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Statistical optimization of asparaginase production by a novel isolated bacterium *Brevibacillus borstelensis* ML12 using Plackett–Burman design and response surface methodology

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ABSTRACT

Asparaginase is widely used in food processing and pharmaceutical industries. It is produced by different types of microorganisms. Applications of this enzyme depend on its source and nature. Furthermore, economic viability depends on enzyme production by fermentation process. There is a need to search potent new microbial strains for higher asparaginase production. In this study, a potent bacterial strain was isolated from different soil samples and selected for maximum asparaginase production. It was identified following the Bergey's Manual of Determinative Bacteriology and phylogenetic analysis by 16S rDNA nucleotide sequencing. The organism was found to be *Brevibacillus borstelensis* ML12. The environmental parameters for asparaginase production include pH (5–10), temperature (25–40°C), inoculum volume (1–10%), fermentation medium volume (25–125 mL), fermentation time (16–48 h), age of culture (16–30 h), and shaking RPM (80–140 rpm). The statistical techniques, Plackett–Burman (PB) design, and response surface methodology (RSM) were further used for the optimization process, using Minitab 18 software. PB design composed of 12 trials and resulted in three significant parameters such as medium volume, inoculum volume, and shaking speed. RSM was employed to detect the optimum conditions for asparaginase production. The maximum production of asparaginase was achieved at media as 50 mL; inoculum as 6%; and shaking RPM as 120 rpm. There is no literature available on the production of asparaginase by *B. borstelensis* ML12; thus, after characterization, it may be used in pharmaceutical and food processing industries.

1. INTRODUCTION

Asparaginase (asparagine amidohydrolase, E.C.3.5.1.1) belongs to the amidase class of enzymes that hydrolyze amino acid asparagine to aspartic acid and ammonia [1]. The utility of this enzyme promotes research to further facilitate the use in pharmaceutical sectors, food industries, biosensors, etc. Lee *et al*. [2] have also shown that *Erwinia carotovora* asparaginase results in significant decrease of asparagine in blood. The clinical action of this enzyme is conferred to the reduction of asparagine, tumor cells unable to synthesize this asparagine are selectively killed by asparagine deprivation [3]. Antitumor activity was demonstrated by Mashburn and Wriston [4] using purified *Escherichia coli* asparaginase. Furthermore, acrylamide production can be potentially inhibited using asparaginase when starch-based foods are baked, roasted, or fried above 120°C [5]. This application is very important because acrylamide is a neurotoxin and classified as potentially carcinogenic to humans [6]. The International Agency for Research on Cancer has identified acrylamide as a probable human carcinogen.

Asparaginase is present in a wide variety of organisms including plants, animals , microbes and in the serum of certain rodents but it is not found in human beings. It's mainly the microbial origin of enzyme that finds utmost application in pharmaceutical and food industry [8]. Bacteria, fungi, yeast, actinomycetes, and algae are very efficient producers of asparaginase, because they can be cultured easily and the isolation and the purification of enzyme from them are also easy, facilitating the large-scale production [9]. Due to difficult downstream extraction processes from animal and plant sources, microorganisms were cultivated for convenient and large scale production of asparaginase [9]. The most of the asparaginase from microbes is found to be intracellular in nature as studied by Narayana *et al*. [10]. Extracellular asparaginase production contributes to a much higher accumulation of enzyme in culture broth under normal conditions and is also easy for extraction and downstream processing [11]. Further, bacteria are capable to produce higher yield of enzyme and a series of preclinical and clinical trials were conducted [12]. Although enzymes from different organisms such as *Erwinia chrysanthemi* [13], *Streptomyces gulbargensis* [11], *Pyrococcus furiosus* [14], *Pectobacterium carotovorum* MTCC1428 [15], and *Yersinia pseudotuberculosis* [16] have been studied for asparaginase production, their therapeutic efficacy is dependent on the genetic composition of the strain used in the study [17].

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However, the current bottleneck of the asparaginase production is screening of microbial species with high selectivity and higher productivity for industrial uses. The objective of this study is to screen potent bacterial strain for the production of asparaginase from different soil sources, isolate and identify the maximum asparaginase yielding strain. Furthermore, optimization of various environmental parameters was performed following one-factor-at-a-time approach to maximize enzyme production. The influence of media volume, inoculum volume, age of inoculum, shaking speed, pH, temperature, and fermentation time on asparaginase production were analyzed using the PB design and RSM.

2. MATERIALS AND METHODS

2.1. Isolation of Bacterial Strains for Asparaginase Production

Potent bacterial strain was isolated from various soil samples collected from places adjacent to Kolkata and their respective locations are mentioned below:

Sample No.	Area of collection	Geographical position
1	East Midnapore, slaughter house-West Bengal	21.9373° N, 87.7763° E
2	Jadavpur University Campus soil-Jadavpur University-Kolkata	22.4968° N, 88.3713° E
3	Park Circus slaughter house-Kolkata	22.5379° N, 88.3682° E
4	Central Leather Institute wastewater water soil E. M. Bypass-Kolkata	22.5527° N, 88.3923° E

