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Production of *Bacillus subtilis* DALX2 Asparaginase: Optimization and Immobilization

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Information of asparaginase has been increasingly forthcoming in recent years because of their applications as therapeutic agents in the treatment of certain types of human cancer. In this study, we report, the production of asparaginase from *Bacillus subtilis* DALX2 using submerged fermentation. An enzyme activity of 130.9 U/ml was reached after 24 h of incubation, at an initial pH of 7.0 and an incubation temperature of 37 C. Plackett-Burman design was employed for optimizing culture conditions. Highest asparaginase was obtained in the medium containing ammonium sulphate as a sole nitrogen source and glucose as the best carbon source. After Plackett-Burman design, an enzymatic titer of 166.1 U/ml was recorded. Moreover, cells were entrapped in Ca-alginate and adsorbed on sponge porous material to increase physical and chemical stability, to avoid leakage of cells or contamination in fermentation medium. Also, evaluation of the efficiency of repeated batch fermentation showed that even after 4 cycles, beads were not disintegrated till the 4th run.

Key words: Asparaginase, optimization, Plackett -Burman, immobilization, *Bacillus subtilis*.

Asparaginase, an enzyme catalyzing the hydrolysis of asparagine to aspartic acid and ammonia has attracted huge attention due to its usage against lymphocytic leukemia and different other kinds of cancer affecting man (Krasotkina *et al*., 2004; Makky *et al*., 2013). The enzyme is present in many animal tissues, bacteria, plants and in the serum of certain rodents but not of man (Shukla *et al*., 2014). The majority of gram-negative and gram-positive bacteria are asparaginase producers (Huang *et al*., 2014; Wakil *et al*., 2015). Several *Bacillus* species are known to produce asparaginase (Makky *et al*., 2013; Shukla *et al*., 2014).

The effect of environmental parameters and culture conditions on microbial production of asparaginase has been studied in bacteria (Makky *et al*., 2013) . The use of immobilized enzymes lowers production costs as these can be readily separated from reaction mixture and hence can be used repeatedly (Khan *et al*., 2010; Di Cosimo *et al*., 2013). Several different methods have been employed for enzyme immobilization which include adsorption on to insoluble materials, entrapment in polymeric gels, encapsulation in membranes, cross linking with bifunctional or multifunctional reagents and linking to an insoluble carrier (Adinarayana *et al*., 2005). Moreover alginate beads permit strong physical and chemical stability, avoiding the chance of leakage of cells or contamination in fermentation medium and also increase the reuse possibilities (Calinescu *et al*., 2012; Zhang *et al*., 2013). The objective of this study was to evaluate the potentiality of a local *Bacillus* species to produce asparaginase to optimize culture conditions and to examine the effect of immobilization on the process.

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MATERIALS AND METHODS

Microorganism

Bacillus subtilis DALX2 used in the present study was locally isolated and identified by 16S rDNA.

Media

M9 medium composed of (g/l): Glucose, 3.0; Asparagine, 6.0; KH_2PO_4, 2.0; $MgSO_4$, 1.0; $CaCl_2$, 1.0 and agar, 20. The medium was supplemented with few drops of phenol red (2.5% in ethanol) (Prakasham *et al*., 2010) and pH was adjusted to 7.0.

Nutrient agar composed of (g/l): Beef extract, 3.0; Peptone,5.0; NaCl, 5.0 and agar, 20).

Medium ²: Glucose, 3.0; $(NH_4)_2SO_4$, 3.0; KH_2PO_4, 1.0; K_2HPO_4, 1.0; $MgSO_4$, 0.5 and $CaCl_2$, 0.5).

Medium Ï: Glucose, 10.0; Peptone, 10.0; KH_2PO_4, 1.0; K_2HPO_4, 1.0; $MgSO_4$, 0.5; $CaCl_2$, 0.5 and $FeSO_4$, traces (0.125).

Medium Ø: Glucose, 10; Peptone, 3.0; Yeast extract, 1.0; KH_2PO_4, 1.0; K_2HPO_4, 1.0; $MgSO_4$, 0.5; $CaCl_2$, 0.5; and $FeSO_4$, traces (0.125).

Medium IV: Glucose, 10; Soy bean, 10; KH_2PO_4, 1.0; K_2HPO_4, 1.0; $MgSO_4$, 0.5; $CaCl_2$, 0.5; and $FeSO_4$, traces (0.125).

Isolation of bacteria: Soil sample (10g) was collected and serially diluted with 100 ml sterile distilled water. Aliquots from each dilution were spread on the surface of M9 agar plates and incubated at 37 °C for 24 h. Colonies displaying pink red color were considered asparaginase - producers. Colonies were purified on nutrient agar plates, sub-cultured on fresh nutrient agar slants and stored at 4°C.

