Vol. 14(1), pp. 16-24, January, 2020
DOI: 10.5897/AJMR2019.9107
Article Number: D4DD27B62686
ISSN: 1996-0808

http://www.academicjournals.org/AJMR

ACADEMIC JOURNALS
expand your knowledge

African Journal of Microbiology Research

Full Length Research Paper

Studies on optimization of L-glutaminase production under submerged fermentation from marine *Bacillus subtilis* JK-79

Jambulingam Kiruthika* and Saranya Murugesan

Department of Industrial Biotechnology, Government College of Technology, Thadagam Road, Coimbatore-641013, Tamil Nadu, India.

Received 25 March, 2019; Accepted 6 May, 2019

In the current study, a marine strain *Bacillus subtilis* JK-79 capable of producing L-glutaminase enzyme, was analyzed for maximal L-glutaminase production. The Plackett-Burman design (PBD) was applied to determine the significant variables. Optimum conditions of the significant variables on L-glutaminase production were determined by the Central Composite Design (CCD) of response surface methodology. Maximum L-glutaminase production of 691.27 U/ml under flask condition was obtained at the predicted optimal values of pH 6.9, fructose 2.1% (w/v), yeast extract 1.25% (w/v), and glutamine 2.47% (w/v). Statistical optimization has enhanced L-glutaminase enzyme production upto 3.48 fold when compared with the basal SWG medium. The results of this study revealed that marine *B. subtilis* JK-79 could be a promising source for L-glutaminase production.

Key words: *Bacillus subtilis* JK-79, L-gltaminase, response surface methodology, submerged fermentation.

INTRODUCTION

Leukemia leads to the lympo-proliferative disorders in particular acute lymphoblastic leukemia (ALL). According to facts and statistics, Leukemia and Lymphomo Society (2018), 60,300 people are expected to be diagnosed with leukemia in United States and 24,370 people are expected to die from leukemia (14,270 males and 10,100 females). In 2010 to 2014, leukemia was the sixth most common cause of cancer deaths in both men and women (American Cancer Society's Cancer Facts and Figures, 2018). The treatment of ALL is very complex with drugs and there is reluctance for use in children and adults as it results in severe toxic reactions (Douer, 2008; Noura et al., 2014). In recent years, cancer therapy is highly relied on enzymes as they are low molecular weight protein molecules specific in their action and have less or no toxic effects. The enzymatic approach was reported to be more promising cancer therapy, due to the discovery of several enzymes with effective anti-cancer activity on various types of cancer (Vellard, 2003; Pandian et al., 2014).

L-glutaminase an amidohydrolase enzyme (E.C 3.5.1.2) has been found to be promising in the treatment

*Corresponding author. E-mail: mailkiruthika@yahoo.com. Tel: +919944935597.

of ALL (Souba, 1993; Vijayan et al., 2017). Cancer cells, especially ALL cells cannot synthesize L-glutamine, as they lack properly functioning glutamine biosynthetic machinery and therefore require large amount of L-glutamine for their rapid growth. These cells depend on the exogenous supply of L-glutamine for their survival and rapid cell division. Hence, the use of L-glutaminase deprives the tumor cells from L-glutamine and causes selective death of L-glutamine dependant tumor cells. Thus, it can act as a possible candidate for enzyme therapy (Hensley et al., 2013; El-Ghonemy, 2014). In recent years, L-glutaminase in combination with or as an alternative to L-asparginase could be used as in enzyme therapy for cancer particularly leukemia (Sabu, 2003).

The production of L-glutaminase by bacteria has attracted great attention owing to their cost effective and eco-friendly nature (Chandrasekaran, 1996; Unissa et al., 2014). Microbial L-glutaminase can be produced by both submerged and solid state fermentation. The improvement in production of L-glutaminase by a microbial strain can be achieved by determining the optimum physico-chemical parameters. Selection of appropriate carbon and nitrogen source is one of the most critical stages in the development of an efficient and economical production process. The methodologies used for screening of the components fall into two major categories: classical and statistical. The screening of various cultural parameters by classical method involves changing one independent variable (component of medium, parameter) at a time while fixing all other at certain level. This single dimensional search is simple, easy and useful technique to evaluate the individual effects of the media components and process conditions (Iyer and Singhal, 2008, 2009, 2010a, b) and has been commonly used for optimization.