The samples were collected in sterile zip lock polythene bags using sterile gloves and transferred to laboratory for the isolation of microorganisms. One gram of each soil sample was separately diluted in 10 mL sterile distilled water in a test tube and vortexed. One milliliter supernatant was taken and serial dilution technique was followed. The primary screening of asparaginase producers was carried out by streak plate method. Asparaginase producers were identified by their pinkish-red colonies on modified M9 media after 24 h. Control plates containing modified M9 medium without asparagine (instead $NaNO_3$) as sole source of nitrogen were used. Isolated colonies on M9 medium were aseptically transferred onto nutrient agar slants for further analysis. All the isolated organisms were studied for their quantitative asparaginase producing capabilities using medium containing $Na_2HPO_4.2H_2O$ – 6.0 g; KH_2PO_4 – 3.0 g; NaCl – 0.5 g; asparagine – 10.0 g; 1 mol/L $MgSO_4.7H_2O$ – 2.0 mL; 0.1 mol/L $CaCl_2.2H_2O$ – 1.0 mL; glucose – 0.2 g; agar – 20.0 g, and phenol red – 0.005% as indicator per liter of distilled water and pH 7.0 [18]. The strain with maximum enzyme productivity was selected for consecutive studies and initial identifications were performed by morphological and biochemical tests, according to Bergey's Manual of Determinative Bacteriology. Phylogenetic and nucleotide sequencing were performed by GCC Biotech, Kolkata. Genomic DNA was extracted from the microbial culture by conventional method and 16S rDNA amplification was done with 8F and 1492R universal primer sequences. The capillary sequencing was done by ABi 3730XL Genetic Analyzer machine. All media components and reagents used for isolation, identification, and fermentative production purposes were procured from HiMedia-India and Merck-Germany.

2.2. Asparaginase Production

Submerged fermentation method was employed for the production of asparaginase in laboratory [19]. Using one-factor-at-a-time method, different environmental parameters such as pH, temperature, inoculum volume, age of culture, volume of fermentation media, fermentation time, and speed of aeration were optimized for maximum enzyme production by the isolated bacterial strain. Inoculum volume varied from 1% to 10% of 16–48 h age of each of the isolates was inoculated in variable amount of medium volume (25–125 mL) of sterile modified M9 medium in 250 mL Erlenmeyer flask and incubated at temperature varied from 25°C to 40°C for different fermentation time (16–48 h) under variable shaking speed (80–140 rpm). pH of the medium was varied from 5.0 to 10.0. After completion of fermentation, the broth was centrifuged at 5000 rpm for 10 min. The cell mass was discarded and the supernatant was used as the source of crude enzyme. All experiments were carried out in triplicates and the mean value is reported in this paper.

2.3. Assay Method of Asparaginase

Modified method of Mashburn and Wriston [4] was used for assaying asparaginase activity. Asparaginase catalyzes the hydrolysis of asparagine and thus producing aspartic acid and ammonia. Ammonia released is quantified by direct Nesslerization method wherein ammonia reacts with the Nessler's reagent to form a yellowish-orange compound [20]. The reaction was started by adding 0.25 mL of 0.04 M L – asparagine and 0.5 mL of 0.025 M sodium borate buffer (pH 8.6) to 0.25 mL of crude enzyme and incubated for 10 min at 37°C. The reaction was stopped by addition of 0.5 mL of 0.1(N) trichloroacetic acid solution. After centrifugation at 10,000 rpm for 10 min, 0.5 mL of supernatant was added to 1 mL of 1(N) NaOH, 0.25 mL of 0.1 M EDTA, and 5.75 mL of sodium borate buffer (pH 8.6). After 2 min, 0.5 mL Nessler's reagent was added and after 5 min, optical density was taken at 450 nm. Amount of ammonia produced was obtained from a standard curve prepared using ammonium sulfate as a standard. One International Unit (IU) of asparaginase is defined as the amount of enzyme required to liberate 1 µmole of ammonia per minute under defined assay conditions [21].

$$\frac{Units}{mL}enzyme = \frac{\mu moles\,of\ NH_3\ liberated \times 1.5}{0.5 \times 10 \times 0.25} \quad (1)$$

1.5 =Initial volume of enzyme mixture (mL); 0.5 = Volume of enzyme mixture used in final reaction (mL); 10 = Incubation time (min); and 0.25 = Volume of enzyme used (mL)

2.4. PB Design

PB design is an effective technique for selection of the significant parameters among large number of operating parameters that impact fermentation process using minimum experimental trials. The seven operating parameters selected for fermentation process were media volume, inoculum volume, age of inoculum, shaking speed, pH, temperature, and fermentation time. Twelve experimental runs were performed to identify the significant parameters. The enzyme production (IU/mL) is known as response [Tables 1 and 2].

2.5. Optimization of parameters by statistical technique (RSM)

Based on the results of screening different operating parameters by PB. design, Box–Behnken method was employed to identify the interaction between different significant parameters. These significant variables were optimized using the RSM. The experimental design consisting of fifteen runs is given in Tables 3 and 4.

Table 1: Optimization study parameters from low to high level.