Identification of bacterial isolate

Morphological, biochemical and physiological tests were done according to Bergey's Manual of Determinative Bacteriology

Molecular identification: The 16S rDNA of the strain was amplified and DNA amplification was performed with a Thermal Cycler. The PCR product was purified by using the QIA quick PCR purification kit according to the manufacturer2 s instructions (Qiagen, Hilden, Germany). The PCR was carried out in a total volume of 50 µL containing 2.5 µL of each primer, 2.5 µL deoxynucleoside triphosphate mix, 3 µL Mg^{++} ion (25 mM), 5 µL buffer (10X buffer provided with the DNA polymerase enzyme), 1 µL template DNA, 5 µL dimethyl sulphoxide (DMSO), 1 µL of DNA polymerase and the reaction was completed to 50 µL with distilled water and mixed gently. To each reaction tube two drops of mineral oil were added. The reaction mixture was incubated at 94°C for 4 min. The following PCR cycle was repeated 30 times: denaturation at 94°C for 1 min, annealing of primers at 55°C for 1 min and DNA synthesis at 2°C for 2 min. This was followed by 4 min incubation at 72°C before the mixture was stored at 4°C (Panday and Das, 2010). Nucleotide sequences thus obtained were assembled using the sequence alignment editor program Bioedit (http://www.mbio). Blast searches for homologous sequences of 16S rDNA in the public data bases were performed in the NCBI web site (Altschul *et al*., 1997). Evolutionary distances to other strains were computed by neighbor joining method (Saitou and Nei, 1987) and the phylogenetic tree was constructed by software MEGA5 (Tamura *et al*., 2007).

Enzyme production

Cultivation was achieved by submerged fermentation as previously reported by Olama & El-Sabaeny (1993). 50 ml of M9 medium were inoculated with 2ml of the prepared bacterial suspension, and incubated at 37°C under static or shacked condition for 24 h. After the incubation period, centrifugation at 5000 rpm for 15 min in a cooling centrifuge was performed and the clear supernatant was assayed for enzymatic activity and estimation of protein. Three flasks were prepared for each experimental variable and the results obtained throughout the work were the arithmetic mean of at least two experiments.

Estimation of protein content

The protein content of the enzyme was determined by the modified Lowry's method (1951).

Enzyme assay

The enzyme was assayed by direct nesslerization method according to the method of Wriston & Yellin (1973). In summary, 0.5 ml of crude enzyme preparation was added to 1.0 ml of 0.05 M borate buffer (pH 7.2) and the reaction was initiated by adding 0.5 ml of 0.08 M asparagine for 30 min, at 37°C. Reaction was stopped by the addition of 0.5 ml of 15 % tri chloro-acetic acid solution and the precipitated protein was removed by centrifugation (5000 rpm) in a cooling centrifuge for 15 min. The

ammonia released in the supernatant was determined using a spectrophotometer at 500 nm. Enzyme and substrate blanks were included in all assays. A standard curve was prepared with ammonium sulphate. One asparaginase unit (U) is defined as that amount of enzyme, which liberates 1 µ mole of ammonia/min. under the optimal assay conditions.

Asparaginase production by immobilized cells

Immobilization was performed by entrapment of bacterial cells in Ca-alginate gel (Jobanputra *et al*., 2011), in agar gel or gelatin. Adsorption of bacterial cells on some solid porous materials (Martins, *et al.*, 2013) was also tried. Cultivation was done in Erlenmeyer flasks containing 50 ml sterilized culture medium and sponge cubes (about 20 cubes), luffa pulp, pumice or clay particles (about 20 particles).

Semi continuous production of asparaginase by alginate entrapped cells and adsorbed cells was also tested (Duarte *et al*., 2013). Immobilized beads and sponge were separately used repeatedly in batch fermentation process to determine the reuse efficiency of immobilization procedure. After attaining maximum production of asparaginase by immobilized cells, the spent medium was replaced and the procedure was repeated until a relatively low activity was obtained.

Plackett-Burman design (Plackett and Burman, 1946)

The matrix and design are presented in the results section. Statistical analysis of data on enzyme activity were subjected to multiple linear regression using MICROSOFT EXCEL 97 to estimate t-value, P-value and confidence level. The significance level (P-value) was determined using the Student's t-test. The t-test for any individual effect allows an evaluation of the probability of finding the observed effect purely by chance. If this probability is sufficiently small, the idea that the effect was caused by varying the level of the variable under test is accepted.

Confidence level is an expression of the P- value in percent

Optimal value of activity was estimated using the solver function of MICROSOFT EXCEL tools. (Yu *et al*., 1997).

RESULTS

Four bacterial isolates (DALX1 ; DALX2; DALX3 and DALX4), which displayed pink red coloured colonies on M9 agar plates were screened quantitatively in flasks inoculated with 2 ml bacterial suspension and incubated under static conditions for 24 h. The results showed that DALX2 supported the highest asparaginase activity (109.5 U/ml) , whereas DALX3 (11.3 U/ml) gave the lowest results. Accordingly DALX2 was selected for identification and optimization experiments. (data not shown).