However, the interaction between components influencing the production of a particular metabolite cannot be studied by varying one factor at a time (OFAT). Moreover, it often does not allow determination of actual optimum level of different components for a particular fermentation. The statistical methods provide an alternative solution to optimize a particular process by considering mutual interactions among the variables and give an estimate of the combined effect of these variables on the final result. Moreover, statistical methods are less laborious and rapid; thus are preferred over conventional OFAT approach for process optimization (Montgomery, 2000). Various statistical designs ranging from 2-factorial to multi-factorial are available for optimization of process parameters (Montgomery, 2000; Myers et al., 2009).

There has been much work reported on the effect of growth medium on the production of L-glutaminase by different marine bacteria. Various carbon and nitrogen sources were found to improve the production of L-glutaminase. Extracellular L-glutaminase producing *Beauveria* species BTMF S10 was isolated from marine sediment (Keerthi et al., 1999) and the yield was improved when the medium was supplemented with 1% yeast extract and sorbitol, 9% sodium chloride and 0.2% methionine at pH 9, 27°C. Optimum production of L-glutaminase from *Streptomyces rimosus* was observed at 27°C, pH 9 and glucose and malt extract as carbon and nitrogen sources, respectively (Sivakumar et al., 2006). Iyer and Singhal (2008, 2009), observed that the carbon and nitrogen sources for L-glutaminase production varied with the organisms. Supplementation of sucrose and yeast extract as carbon and nitrogen source improved L-glutaminase production by *Zygosaccharomyces rouxii* while higher L-glutaminase production was noticed in *Providencia* species with glucose and urea as carbon and nitrogen source, respectively. Recently, there are several reports which confirm the fact that the carbon and nitrogen sources significantly affect the production of L-glutaminase by microorganisms (Sathish and Prakasham, 2013; Krishnakumar et al., 2011; Pandian et al., 2014; Jesuraj et al., 2017).

In the present investigation, the potent marine strain *Bacillus subtilis* JK-79 was evaluated for maximal production of the L-glutaminase enzyme. In this context, different production media, carbon and nitrogen sources were tested for the highest yield of the L-glutaminase production.

MATERIALS AND METHODS

Microorganism and culture maintenance

The strain *B. subtilis* JK-79 (KC492745) used in this study was isolated from marine soil collected from Parangipettai costal area (Kiruthika and Saraswathy, 2014).

L-Glutaminase assay and protein estimation

L-glutaminase was assayed by the method of Imada et al. (1973). One international unit of L-glutaminase was defined as the amount of enzyme that liberates 1 µmol of ammonia under optimal conditions. The enzyme yield was expressed as Units/ml (U/ml of culture supernatant). Protein content in the crude enzyme source was estimated by Lowry's method (Lowry et al., 1951) using bovine serum albumin as the standard and the values were expressed as mg/ml.

Optimization by statistical design

Identifying significant variables by Plackett-Burman Design (PBD)

The Plackett-Burman Design (PBD; Mini Tab, trial version 17) was used to identify the significant variables affecting the L-glutaminase production. A total of eight factors such as pH (A), temperature (B), agitation speed (C), inoculum size (D), incubation time (E), carbon source (F), nitrogen source (G), and glutamine level (H), were considered and their high and low levels are shown in Table 1.

The responses from the 12 individual experiments were utilized for generating regression co-efficient values and the significant variables were further optimization by central composite design (CCD).

Table 1. High and low levels of variables used in PBD.

S/N	Coded variable	Factor	Low level (-1)	High level (+1)
1	A	pH	5	9
2	B	Temperature	25°C	45°C
3	C	Agitation speed	50 rpm	200 rpm
4	D	Incubation time	6 h	24 h
5	E	Inoculum size	1% (v/v)	7% (v/v)
6	F	Carbon source (fructose)	1% (w/v)	5% (w/v)
7	G	Nitrogen source (Yeast extract)	1% (w/v)	5% (w/v)
8	H	Glutamine	1% (w/v)	5% (w/v)

Table 2. PB experimental design for evaluating factors influencing L-glutaminase production by *Bacillus subtilis* JK-79.