Variable name	Variable code	Units	Low level (−1)	High level (+1)
pH	A	-	7	9
Temperature	B	°C	25	37
Fermentation time	C	Hours	24	40
Age of culture	D	Hours	16	24
Inoculum volume	E	%	4	8
Medium volume	F	Ml	25	75
Shaking speed	G	Rpm	100	140

3. RESULTS AND DISCUSSION

Asparaginase producing bacterial strains were isolated using modified M9 media. Enzyme producing ability of isolated strain after primary screening is presented in Figure 1. A total of 78 microorganisms were found as asparaginase producers. This observation is in concord with the work of Gulati *et al*. [18], wherein it was depicted that the change in color from orange to red was because of asparaginase production. Isolate no.-55 from soil sample 4 was observed to be the highest asparaginase producer and secondary screening revealed extracellular enzyme production of 102.61 IU/mL. Kamble and Khade [22] showed that *Bacillus subtilis* is a potent asparaginase producer. The enzyme production by the selected bacterial strain is considerably higher as compared to 23.8 IU/mL reported by Pradhan *et al*. [20] and his associates and 47 IU/mL reported by Sanghvi *et al*. [23]. *Streptomyces ginsengisoli* produces an optimum enzyme activity of 3.32 IU/mL [24] and *Trichosporon asahii* IBBLA1 yielded 20.57 IU/mL [25].

3.1. Identification of the Most Potent Strain

This isolated organism was identified by various morphological, biochemical, and physiological tests as per Bergey's Manual of Determinative Bacteriology [26]. Similar studies were also performed by Audipudi *et al*. [27] using mangrove soil. The characteristics are summarized in Table 5.

It can most conclusively be derived that the isolated microorganism belongs to the genera of *Bacillus*.

3.2. Bacterial Identification by 16S rDNA Analysis

16S rDNA sequencing is particularly useful in identifying bacteria that are difficult to identify by conventional methods and identification of "routine" bacterial strains is most useful in the context of bacterial species. Phylogenetic tree is shown in Figure 2.

The evolutionary history was inferred using the UPGMA method. The optimal tree is shown (next to the branches). The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA X as reported by GCC Biotech, Kolkata. Based on nucleotide sequencing and phylogenetic data analysis, the isolated bacterial strain was identified as *Brevibacillus borstelensis* ML12 by 16S ribosomal RNA gene, partially sequenced by GCC Biotech, Kolkata. So far, to the best of our knowledge, this is the first reported work on *B. borstelensis* ML12 for asparaginase production.

3.3. Effect of pH

pH of the fermentation media plays a key role in the change of enzyme production. The bacterial isolate was grown in sterile modified M-9

Table 2: Plackett–Burman design and asparaginase activity in fermentation broth.

Run no.	A	B	C	D	E	F	G
1	+1	+1	−1	+1	−1	−1	−1
2	−1	+1	+1	+1	−1	+1	+1
3	−1	+1	−1	−1	−1	+1	+1
4	−1	+1	+1	−1	+1	−1	−1
5	+1	+1	+1	−1	+1	+1	−1
6	+1	+1	−1	+1	+1	−1	+1
7	+1	−1	+1	−1	−1	−1	+1
8	−1	−1	−1	−1	−1	−1	−1
9	−1	−1	+1	+1	+1	−1	+1
10	−1	−1	−1	+1	+1	+1	−1
11	+1	−1	−1	−1	+1	+1	+1
12	+1	−1	+1	+1	−1	+1	−1

Table 3: Optimization study parameters for RSM from low to high level.

Parameters	Factor code	Levels		
		−1 (low)	0 (mean)	+1 (high)
Shaking speed (rpm)	A	100	120	140
Inoculum volume (%)	B	4	6	8
Medium volume (ml)	C	25	50	75

RSM: Response surface methodology

Table 4: Experimental design for RSM.

Run no.	A	B	C
1	100	6	25
2	120	8	25
3	140	6	75
4	100	8	50
5	120	6	50
6	120	6	50
7	100	4	50
8	120	8	75
9	120	6	50
10	100	6	75
11	120	4	25
12	120	4	75
13	140	8	50
14	140	4	50
15	140	6	25

RSM: Response surface methodology

medium of different pH – 5, 6, 7, 8, 9, and 10. About 2% of 24 h old inoculum was transferred into 50 mL of M-9 media of different pH and incubated at 37°C for 48 h at 120 rpm and assayed to observe the enzyme activity. Maximum yield was reported at pH 8 which was 115.106 IU/mL. Further increase in pH resulted in decrease in production of enzyme as observed from Figure 3a. Cell mass was found to be gradually increasing with the increase in pH. During cell mass production, enzymes involved in different biochemical pathways and enzymatic activity are highly dependent on optimum pH. In this case, optimum pH was 8.0, hence, enzyme production was increased from 5.0 to 8.0 and then decreased. Asparaginase from *E. coli* was found to have a wide stability in the pH range 4.5–11.5 [28]. A study by Muneer *et al*. [29] has stated that microbial asparaginases have an optimum pH in the range of 6–10. Dejong [30] revealed that asparaginase from *Streptomyces griseus* shows a pH optimum at 8.5 which is quite comparable to that of the asparaginase derived from *Escherichia coli* that also has anti-tumor properties [31]. *S. ginsengisoli* has an optimal enzyme activity 2.4 IU/mL at pH 8.0 as reported by Deshpande *et al*. [24]. Furthermore, *Bacillus polymyxa* has its highest enzyme activity at pH 8 and *Bacillus firmus* at pH 7 [1]. This reflects that both acidic and an alkaline pH could inhibit asparaginase enzyme production.