Identification of DALX2

Cells of DALX2 were gram positive, endospore forming large rods. Physiological and biochemical characteristics showed that, they were facultative anaerobes. Positive with catalase, Voges–Proskauer, gelatinase enzyme production, nitrate reduction and citrate utilization. The bacterium grew over a temperature range between 30 C – 55 C and pH 5.7-6.8 in nutrient broth and

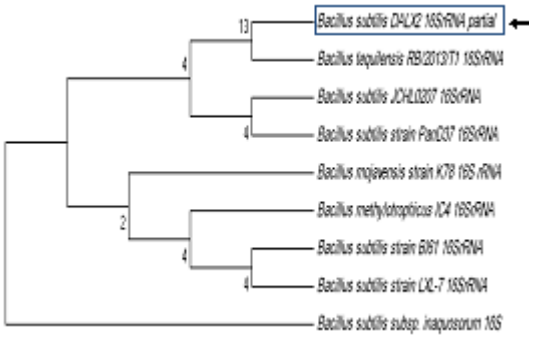

Fig. 1. Phylogenetic tree showing the relationship between *Bacillus subtilis* DALX2 and its homology strains using MEGA4 program

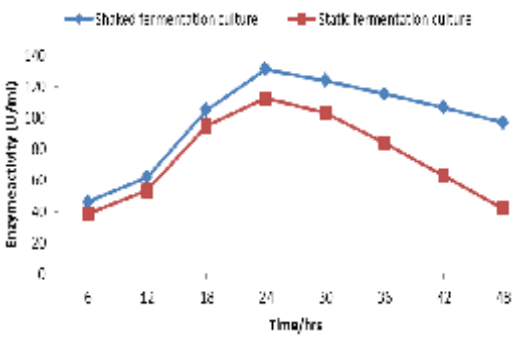

Fig. 2. Effect of aeration on enzyme production by *B. subtilis* DALX2 grown in 50 ml M 1 medium for 48 h at 37 C and pH 7

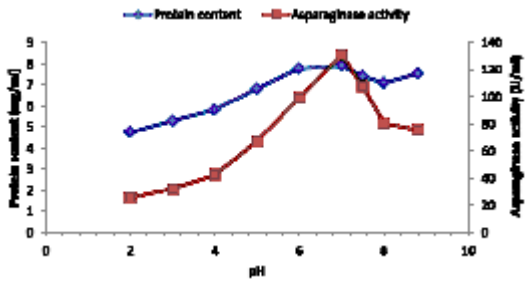

Fig. 3. Effect of initial pH on asparaginase activity of *Bacillus subtilis* DALX2 grown in 50 ml of M1 medium receiving 2 ml inoculum and incubated at 37 C

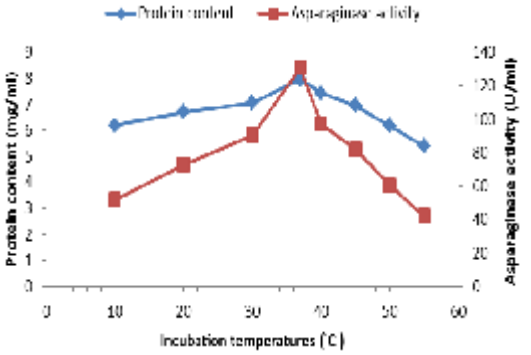

Fig. 4. Effect of temperature on asparaginase activity by *Bacillus subtilis* DALX2 grown in 50 ml of M1 medium, receiving 2 ml inoculum, at pH 7

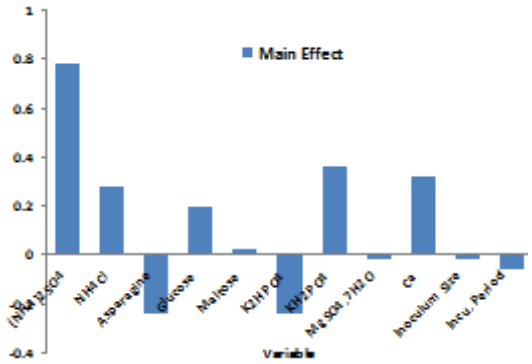

Fig. 5. Main Effect of variables influencing asparaginase production from *Bacillus subtilis* DALX2

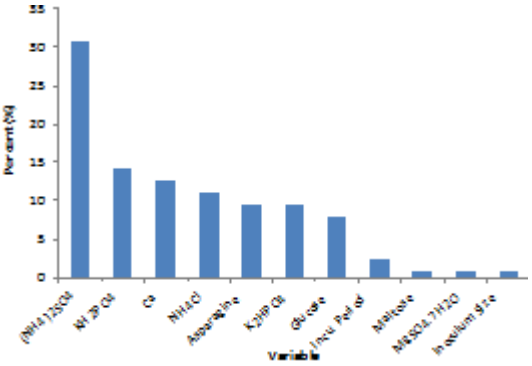

Fig. 6 . Variable percent for production of asparaginase from *Bacillus subtilis* DALX2