Run order	A (pH)	B (Temperature)	C (Agitation speed)	D (Incubation time)	E (Inoculum size)	F (Carbon source)	G (Nitrogen source)	H (Glutamine)	L-glutaminase activity (U/ml)	Protein concentration (mg/ml)
1	-1	-1	1	1	1	-1	1	1	566.67	11.4
2	1	-1	1	1	-1	1	-1	-1	233.33	4.11
3	1	1	1	-1	1	1	-1	1	450	9.12
4	-1	1	-1	-1	-1	1	1	1	666.67	13.4
5	-1	-1	-1	1	1	1	-1	1	466.67	10
6	-1	1	1	1	-1	1	1	-1	412.22	8.78
7	1	1	-1	1	1	-1	1	-1	192.33	4
8	1	-1	-1	-1	1	1	1	-1	383.33	8.82
9	1	-1	1	-1	-1	-1	1	1	366.67	8.1
10	-1	-1	-1	-1	-1	-1	-1	-1	150	3.23
11	1	1	-1	1	-1	-1	-1	1	175	3.86
12	-1	1	1	-1	1	-1	-1	-1	183.33	3.98

Response surface methodology

The significant variables screened through PBD technique were subjected to CCD Software Design Expert Release 9 (Stat-Ease INC. Minneapolis MN, USA), a popular second order experimental design for developing sequential experimentation and predicting the levels of factors to get an optimal response through regression analysis. The effects of four independent variables *viz.* pH, carbon source, nitrogen source and glutamine on the production of L-glutaminase were studied at five different levels (-2, -1, 0, 1, 2). A full factorial central composite design was performed to build a total of 30 experiments, having $2^4 = 16$ cube points and 6 centre points (4 in cube and 2 in axial positions) and 4 × 2= 8 star points. The second-degree polynomial equation was used to determine the relationship between the independent variables and the response.

RESULTS AND DISCUSSION

Identification of significant factors using pbd

The influence of eight variables, in the production of l-glutaminase by the strain *b. Subtilis* jk-79 was investigated in 12 runs using pbd. Table 2 represents the PBD for the selected eight variables

Table 3. ANOVA for PBD.

Source	DF	Adj SS	Adj MS	F-Value	p-Value
Model	8	304153	38019	13.20	0.029*
Linear	8	304153	38019	13.20	0.029*
pH	1	34658	34658	12.03	0.040*
Temperature	1	632	632	0.22	0.671
Agitation speed	1	2647	2647	0.92	0.409
Incubation time	1	1971	1971	0.68	0.469
Inoculum size	1	4738	4738	1.64	0.290
Carbon source	1	79743	79743	27.68	0.013*
Nitrogen source	1	72007	72007	25.00	0.015*
Glutamine	1	107757	107757	37.40	0.009*
Error	3	8643	2881	-	-
Total	11	312795	-	-	-

*Significant. R^2 = 97.24%, Adj. R^2=89.87%, Pred. R^2= 55.79%.

Table 4. Actual and coded values of the factors employed in CCD.

Factor	Range of levels					
	Code	-2	-1	0	1	2
pH	A	3	5	7	9	11
Carbon source (fructose % w/v)	B	1.50	1.75	2.0	2.25	2.50
Nitrogen source (yeast extract % w/v)	C	0.5	1.0	1.5	2.0	2.5
Glutamine (% w/v)	D	0.5	1.5	2.5	3.5	4.5

and the corresponding response for L-glutaminase production (U/ml). Variations were observed in the production of L-glutaminase ranging from 150 to 666.67 U/ml. On the basis of analysis of variance (ANOVA), the most effective factors with high significance were in the order glutamine (H), fructose (F), yeast extract (G) and pH (A) (Table 3). The first order polynomial equation was derived representing L-glutaminase production as a function of independent variables

$$Y = 353.9 + 94.8\,H + 77.5\,G + 81.5\,F - 53.7\,A$$

Where, Y is the response (L-glutaminase production U/ml); H, G, F and A are independent variables of glutamine, fructose, yeast extract and pH, respectively with their corresponding co-efficients.

Statistical analysis of PBD demonstrated that the model F value of 13.2 is significant and among the significant factors, glutamine showed a remarkable effect on the production of L-glutaminase by *B. subtilis* JK-79. In the present model, R^2 was 97.24%, and only 2.77% variability was not explained. Thus, the present R^2 value reflected reliability of the model for predicting L-glutaminase production. The value of the adjusted determination coefficient (Adj R^2 = 0.8987) confirmed the significance of the model as well.

