate for protease production by *B.cereus* were pH 7 at 37°C for 72 h at 150 rpm and glucose was found to be an important media component. Thus the Protease yields vary considerably with temperature and pH, which may be attributed to other components of the medium and their combined influence on the metabolism of the bacterial species (Maghsoodi *et al.,* 2013). *B. cereus* GVK21 yielded 606.5 U/mL of protease at optimum conditions, which is better than many reports, and almost similar to the one recently reported by Mehakbaweja *et al.,* (2018).

In recent years the application of modern statistical models was widely increased to optimize the physicochemical components of the cultural conditions in the field of industrial biotechnology due to its universal applicability and suitability (Govarthanan *et al.,* 2014). The BBD was applied to identify the optimal conditions for the enhanced production of the protease production. The results of experimental design are presented in Table 1. The

ANOVA of the quadratic regression model exhibits that it was a highly significant model, as evident from the Fisher's F test with a very low probability value (F value = 14.98) (Table 2). The model F value of 14.28 implies that the model was significant. The values of 'Prob 'P' (0.0001) indicate that the term of the model was significant. The predicted R^2 (0.6157) and adjusted R^2 (0.8710) values for protease production were in reasonable agreement with the value of R^2 (0.9320), which is closer to 1.0, indicating the better fitness of the model in the experimental data.

Table 1 Results of Box–Behnken design for the variables and the experimentally observed responses

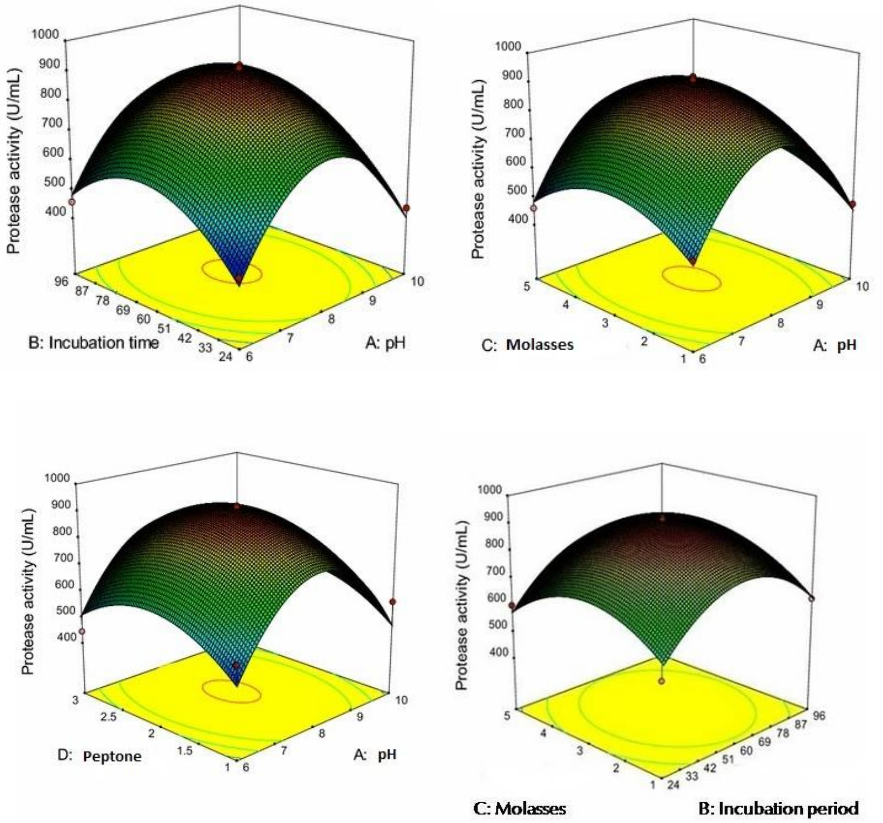
Run	pH	Incubation period	Molasses (%)	Peptone (%)	Protease activity (U/mL)
1	6	48	1.00	1.00	512
2	8	72	3.00	1.50	519
3	9	48	2.00	1.50	584
4	9	48	1.00	3.00	634
5	9	96	3.00	2.00	812
6	7	48	2.00	2.00	631
7	9	36	1.00	3.00	658
8	9	36	3.00	1.00	564
9	9	72	1.00	2.00	592
10	10	60	2.00	2.50	612
11	9	60	1.00	1.00	581
12	6	96	1.00	2.00	493
13	7	48	5.00	3.00	437
14	7	48	2.00	2.00	472
15	9	72	4.00	2.50	798
16	9	72	1.00	3.00	721
17	9	48	1.00	3.00	653
18	9	48	5.00	2.00	981
19	7	24	4.00	1.50	489
20	10	36	2.00	2.00	485
21	9	48	3.00	2.50	898
22	7	48	4.00	1.00	531
23	9	36	2.00	2.00	844
24	8	48	1.00	1.00	584
25	9	48	0.75	1.00	912
26	9	24	2.00	2.00	664
27	10	48	5.00	2.50	593
28	6	36	3.00	2.00	485
29	9	72	1.00	1.00	682