Table 1. Variables and their levels employed in Plackett- Burman design for screening of free culture conditions affecting asparaginase production by *Bacillus subtilis* DALX2 on M1 medium

Value				
1 +	0	1 -	Variables	Code
4.5	3	1.5	(NH4)2SO4 (g/l)	X1
4.5	3	1.5	NH4Cl (g/l)	X2
4.5	3	1.5	Asparagine (g/l)	X3
4.5	3	1.5	Glucose (g/l)	X4
4.5	3	1.5	Maltose (g/l)	X5
1.5	1.0	0.5	K2HPO4 (g/l)	X6
1.5	1.0	0.5	KH2PO4 (g/l)	X7
0.75	0.5	0.25	MgSO4 (g/l)	X8
0.75	0.5	0.25	CaCl2 (g/l)	X9
3	2	1	Inoculum Size (% v/v)	X10
48	24	18	Incubation period (hour)	X11

tolerated up to 2 to 7 % NaCL. Negative with indole, urease production and formation of gas from glucose fermentation and for nitrate reductase . Formed acid with D(-) Glucose, L(+) Arabinose, D(-) Mannitol and D(-) Xylose.

Sequence analysis of 16S rDNA showed 96 % similarity to *Bacillus subtilis* and was thus designated as *B.subtilis* DALX2. The sequence was deposited in Gen Bank database with an accession number of KR259637. The Phylogenetic tree is shown in Fig 1. .

subtilis DAX2 asparaginase

Effect of medium composition

Five different media (M I, M II, M III, M IV and M 9) were examined for asparaginase production by *B. subtilis* DALX2. Flasks received 2 ml inoculum and were incubated for 24 h at 37 C under static conditions.

Results revealed that, the highest value of asparaginase activity (112.4 U/ml was achieved with M I medium, whereas M3 resulted in the lowest enzyme activity (46.1 U/ml). (data not shown)

Effect of aeration and incubation time

The data shown in Fig 2, depict that the enzyme activity increased till its maximum value (130.9 U/ml) after 24 h of incubation under shacked conditions. On the other hand, lower activity (112.4 U/ml) was recorded under static conditions at the same incubation time.

Effect of carbon and nitrogen sources

Among different carbon and nitrogen sources examined, the highest enzymatic titer (131.1 U/ml) was recorded in presence of glucose and ammonium sulphate (data not shown).

Effect of initial pH and temperature

Asparaginase activity was estimated in M1 medium containing ammonium sulphate and glucose and adjusted to different pHs from 2.0 to 8.8. As shown in Fig 3. maximum activity (130.9 U/ml) was recorded at pH 7.

Incubation at different temperatures (10-55 C) showed a variation in enzyme activity with a maximum activity (130.9 U/ml) at 37 C (Fig 4).

Table 2. Experiment combination variables (showing the design plan and the averages of asparaginase activity (U/ml) for the different trials).

Exp. No.	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	Asparaginase activity(U/ml)
1	-	-	-	-	+	+	+	-	+	+	-	124.1
2	+	+	+	-	+	+	-	+	-	-	-	138.3
3	-	-	-	-	-	-	-	-	-	-	-	113.6
4	0	0	0	0	0	0	0	0	0	0	0	153.2
5	+	-	+	+	-	+	-	-	-	+	+	159.2
6	-	+	+	-	+	-	-	-	+	+	+	152.1
7	+	+	-	+	+	-	+	-	-	-	+	166.1
8	-	+	-	+	-	-	-	+	+	+	-	143.3
9	+	-	-	+	+	+	-	+	+	-	+	148.6
10	+	-	+	-	-	-	+	+	+	-	+	157.8
11	-	-	+	+	+	-	+	+	-	+	-	143.4
12	0	0	0	0	0	0	0	0	0	0	0	153.2
13	-	+	-	-	-	+	+	+	-	+	+	126.9
14	-	+	+	+	-	+	+	-	+	-	-	122.1

Note: X1; $(NH_4)_2SO_4$, X2; NH_4Cl, X3; Asparagine, X4; glucose, X5; maltose, X6; K_2HPO_4, X7; KH_2PO_4, X8; $MgSO_4.7H_2O$, X9; $CaCl_2.2H_2O$, X10; inoculum size and X11; incubation period.