Optimization using CCD

RSM using CCD was adopted to understand the interactive effects of these four significant variables. Table 4 illustrates the details of actual and coded values employed in the CCD. The experimental trials were performed based on the CCD (Table 5) and the results obtained were fitted to a second order polynomial equation to explain the dependence of L-glutaminase production with the independent variables.

$$Y = +683.08 - 13.88 \times A + 10.05 \times B - 32.83 \times C - 5.88 \times D + 3.05 \times AB - 8.86 \times AC + 27.78 \times AD + 12.41 \times BC - 20.47 \times BD + 11.88 \times CD - 159.62 \times A^2 - 27.55 \times B^2 - 49.30 \times C^2 - 100.93 \times D^2$$

Where, Y is the response of L-glutaminase production, A, B, C and D are the coded values of pH, fructose, yeast extract and glutamine, respectively.

The analysis of variance of the quadratic regression model (Table 6) suggested that the model was very significant which was evident from the Fisher's F-test (F_{model} = 588.38) and a low probability value (P_{model}<0.0001). The p value for "lack of fit" (0.0957) also indicated that the quadratic model adequately fitted the data. In this model, A, B, C, D, AC, AD, BC, BD, CD, A^2, B^2, C^2, and D^2 are significant model terms. R^2 was found

Table 5. Observed response of CCD using four independent variables and six centre points.

Std.	Run	Factor 1	Factor 2	Factor 3	Factor 4	Response	Protein concentration (mg/ml)
		A:pH	B:Carbon source (% w/v)	C:Nitrogen source (% w/v)	D: Glutamine (%)	L-glutaminase activity (U/ml)	
26	1	0	0	0	0	686.67	14.12
2	2	1	-1	-1	-1	335.58	7.91
18	3	2	0	0	0	22.23	0.97
11	4	-1	1	-1	1	305.17	6.92
22	5	0	0	2	0	433.38	9.36
3	6	-1	1	-1	-1	458.87	9.98
24	7	0	0	0	2	268.38	5.28
29	8	0	0	0	0	691.27	14.98
20	9	0	2	0	0	592.28	12.23
9	10	-1	-1	-1	1	366.67	8.16
7	11	-1	1	1	-1	389.87	8.89
27	12	0	0	0	0	683.38	13.86
30	13	0	0	0	0	686.33	13.54
12	14	1	1	-1	1	372.27	8.38
14	15	1	-1	1	1	303.47	6.02
17	16	-2	0	0	0	68.33	3.42
25	17	0	0	0	0	677.87	13.02
23	18	0	0	0	-2	290.29	5.82
13	19	-1	-1	1	1	317.77	7.18
16	20	1	1	1	1	322.27	7.89
1	21	-1	-1	-1	-1	422.27	9.36
5	22	-1	-1	1	-1	308.33	6.02
6	23	1	-1	1	-1	218.87	4.33
8	24	1	1	1	-1	298.87	5.81
15	25	-1	1	1	1	318.87	7.61
21	26	0	0	-2	0	538.33	11.38
4	27	1	1	-1	-1	383.33	8.71
10	28	1	-1	-1	1	412.27	8.89
19	29	0	-2	0	0	553.83	11.41
28	30	0	0	0	0	672.97	13.34

to be 0.9982 indicating that the model was reliable.

3D response surfaces were generated to understand the interaction between independent variables. Figure 1*i-vi* shows the response surfaces and contour plots generated for the variation in the yields of L-glutaminase as a function of concentrations of two variables with the other two variables at their central value. The coordinates of the central point within the highest contour levels in each of the figures correspond to the optimum concentrations of the respective components. Evaluation of response surface curves and contour plots indicate the range of optimum conditions within the experimental area covered or show the way to conduct further experiments to achieve better results.

From the Figure 1i, it was evident that when the concentration of glutamine and nitrogen source were held at their middle values, the pH showed a parabolic response at the different concentrations of fructose with the highest yield of L-glutaminase obtained in the range of pH 7. Very low and high pH values were not favorable for enzyme production. Variation in fructose concentration has also followed a parabolic curve and optimum yield was in the range of 2.0 to 2.25%.

Similarly, the response behavior was analyzed between pH and yeast extract with the other two factors (glutamine and fructose) kept at their middle values. The production of L-glutaminae was affected by pH and followed a parabolic curve. Extreme conditions of pH decreased the production of the enzyme and the optimum pH was 7. However, the concentration of yeast extract also affected the response and the maximum L-glutaminase production was obtained in the range of 1 to 1.5% (Figure 1ii).

The 3D response surface and contour plot between pH and glutamine is represented in Figure 1iii. From these

Table 6. ANOVA for the CCD quadratic model.