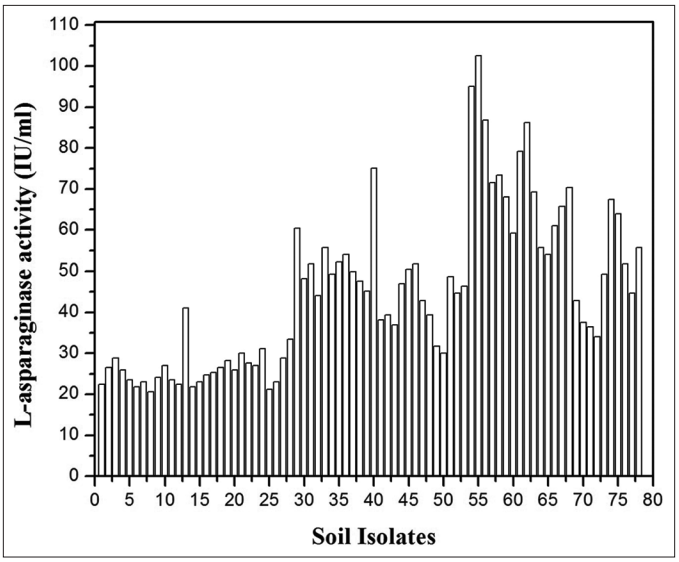

Figure 1: Screening of bacteria from different soil samples.

3.4. Effect of Temperature

The bacterial isolates were grown in sterile modified M-9 medium. About 2% of 24 h old inoculum was transferred into 50 mL of modified M-9 medium and incubated at four different temperatures: 25°C, 30°C,

Table 5: Morphological characteristics and biochemical tests of isolated organism.

Morphological characteristics					
Size and shape	**Gram staining**	**Spore staining**		**Motility test**	
2.4 µm×1.2 µm, distinct rods.	Positive	Positive		Positive	
Biochemical tests		**Carbohydrate fermentation**			
		Sugar		**Acid formation**	**Gas formation**
Ammonia from arginine		Positive	Arabinose	+++	+++
Arginine used as sole source of carbon		Positive	Salicin	+	–
Nitrate reduction		Positive	Sorbitol	+	–
Catalase		Positive	Galactose	+	+
Oxidase		Negative	Mannitol	++	–
Amylase		Positive	Xylose	++	+
Gelatin liquefaction		Positive	Fructose	++	+++
NaCl tolerance		Negative	Dextrin	+	+
Lysine decarboxylase		Negative	Glucose	++	++
Phenylalanine deaminase		Negative	Adonitol	–	–
Ornithine decarboxylase		Negative	Dulcitol	–	–
Arginine dihydrolase		Negative	Erythritol	–	–
Tyrosine hydrolysis		Negative	Rhamnose	–	–
Casein hydrolysis		Positive	Inositol	++	+
Lecithinase		Negative	Maltose	+	+
Esculin hydrolysis		Positive	Mannose	+	–
Urease		Negative	Raffinose	+	–
Litmus milk		Positive	Trehalose	+	–
Indole		Negative	Inulin	–	–
Methyl red		Negative	Melibiose	+	+
Vogues–Proskauer		Positive	Glycerol	+	–
Citrate		Positive	Sucrose	+	+
Starch				+	–
Lactose				–	–

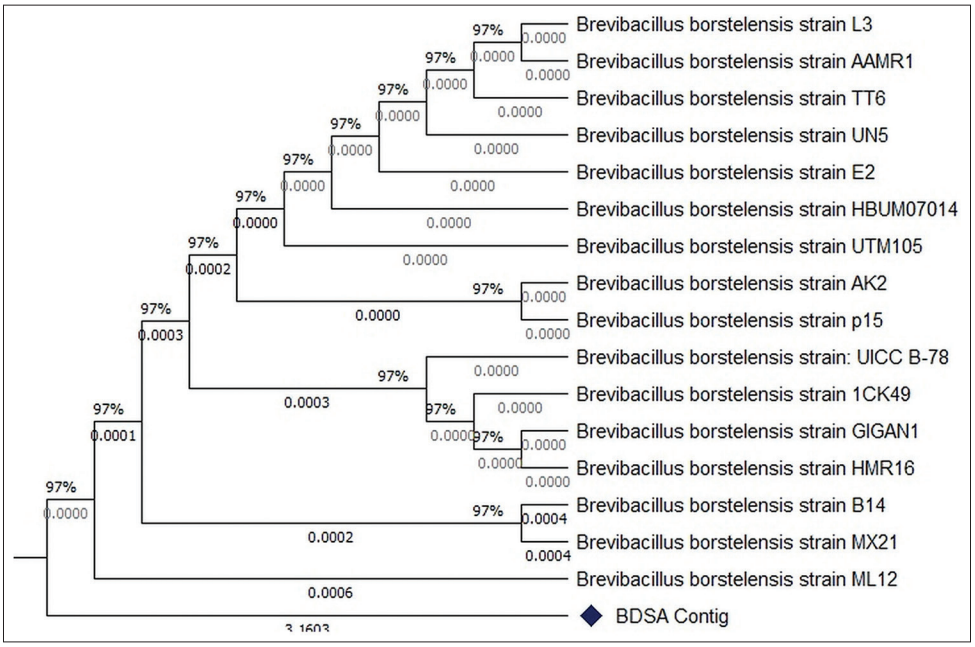

Figure 2: Phylogenetic tree – Evolutionary relationships of taxa.