Table 2: Results of ANOVA obtained for the protease production by *Bacillus cereus* GVK21 for designed optimization experiment

Source	Sum of squares	Df	Mean square	F value	P value prob >F
Model	6.877E+ 004	14	53830.12	14.98	<0.0001
A—pH	3.00	1	3.00	8.578E-003	0.9672
B—incubation time	11840.06	1	11840.08	3.39	0.0798
C—molasses	468.03	1	468.03	0.14	0.6303
D—peptone	4393.23	1	4393.23	1.12	0.2324
AB	9.00	1	9.00	2.600E-003	0.8100
AC	621.00	1	621.00	0.18	0.6640
AD	63.00	1	63.00	0.019	0.8146

Source	Sum of squares	Df	Mean square	F value	P value prob >F
BC	746.21	1	746.21	0.21	0.5540
BD	255981.20	1	255981.20	7.38	0.0121
CD	23526.00	1	23526.00	6.73	0.0201
A^2	5.445E+005	1	5.445E+005	157.00	<0.0001
B^2	1.853E+004	1	1.853E+004	54.10	<0.0001
C^2	1.077E+005	1	1.077E +005	30.80	<0.0001
D^2	1.185E+005	1	1.185E+005	31.46	<0.0001
Residual	48298.38	14	3351.68	–	–
Lack of fit	48039.02	10	4794.98	75.67	0.0003
Pure error	248.20	4	62.42	–	–
Core total	7.980E+005	28	–	–	–

Figure 11 shows the 3-D plot graphical representations of RSM. From the results it can be inferred that there was a significant relation of pH, incubation time, molasses and peptone concentrations for protease production. The optimum levels of the variables were obtained by using Box-Behnken design. The model predicted a maximum protease activity of 912 U/mL at 48h of incubation period with molasses of 1.0%, peptone of 1.50%, pH 9 and temperature 40°C. The results obtained are agreement with the earlier work using *Bacillus* sp. SKK11 showing protease activity of 821 U/mL (Govarthanan *et al.,* 2014). The predicted model values were in good agreement with the values measured in these experiments, thus mitigating the validity of the response model and the necessity for optimal conditions. The 3D plots highlighted the roles played by the variables in the production of protease. Optimization of fermentation conditions using RSM for the production of protease showed the progress in the rate of production, reduction in the overall cost of production.



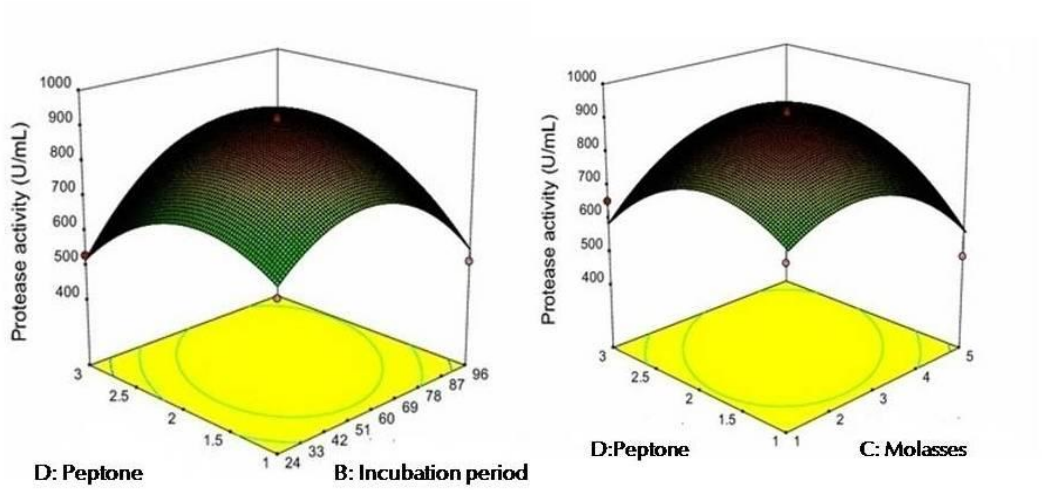


Fig. 11 Response surface plots of combined effects of two variables on the production of protease

CONCLUSION:

The bacterium, *B. cereus* GVK21 produced a high level of protease under the physiological fermentation factors such as the incubation time (48 h), pH (9), temperature (40°C), the inoculum level (1.5 %), inoculum age (12 h), and the agitation rate (140 rpm). The results of the study show that this isolate can be further exploited for commercial production of protease.

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