Source	Analysis of variance table [Partial sum of squares - Type III]					
	Sum of squares	df	Mean Square	F Value	p-value Prob > F	Significance
Model	9.366E+005	14	66900.18	588.38	< 0.0001	Significant
A-pH	4622.87	1	4622.87	40.66	< 0.0001	-
B-Carbon source	2423.86	1	2423.86	21.32	0.0003	-
C-Nitrogen source	25873.32	1	25873.32	227.55	< 0.0001	-
D-Glutamine	828.96	1	828.96	7.29	0.0165	-
AB	148.90	1	148.90	1.31	0.2704	-
AC	1257.23	1	1257.23	11.06	0.0046	-
AD	12348.21	1	12348.21	108.60	< 0.0001	-
BC	2464.87	1	2464.87	21.68	0.0003	-
BD	6703.11	1	6703.11	58.95	< 0.0001	-
CD	2258.86	1	2258.86	19.87	0.0005	-
A^2	6.973E+005	1	6.973E+005	6132.63	< 0.0001	-
B^2	20742.70	1	20742.70	182.43	< 0.0001	-
C^2	66664.59	1	66664.59	586.31	< 0.0001	-
D^2	2.794E+005	1	2.794E+005	2457.38	< 0.0001	-
Residual	1705.54	15	113.70	-	-	-
Lack of Fit	1485.57	10	148.56	3.38	0.0957	Not significant
Pure Error	219.97	5	43.99	-	-	-
Cor Total	9.383E+005	29	-	-	-	-
Std. Dev.			10.66			
Mean			413.3			
C.V. %			2.58			
PRESS			8874			
R-Squared			0.9982			
Adj R-Squared			0.9965			
Pred R-Squared			0.9905			
Adeq Precision			88.267			

plots it was evident that lowest yield of L-glutaminase was obtained in the extreme pH conditions, that is, highly acidic and highly alkaline pH. The L-glutaminase production increased considerably when the pH approached its middle values and the optimum was in the range of 7. The L-glutaminase production was also affected by glutamine concentration and followed a parabolic curve. The yield was minimum at the very low and high concentration of glutamine and the highest production was obtained in the range of 2.5 and 3%.

The fructose and yeast extract concentration affected the L-glutaminase production and the optimum enzyme production was obtained in the fructose and yeast extract concentration of 2 to 2.5% and 1 to 1.5%, respectively (Figure 1iv). In the response behaviour of different concentrations of fructose and yeast extract (Figure 1v), the response pattern was found to be parabolic and the optimum production of L-glutaminase was obtained at the middle values. Figure 1vi demonstrates that the production pattern of L-glutaminase was parabolic with respect to glutamine concentration. In the case of yeast extract, the highest yield of the L-glutaminase was attained at the mid concentration and further increase in yeast extract concentration did not significantly change the production of L-glutaminase.

The optimum values of the independent variables were predicted using point prediction tool of design of expert software (Figure 2). Maximum L-glutaminase production of 688.5 U/ml under flask condition was obtained at the predicted optimal values of pH 6.9, fructose 2.1% (w/v), yeast extract 1.25% (w/v), and glutamine 2.47% (w/v). The maximum experimental L-glutaminase production was 691.27 U/ml thus indicating a strong correlation between them.

The L-glutaminase production under submerged fermentation by marine *B. subtilis* JK-79 was determined in optimized and unoptimized base medium, that is, Sea Water Glutamine (SWG) medium (Kiruthika and Saraswathy, 2014) and a 3.48 fold increase in glutaminase production was obtained by phase-wise