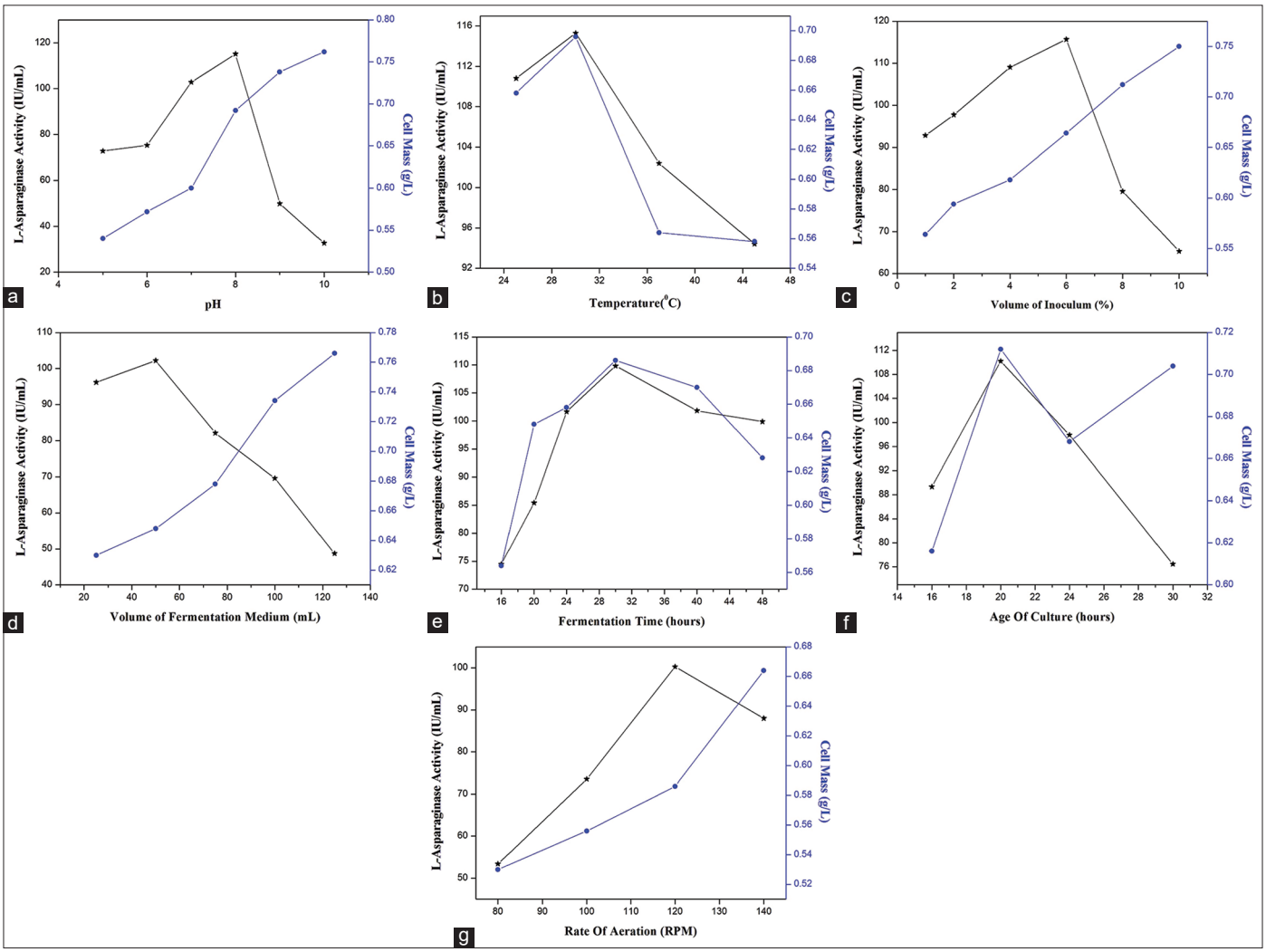

Figure 3: Effect of environmental parameters on asparaginase production. (a) effect of pH; (b) effect of temperature; (c) effect of inoculum volume; (d) effect of media volume; (e) effect of fermentation time; (f) effect of age of culture; and (g) effect of shaking speed.

35°C, and 40°C in shaking condition (120 rpm) for 48 h. The optimum temperature for the production of asparaginase was found to be 30°C [Figure 3b]. Further increase in temperature decreases the production of asparaginase. The incubation temperature of 30°C shows that the maximum enzyme activity is 115.30 IU/mL. The highest activity is at 30°C and the lowest activity is at 45°C. Cell mass production was increased with increase in temperature from 25°C to 30°C but then decreased as the temperature is further increased. A temperature optimum at 37°C has been shown by Kushwaha *et al*. [32] in *B. subtilis* and by El-Naggar *et al*. [33] in *Streptomyces brollosae*. El-Hadi *et al*. [34] have shown that at 30°C, *Streptomyces* spp. strain produces maximum asparaginase. This indicates that temperature around 30°C–37°C proves to be optimum for enzyme production. Low enzyme production with a decrease in cell mass beyond the optimal temperature indicates that this enzyme is a primary metabolite.