Table 3. Coefficient value of the factor

	Coefficients	t Stat	P- value
$(NH_4)_2SO_4$	0.389	13.85	0.005
NH_4Cl	0.143	5.12	0.036
Asparagine	-0.121	-4.33	0.049
Glucose	0.081	2.89	0.101
Maltose	0.008	0.29	0.802
K_2HPO_4	-0.116	-4.15	0.053
KH_2PO_4	0.284	10.12	0.009
$MgSO_4.7H_2O$	-0.008	-0.31	0.787
$CaCl_2.2H_2O$	0.155	5.91	0.027
Inoculum size	-0.001	-0.04	0.969
Incubation period	-0.027	-0.99	0.427

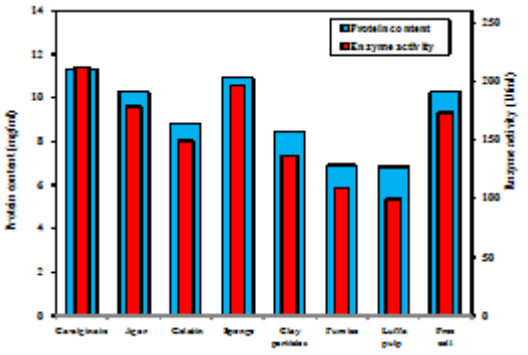

Fig. 7. Effect of different immobilization materials on the production of asparaginase by *Bacillus subtilis* DALX2

Application of Plackett - Burman for optimization of *Bacillus subtilis* DALX2 asparaginase production

The main aim of this experiment was the optimization of cultivation conditions that could affect asparaginase production. For this reason, screening design namely; Plackett - Burman experimental design was applied to investigate the significance of various factors on asparaginase production. Eleven chemical and environmental independent variables (including fermentation conditions and medium constitution) were examined in two levels: -1 for low and +1 for high level based on Plackett - Burman design (Table 1). According to the matrix illustrated in Table 2, the independent variables were screened in 14 combinations. The main effect of each variable is the difference between the average of measurements at high setting (+1) and the average of measurements observed at low setting (-1) of that factor. Plackett-Burman experimental design was based on the first order model (Equation 1):

$$Y=\beta 0+\Sigma\beta iXi \quad ...(1)$$

Where, Y is the predicted response, β0 and βi are constant coefficients, and Xi is the coded independent variables estimates or factors. The quality of fit of the polynomial model equation was expressed by the coefficient of determination R^2 .

Selected Variables, listed in Table 1 show the ability of *Bacillus subtilis* DALX2 to produce asparaginase at the optimum values.

Eleven different factors including fermentation conditions and medium constitution were screened for their effect on asparaginase production using the Plackett – Burman design.

The design plan and the averages of asparaginase activity for the different trials are given in U/ml and shown in Table 2. The main effect of each variable was estimated as the difference between both averages of measurements made at the high level (+1) and at the low level (-1) of that factor. The data in Table 2 show a wide variation from 113.6 to 166.1 U/ml of asparaginase activity. This variation reflects the importance of medium optimization to attain higher productivity. The analysis of the data from the Plackett– Burman experiments involved a first order (main effects) model. The main effects of the examined factors on the enzyme activity were calculated and presented graphically in Fig. 5 and also the regression analysis. On the basis of the analysis of the regression coefficients of the 11 variables after 48 h of incubation, $(NH_4)_2SO_4$, NH_4Cl, glucose, KH_2PO_4 and $CaCl_2.2H_2O$ showed the highest positive significance on asparaginase activity. Asparagine and K_2HPO_4 showed negative effect. Nearly non- significant effect in enzyme activity was recorded due to change in maltose, inoculum size and incubation period. Furthermore, Figure 6 shows the ranking of factor estimates in a Pareto chart. The Pareto chart (displaying the magnitude of each factor estimate) and it is a convenient way to view the results of a Plackett - Burman design.

On the basis of calculated P- and t- values as well as confidence level (Table 3), $(NH_4)_2SO_4$ and KH_2PO_4 were the most significant variables supported by the lowest P-values recorded (0.005 and 0.009, respectively).

Asparaginase production by entrapment and adsorption cells

Bacillus subtilis DALX2 cells were immobilized on different materials (luffa pulp, synthetic sponge cubes, clay and pumice particles as adsorption porous material and Ca-alginate, agar and gelatin were used as entrapment materials. The results presented in Fig.7, reveal that, immobilization by Ca-alginate showed the highest asparaginase activity among the immobilization material under test (211.6 U/ml).

The culture medium with sponge adsorbed cells revealed the second highest yield (196.3 U/ ml). Also agar showed a fairly high activity reading (178.6 U/ml), but still higher than the free culture. However, gelatin, clay particles, pumice and luffa pulp yielded relatively low asparaginase activities.

According to these results the immobilization with Ca-alginate entrapped cells, was chosen for the enzyme production.

Production of asparaginase by different concentrations of Ca-alginate entrapped cells:

Using 4% Ca-alginate concentration yielded the highest enzyme activity and recorded 267.9 U/ml. (data not shown).

Semi continuous production of asparaginase by Ca-alginate entrapped *Bacillus subtilis* DALX2:

The reuse of Ca-alginate beads 4% lead to the increase in enzyme activity gradually until the 4th run, then it decreased again in a gradual

manner, but still it was higher than the free activity of the free culture cells(data not shown).

DISCUSSION

Asparaginase (asparagine aminohydrolase, E.C. 3.5.1.1.) attracted much attention many years ago, because of its antineoplastic activity (Shukla *et al*., 2014; Jha *et al*., 2015). The enzyme has a particularly high therapeutic index when used in the treatment of acute lymphocytic leukemia (Jayam and Kannan, 2014).