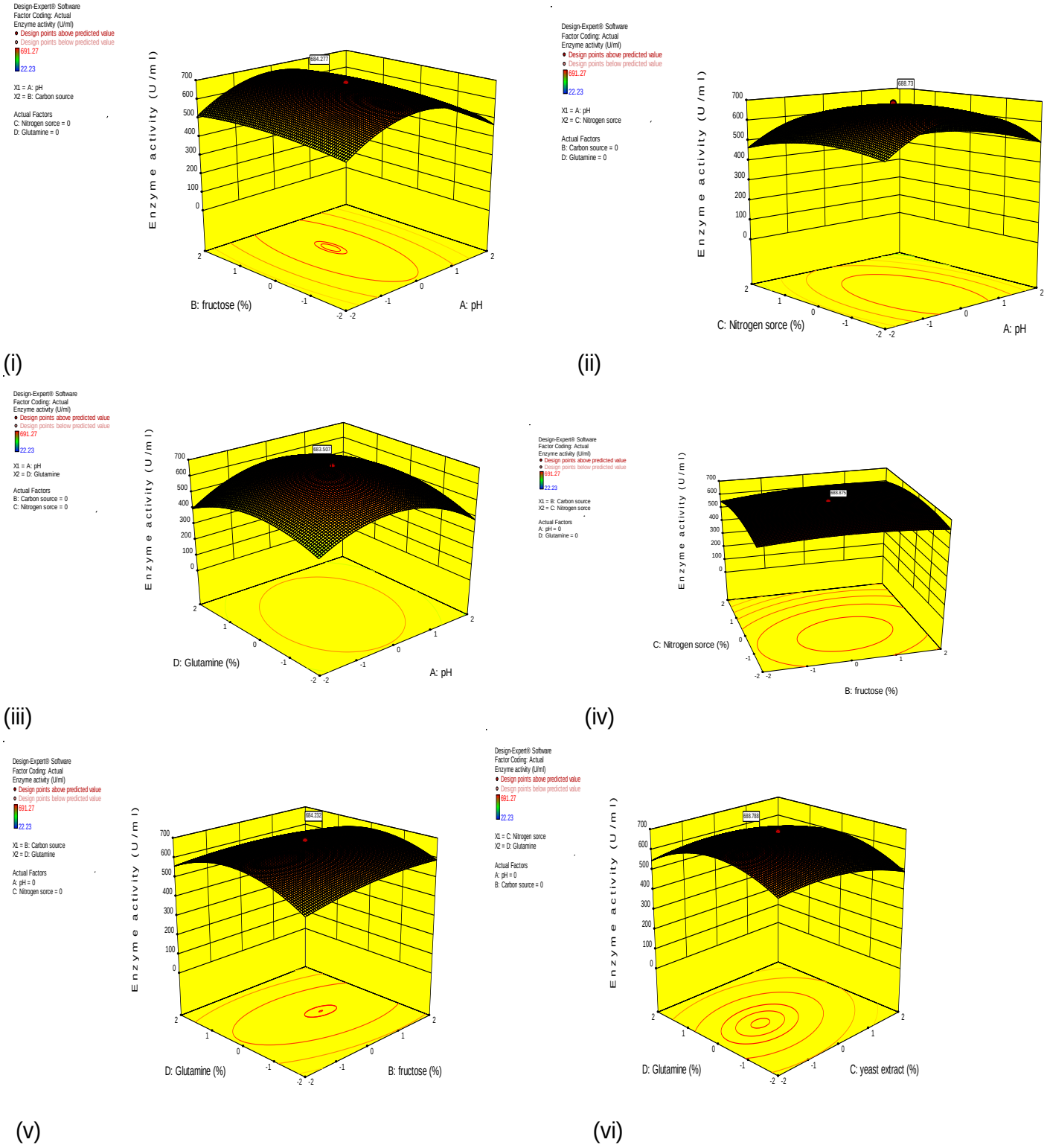

Figure 1. 3D response surface plots showing the interaction between the various components considered in the optimization. (i) Response behaviour of pH and fructose concentration under constant level of glutamine and nitrogen concentration. (ii) Response behaviour of pH and yeast extract concentration under constant level of glutamine and fructose concentration. (iii) Response behaviour of pH and glutamine concentration under constant level of yeast extract and fructose concentration. (iv) Response behaviour of fructose and yeast extract concentration under constant level of pH and glutamine concentration. (v) Response behaviour of fructose and glutamine concentration under constant level of pH and yeast extract concentration. (vi) Response behaviour of yeast extract and glutamine concentration under constant level of pH and fructose concentration.