3.5. Effect of Inoculum Volume

Different inoculum volumes were considered to observe the changes in enzyme production. Twenty-four hours old culture of different volumes such as 1%, 2%, 4%, 6%, 8%, and 10% was transferred into 50 mL sterile modified M-9 media and incubated at 30°C in shaking condition (120 rpm) in incubator for 48 h. Figure 3c shows that 6% of inoculum volume gave the maximum asparaginase production of 115.693 IU/mL. The enzymatic activity was increased with inoculum volume from 1 % to 6%, where it gave the highest activity, and then, the activity declined with inoculum volume. Enzyme production rate increased with an increase in the number of cell or inoculum volume but above a certain cell mass concentration, agglomeration occurred. As a result of which effective cell mass concentration decreased. Cell mass was found to increase with increase in volume of inoculum up to 10%. Khaleed and Khobragade observed optimum asparaginase production (100 IU/mL) at 10% inoculum of isolated bacteria [35].

Trilokchandran *et al*. performed the experiment using varied inoculum volumes of 5%, 7.5%, 10%, 12.5%, and 15%, and the maximum activity of 2.20 IU/ml was observed at 10% inoculum volume of isolated bacterial strain [36].

3.6. Effect of Fermentation Medium Volume

Five different volumes of modified M-9 media such as – 25 mL, 50 mL, 75 mL, 100 mL, and 125 mL were prepared in five different conical flasks. Then, 24 h old culture was added into each five sterile M-9 media and incubated at 37°C at 120 rpm in incubator for 48 h. The highest enzyme activity was observed at 50 mL of media whereas the lowest enzyme activity was observed at 125 mL of media. Figure 3d depicts that the enzyme activity showed an increase from 25 to 50 mL media volume, then, it decreased with an increase in the media volume. In this case also, cell mass was found to increase gradually with the increase in volume of fermentation medium. Oxygen supply rate gradually decreases with medium volume in Erlenmeyer flask. Probably asparaginase production pathway is oxygen dependent. Hence, enzyme production is decreased with medium volume above 50 mL.

3.7. Effect of Fermentation Time

The bacterial isolate was grown in sterile modified M-9 medium. About 6% of 24 h old inoculum was transferred into five different conical flasks each containing 50 mL of modified M-9 medium and was incubated for 16 h, 20 h, 24 h, 30 h, 40 h, and 48 h at 30°C in shaking condition (120 rpm) in incubator. Enzyme production gradually increased, showing a maximum at 30 h (109.833 IU/mL). Further beyond 30 h, enzyme production declined to a smaller extent (99.876 IU/mL) till 48 h, as noticed in Figure 3e. Here, cell mass constantly increased as the fermentation time increased from 16 h to 30 h and after that its value decreased till 48 h. This may indicate that as the time interval is increased, enzyme production decreases. It is also possible that with further increase in time, autolysis of cell debris happened, which is shown by a decrease in cell mass after 30 h. Khaleed and Khobragade studied that the maximum asparaginase was produced by isolated bacterial strain after 48 h fermentation (103.7 U/ml) [35].

In general, enzymes are active at the range of 24–72 h. Maximum enzyme production could be obtained only after a certain fermentation time which allows the organism to grow at a steady state [37].

3.8. Effect of Age of Culture

About 6% of 16 h, 20 h, 24 h, and 30 h old culture were transferred into four different conical flasks, each containing 50 mL of sterile modified M-9 media and incubated for 30 h in shaking condition at 30°C. Among four different time intervals considered (16, 20, 24, and 30 h), 20 h old inoculum resulted in maximum enzyme production (110.22 IU/mL) which decreased to 76.45 IU/mL for 30 h old inoculum. It was observed that cell mass rises till 20 h and then decreases at 24 h but again increases at 30 h. Beyond 20 h, the viability of bacterial cells decreases and hence its efficacy in degrading fermentation medium also decreases, thus enzyme production gradually reduces. Enzyme productivity depends on growth phase of microbial cell. Figure 3f reveals that inoculum of early stationary phase is suitable for asparaginase production.

3.9. Effect of Shaking Speed

About 6% of 20 h old inoculum was transferred to five conical flasks, each containing 50 mL of modified M-9 medium and incubated for 30 h at 30°C under different shaking conditions – 80 rpm, 100 rpm, 120 rpm, and 140 rpm. Asparaginase production gradually increased with increase in speed of rotation up till 120 rpm, showing a maximum activity of 100.27 IU/mL as evident from Figure 3g, beyond which activity decreased to 87.97 IU/mL at 140 rpm. Here, cell mass gradually increased with increase in rate of aeration. This shows that the organism may be aerobic and requires oxygen for metabolic processes.