Strains producing asparaginase were identified by the formation of pink colored colonies on M9 agar medium with phenol red as an indicator (Prakasham *et al*., 2010).

In this study, among the bacterial isolates tested, DALX2 was the most promising and was thus selected and identified as *Bacillus subtilis* DALX2.

Bacillus subtilis DALX2 proved to be a potent bacteria producing asparaginase under both static and shacked conditions using M 9 medium containing asparagine, at pH 7.0, after 24 h at 37 C. Production of L-asparaginase by submerged fermentation was reported with *Bacillus cereus* MNTG-7 (Audipudi *et al*., 2013).

Asparaginase activity was monitored during 48 h under shacked and static conditions using the medium containing ammonium sulphate. The enzyme activity increased by extending the fermentation till 24 h.

Media containing ammonium sulphate (3g/l) and glucose (3g/l) showed highest asparaginase activity (131.1 U/ml). These results suggest that regulation of *Bacillus subtilis* DALX2 asparaginase is similar to data obtained by other investigators (Sunitha *et al*., 2010; Moorthy *et al*., 2010).

Several workers used ammonium sulphate for asparaginase production by different bacterial species namely, *Enterobacter cloacae* and *E.coli* (Mercado & Arenas, 1999). Whereas, it was found that asparaginase activity was highest in cultures grown with ammonium nitrate (Nawaz *et al*., 1998). Wakil *et al*., (2015) observed that yeast extract supported the highest titers of L-asparaginase by *Bacillus firmus* and *Bacillus polymyxa*. Moreover, Kenari *et al*., (2011) showed that yeast extract was the best nitrogen source for *E. coli*.

The action of glucose as catabolic repressor was reported by some investigators (Mukherjee *et al*., 2000; Wakil *et al*., 2015).

An initial pH value of 7 was found to be the best for maximal *Bacillus subtilis* DALX2 asparaginase. These results coincide with the results of other workers (Mukherjee *et al*., 2000; Makky *et al*., 2013 ; Joshi *et al*., 2015).

Incubation temperature of 37°C was favourable for maximal asparaginase activity by *Bacillus subtilis* DALX2. Similar data were reported (Mahajan *et al*., 2014 ; Joshi *et al*., 2015). In contrast, (Wakil *et al*., 2015) observed highest aspaginase activity at an incubation temperature of 45°C for *Bacillus firmus*, *Streptococcus* spp. D1 and *Bacillus circulans*.

Eleven different factors including fermentation conditions and medium constituents were screened for their effect on asparaginase production using Plackett – Burman design. Among the selected variables; $(NH_4)_2SO_4$, NH_4Cl, glucose, $CaCl_2.2H_2O$ and KH_2PO_4 showed the highest positive effect. These results are in good agreement with those reported by Strobel and Sullivan (1999) and Gupta *et al*., (2004).

On the basis of calculated *P*- and *t*- values as well as confidence level, $(NH_4)_2SO_4$ and KH_2PO_4 were the most significant variables affecting asparaginase activity by *Bacillus subtilis* DALX2 as revealed by the lowest P -value (0.005 and 0.009, respectively). (Gupta *et al*., 2004)).

Cell immobilization is a technique generally used for higher productivity by protecting the cells from shear forces. Among the tested immobilization materials used in this work, Ca-alginate supported the highest asparaginase activity (211.6 U/ml), followed by sponge (196.3 U/ml) and agar (178.6 U/ml). Similar observation was reported by Ray *et al*., (1995) and Kumar, (2010) who found that Ca- alginate was reported as a good immobilizing matrix. Immobilization induces cellular or genetic modification that helps to produce maximum production of the enzyme titer.

Ramakrishnan *et al*., (2011) and Kumar, (2010), reported that alginate beads permit strong physical and chemical stability, avoiding the chance of leakage of cells or contamination in fermentation medium and also increase the reuse possibilities. Therefore, Ca-alginate entrapped cells

has been used to evaluate the efficiency of repeated batch fermentation. By using 4 % Ca-alginate entrapped cells, a significant amount of asparaginase titer (20.04 U/mg protein) was attained .

When Ca-alginate (4%) was reused, gel beads remained without disintegration till the 4th cycle. In each cycle, the enzyme titer was lowered very slowly. This is similar to the results obtained by Amena *et al*., (2012), who studied the possibility for reuse of the immobilized *S.gulbargensis* to produce L-asparaginase in a semi-continuous mode.

The results revealed that the immobilized cells continued to produce significant enzyme titers for four successive batches. Further repetition, after 4 cycles, cell leakage occured. In order to confirm reproducibility of repeated batch study, this test was repeated three times under the same conditions.