3.10. PB Design

PB design was performed in the present study considering seven operating conditions (media volume, inoculum volume, age of inoculum, pH, temperature, fermentation time, and shaking speed). The effect of seven parameters gave 12 runs using PB design [Tables 6 and 7]. $P < 0.05$ is considered as statistically significant. From Tables 6 to 7, the significant parameters were medium volume, inoculum volume, and shaking speed. The Pareto chart displays the significant experimental parameters for enzyme production. It reflects the standardized effect of individual or the combination of parameters [Figure 4].

$$\text{Experimental value (Y)} = 92.80 - 0.73\,A + 0.54\,B - 0.05\,C + 3.95\,D - 5.12\,E - 5.42\,F + 4.73\,G \quad (2)$$

Y is the experimental response (enzyme production) and the coefficients indicate the effect of the parameters on asparaginase production. The R^2 value is the indicator of variability in the experimental output. In this

case, the R-square value was found to be 89.57% for the asparaginase production.

The goodness-of-fit model was verified by R^2 which indicated that the model could explain 89.57% variation of the data. Using PB design, the effect of operating parameters on asparaginase production is formulated by first-order linear model and is given in Eq. 2. Table 7 reviews the analysis of variance for a linear model on the effect of operating parameters on asparaginase production from *B. borstelensis* ML12.

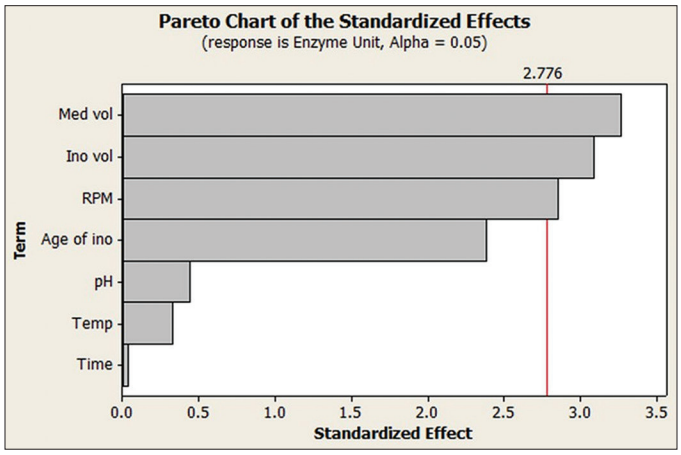

Figure 4: Pareto chart indicating the significant parameters.

Table 6: Plackett–Burman design matrixes for seven variables and the observed response for asparaginase production.

Run no.	L-asparaginase activity (IU/ml)	
	Experimental	Predicted
1	96.755	102.42
2	102.612	102.40
3	94.412	94.60
4	87.97	85.64
5	70.985	81.24
6	107.297	101.64
7	100.269	102.80
8	97.926	94.90
9	99.098	101.92
10	79.098	81.72
11	81.527	81.82
12	95.583	90.40

Table 7: ANOVA of asparaginase production generated by Minitab software.

Source	DF	Adj SS	Adj MS	F-value	*P*-value
Model	7	1133.79	161.971	4.91	0.072
pH	1	6.43	6.433	0.19	0.682
Temp	1	3.46	3.460	0.10	0.762
Time	1	0.03	0.028	0.00	0.978
Age of inoculum	1	187.55	187.546	5.68	0.076
Inoculum volume	1	315.15	315.147	9.55	0.037
Medium volume	1	352.21	352.213	10.67	0.031
Shaking speed	1	268.97	268.967	8.15	0.046

ANOVA: Analysis of variance

3.11. RSM

RSM is a combination of mathematical and statistical techniques through which we can determine the optimum experimental conditions using Box–Behnken design [38,39]. The effect of medium volume, inoculum volume, and shaking speed was studied using RSM by Box–Behnken design [Tables 8 and 9]. From Tables 8 and 9, the model F value 5.82 and *P* value 0.033 were found significant. The model equation is significant since $P < 0.05$ was considered.

$$\text{Experimental value (Y)} = 123.50 + 2.49\,A - 8.27\,B - 1.39\,C - 9.50\,A^*A - 12.86\,B^*B - 13.45\,C^*C - 0.88\,A^*B + 2.05\,A^*C - 0.73\,B^*C \quad (3)$$

R^2 value was found to be 91.29%. It indicates that there is a good agreement between the experimental and the predicted values. The interaction between the shaking speed and medium volume [Figure 5a] shows that at shaking speed from 112 to 126 rpm and medium volume 40–58 mL, the interaction was reported significant. The interaction between the inoculum volume 4.5–6.2% and medium volume 40–60 mL also indicates significant interaction [Figure 5b].

Table 8: Box–Behnken design and results for asparaginase production.

Run no.	Actual values			Enzyme activity (IU/ml)	
	A	B	C	Observed	Predicted
1	100	6	25	97.920	101.50
2	120	8	25	97.340	91.04
3	140	6	75	107.290	103.70
4	100	8	50	88.550	91.26
5	120	6	50	127.790	123.50
6	120	6	50	122.525	123.50
7	100	4	50	110.220	106.04
8	120	8	75	87.380	86.80
9	120	6	50	120.182	123.50
10	100	6	75	96.750	94.62
11	120	4	25	105.540	106.12
12	120	4	75	98.510	104.80
13	140	8	50	90.310	94.48
14	140	4	50	115.497	112.78
15	140	6	25	100.269	102.38

Table 9: Regression ANOVA for RSM of asparaginase production.