CONCLUSION

The present study reveals that soil is a rich source for the isolation of L- asparaginase producing bacteria. A reasonable amount of enzyme was achieved with *Bacillus subtilis* DALX2 in submerged fermentation at 37°C temperature, and neutral pH using glucose and $(NH_4)_2SO_4$ as a carbon and nitrogen source, respectively.

REFERENCES

1. Adinarayana, K., Jyothi, B., Ellaiah, P. Production of alkaline protease with immobilized cells of *Bacillus subtilis* PE-11 in various matrices by entrapment technique. *AAPS Pharma Sci Tech*, 2005; **6**:391-397.
2. Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 1997; **25**: 3389- 402.
3. Amena, S., Lingappa, K., Prabhakar, M., Vishalakshi, N. Production Of L-Asparaginase By Strain Improvement and Whole-Cell Immobilization of *Streptomyces gulbargensis*. *Journal Of Recent Advances In Applied Sciences* 2012; **27**:93-8.
4. Audipudi, A.V., Pallavi, R., Supriya, G.N.R. Characterization of L-Asparaginase Producing Bacteria From Mangrove Soil. *International Journal of Chem Tech Research*. 2013; **5**(1): 109-112.
5. Calinescu, R., Ghezzi, C., Kwiatkowska, M., Nirandola, R. Self-adaptive software beeds quantitative verification at runtime. *Comm. ACM* 2012; **55**(9): 69-77.
6. DiCosimo, R., McAuliffe, J., Poulose, A.J., Bohlmann, G. Industrial use of immobilized enzymes. *Chem Soc Rev.* 2013; **42**(15): 6437-74.
7. Duarte, J.C., Rodrigues, J.A.R., A.R, Moran, P.J.S., Valença, G.P., Nunhez J.R. Effect of immobilized cells in calcium alginate beads in alcoholic fermentation. *AMB Express*. 2013; **3**: 31.
8. Gupta, M., Upal Kanti Mazumder, U.K., Kumar, R.S., Kumar, T.V. Antitumor activity and antioxident role of *Bauhinia racemosa* against Ehrlich ascites carcinoma in Swiss albino mice. *Acta Pharmacol Sin* 2004; **25** (8): 1070-6.
9. Huang, L., Liu Y., Yan Sun, Qiaojuan Yan, Zhengqiang Jiang. Biochemical Characterization of a Novel L-Asparaginase with Low Glutaminase Activity from *Rhizomucor miehei* and Its Application in Food Safety and Leukemia Treatment. *Applied and Environmental Microbiology*. 2014; **80**(5): 1561–1569.
10. Jayam, D.G., Kannan, S. Screening and characterization of L-asparaginases producing *Streptomyces* isolated from soil samples of Periyar Lake, Kumily. *Biosci. Discover.* 2014; **5**: 50-4.
11. Jha, S.K., Singh, R.H., Nigam, V.K., Vidyarthi, A.S. A Heuristic Approach for the Enhanced Recovery of Anti-leukemic L-Asparaginase from *Bacillus licheniformis* by the Statistical Optimization of Three-Phase Partitioning System. *International Journal of Pharmaceutical and Clinical Research* 2015; **7**(2): 144-7.
12. Jobanputra, A.H., Karode, B.A., Chincholkar S.B. Calcium alginate as supporting material for the immobilization of rifamycin oxidase from *Chryseobacterium* species. *Research Article, Biotechnol. Bioinf. Bioeng.* 2011; **1**(4):529-35.
13. Joshi, R.D., Umrikar, A.M., Bhate, M.A., Kulkarni, S.S. Production, Purification and Characterization of L- Asparaginase From Soil Isolate Of *Bacillus* species. *International Journal of Recent Scientific Research* 2015; **6**: 2472-6.
14. Kenari, S.L.D., Alemzadeh, I., Maghsodi, V. Production of L-asparaginase from *Escherichia coli* ATCC 11303: Optimization by response surface methodology. *Food Bioprod. Process* 2011; **86**:315-321.
15. Khan, A.A., Alzohairy, M.A. Recent Advances

and Applications of Immobilized Enzyme Technologies: A Review. *Research Journal of Biological Sciences.* 2010; **5**(8):565-75