Source	DF	Adj SS	Adj MS	F-value	*P*-value
Model	9	2038.32	226.480	5.82	0.033
A	1	292.53	292.526	7.52	0.041
B	1	21.78	21.776	0.56	0.488
C	1	193.18	193.184	4.96	0.076
A*A	1	332.88	332.880	8.55	0.033
B*B	1	610.61	610.609	15.69	0.011
C*C	1	667.62	667.625	17.16	0.009
A*B	1	3.09	3.092	0.08	0.789
A*C	1	16.77	16.773	0.43	0.540
B*C	1	2.15	2.146	0.06	0.824

ANOVA: Analysis of variance, RSM: Response surface methodology

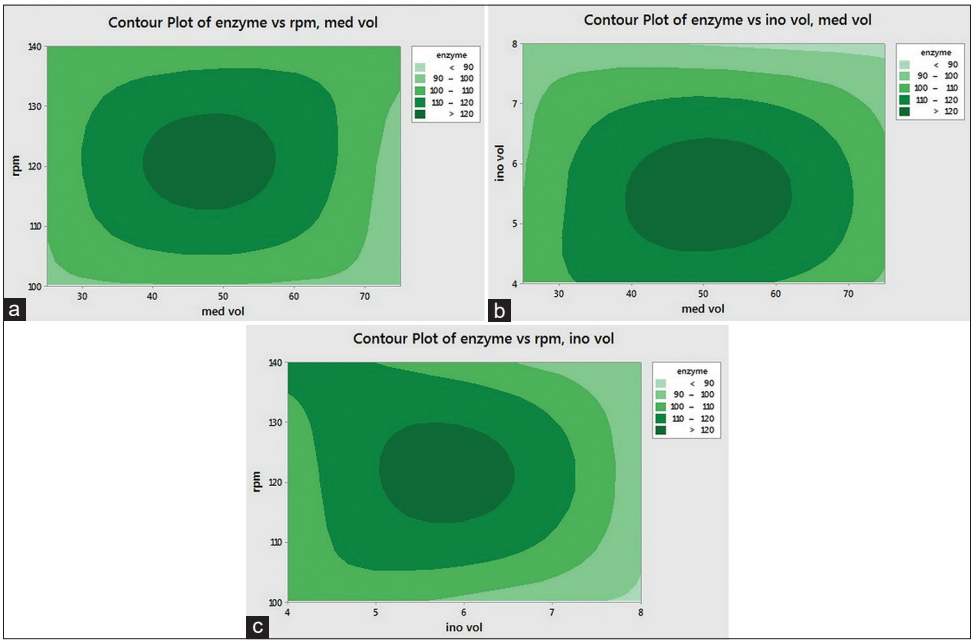

Figure 5: (a) Contour plot showing interaction between medium volume and shaking speed for asparaginase production, (b) Contour plot showing interaction between medium volume and inoculum volume for asparaginase production, (c) Contour plot showing interaction between inoculum volume and shaking speed for asparaginase production.

Furthermore, the interaction between shaking speed 115–126 rpm and inoculum volume 5.2–6.5 % is significant [Figure 5c]. The optimum condition of response analyzed using response optimization for the enzyme production was obtained at medium volume = 50 mL; inoculum volume = 6%; and shaking speed = 120 rpm.

Validation was performed under conditions predicted by the model [Table 6]. The values predicted by the model were in agreement with the results obtained which indicate that the model was successfully validated [Tables 7 and 8]. The optimum levels of the operating parameters for asparaginase production under submerged fermentation were rate of aeration at 120 rpm, inoculum volume at 6%, and medium volume at 50 ml. A production value of 127.79 IU/ml was observed in the verification experiment that was in agreement with the predicted value. Compared to the production of original level (102.61 IU/ml), a 1.245-fold increase in enzyme production was obtained. Experimental results were quite close to the predicted value (within 5%).

4. CONCLUSION

In the present study, 78 bacterial strains were isolated from soil samples using modified M9 media, out of which isolate no. 55 was highest asparaginase producer (102.61 IU/ml). After physiological properties study and 16s RNA analysis, the isolate no. 55 was identified as *B. borstelensis* ML12. It was observed that *B. borstelensis* ML12 produced 115.69 IU/ml asparaginase by two-dimensional optimization of environmental parameters, that is, pH – 8, temperature – 30°C, inoculum volume – 6%, fermentation medium volume – 50 mL, fermentation time – 30 h, age of culture – 20 h, and shaking RPM – 120 rpm. Further, statistical optimizations were accomplished using PB design and RSM. A total of seven parameters were incorporated in the study and the significant parameters identified were medium volume, inoculum volume, and shaking speed. RSM depicts the optimum response of the production of asparaginase. In this case, *B. borstelensis* ML12 produced extracellular asparaginase. Extracellular asparaginase possesses more advantages as compared to the intracellular type due to the ease in extraction, downstream processing, and higher accumulation in culture broth, more solubility, biologically active, and relatively free from endotoxins which reduce adverse effects. Specific application of asparaginase depends on source and characteristics of enzyme. As per our knowledge, asparaginase production by *B. borstelensis* ML12 is not reported. Hence, after purification and characterization, this enzyme may be utilized in food processing industries to reduce acrylamide in baked products and as anti-leukemic agent in pharmaceutical sectors.

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6. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This article does not contain any studies with human participants or animals performed by any of the authors.

10. DATA AVAILABILITY

Data generated or analyzed during this study are included in this published article.

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