16. Krasotkina, J., Anna, A., Gervaziev, B.Y.V., Sokolov, N.N. One step purification and kinetic properties of the recombinant L-asparaginase from *Erwinia caratovora. Biotechnol. Appl. Biochem.* 2004; **39**: 215-22.
17. Kumar, R., Vats, R.. Protease production by *Bacillus subtilis* immobilized on different matrices. *New York Science Journal,* 2010; **20**: 3-24.
18. Lowry,O.H., Rosebrough,N.J.,Farr A.L., Randall,.J. Protein measurement with the folin phenol reagent. *J. Biol. Chem.,* 1951; **193**: 265- 75.
19. Mahajan, R.V., Mihooliya, K.N., Saran, S., Saxena, R.K. L-Asparaginase From *Bacillus* sp. Rks-20: Process Optimization and Application in the Inhibition of Acrylamide Formation In Fried Foods. *Journal of Proteins and Proteomics.* 2014; **5**(2): 125-32.
20. Makky, E.A., Ong J.J., Karim, R., Lee, C.M. Production and optimization of L-asparaginase by *Bacillus* sp. KK2S4 from corn cob. *African Journal of Biotechnology* 2013; **12**(19): 2654-8.
21. Martins, S.C.S, Martins, C.M., Guedes Fiúza L.M.C., Santaella, S.T. Immobilization of microbial cells: A promising tool for treatment of toxic pollutants in industrial wastewater. *African Journal of Biotechnology.* 2013; **12**(28): 4412-8.
22. Mercado, L., Arenas, G. *Escherichia coli* asparaginase induced phosphorylation of endogenous polypeptide in human immune cells. *Sangre (Brac).* 1999; **44**:438-42.
23. Moorthy, V., Ramalingam, A., Sumantha, A., Shankaranaya, R.T. Production, purification and characterisation of extracellular L-asparaginase from a soil isolate of *Bacillus* sp. *African Journal of Microbiology Research* 2010; **4**(18): 1862-7.
24. Mukherjee, J., Majumadar, S., Scheper, T. Studies on nutritional and oxygen requirements for production of asparaginase by *Enterobacter aerogenes. Appl. Microbiol Biotechnol.* 2000; **53**: 180-4.
25. Nawaz, M.S., Zhang, D., Khan, A.A., Cerniglia, C.E. Isolation and characterization of *Enterobacter*. Capable of metabolizing asparagine. *Appl. Microbiol. Biotechnol.* 1998; **568**: 50-72.
26. Olama, Z.A., El-Sabaeny, A.H. Lipase production by *Aspergillus niger* under various growth conditions using solid state fermentation. *Microbiologia SEM* 1993; **9**: 134-41.
27. Panday, D., Das, S.K. *Chelatococcus sambhunathiisp*. nov., a moderately thermophilic alphaproteobacterium isolated from hot spring sediment. *Int J Syst Evol Microbiol,* 2010; **60**: 861- 865.
28. Plackett, R.L., Burman J.P., *Biometrica.* 1946; **33**: 305-25.
29. Prakasham, R.S., Hymavathi, M., Rao Ch, S., Arepalli, S.K., RaoJ, V., Kennady, P.K., Nasaruddin, K., Vijayakumar, J.B., Sarma, P.N. Evaluation of antineoplastic activity of extracellular asparaginase produced by isolated *Bacillus circulans. Appl. Biochem. Biotechnol* 2010; **160**: 72-80.
30. Ramakrishnan, S.V., Prakasham, R.S. Microbial fermentation with immobilized cells. *Curr Sci,* 2011; **77**: 87-100.
31. Ray, R.R., Jana, S.C., Nanda, G., Beta - amylase production by immobilized cell of *Bacillus megaterium* B6. *J Basic Microbiol*, 1995; **35**: 113-6.
32. Saitou, N., Nei, M. The neighbor- joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution,* 1987; **4**: 406-25.
33. Shukla, D., Shrivastav, V.K., Jana, A.M., Shrivastav, A. Exploration of the potential L-asparaginase producing bacteria from the soil of Gwalior (India). *Int. J. Curr. Microbiol. App.Sci.* 2014; **3**(5): 665-72.
34. Strobel, R.J., Sullivan, G.R. Experimental design for improvement of fermentations. In Manual of industrial Microbiology and Biotechnology, A.L. Demain and J.E. Davies, eds., ASM Press, Washington, DC, 1999; 80-93.
35. Sunitha, M., Ellaiah, P., Bhavani, Devi, R. Screening and optimization of nutrients for L-asparaginase production by *Bacillus cereus* MNTG-7 in SmF by Plackett-Burmann design. *Afr. J. Microbiol. Res.* 2010; **4**: 297-303.
36. Tamura, K., Dudley, J., Nei, M., Kumar S.: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution,* 2007; **24**: 1596- 9.
37. Wakil, S.M., Adelegan A.A. Screening, Production and Optimization of L-Asparaginase From Soil Bacteria Isolated in Ibadan, South-western Nigeria, *Journal of Basic & Applied Sciences,* 2015; **11**: 39-51.
38. Wriston, J.C. and Yellin, T.O. (1973). Asparaginase: A review. *Adv. Enzymol.* 1973; **39**: 185-248.
39. Yu, X., Hallett, S.G., Sheppard, J., Watson, A.K. Application of the Plackett-Burman experiment design to evaluate nutritional requirements for the production of *Colletotrichum coccodes*

spores. 1997; **47**:301-5.
40. Zhang, S., Shang, W., Yang, X., Zhang, S., Zhang, X., Chen, J. Immobilization of Lipase using Alginate Hydrogel Beads and Enzymatic Evaluation in Hydrolysis of p-Nitrophenol Butyrate. *Bull. Korean Chem. Soc.* 2013; **34**(9): 2741.