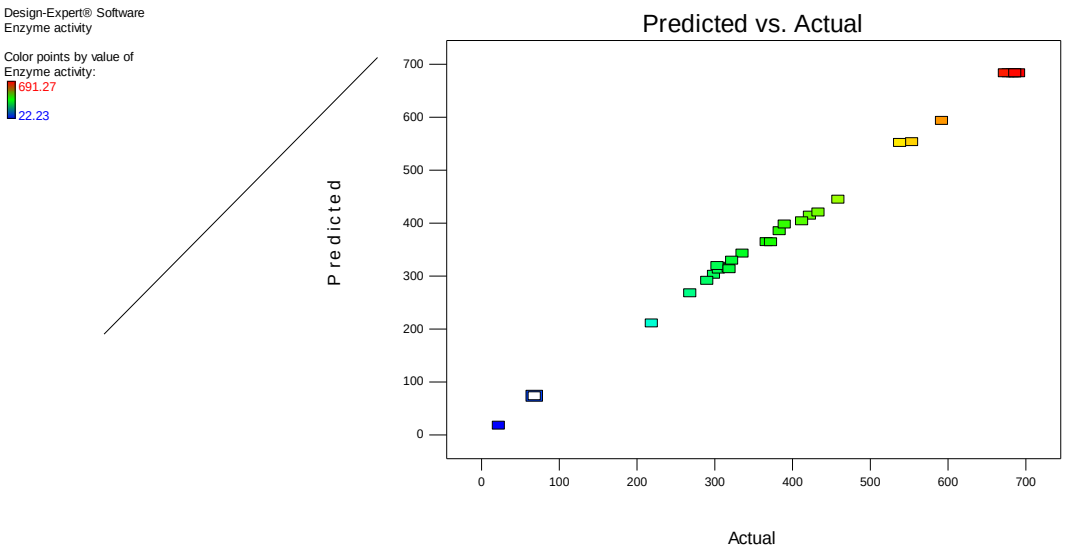

Figure 2. Plot between predicted and actual response of L-glutaminase production under submerged fermentation.

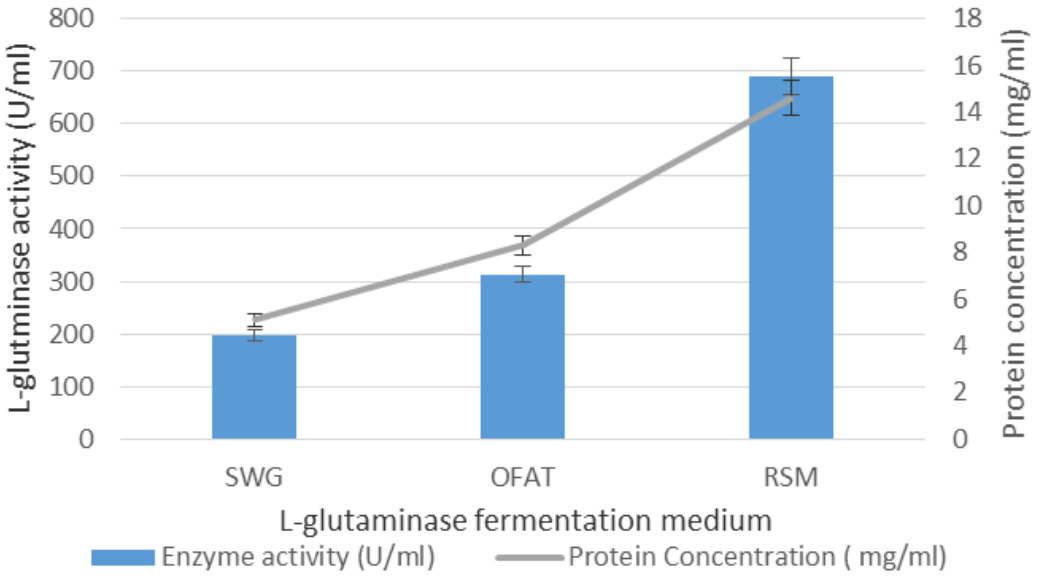

Figure 3. Comparision of L-glutaminase production by marine *Bacillus subtilis* JK-79 in basal and optimized medium.

optimizing the medium (Figure 3).

Several authors have reported an increased fold of L-glutaminase production by the application of RSM. Iyer and Singhal (2008) used a face centered central composite design (FCCCD) to enhance the L-glutaminase production and specific activity by 2.94 and 3.58 folds, respectively with *Z. rouxii*. Similarly, the authors have employed One Factor at a Time (OFAT) and FCCCD to design the optimized medium for *Providencia* spp. (Iyer and Singhal, 2009). Sathish and Prakasham (2010) found that a hybrid methodology adopted resulted in a significant improvement (47%) in the L-glutaminase yield by *B. subtilis* RSP-GLU.

Suresh et al. (2013) reported optimization of medium components through OFAT approach and FCCCD for the submerged production of L-glutaminase by *Serratia marcescens*. Pandian et al. (2014) reported the statistical optimization of medium composition for the production of glutaminase from *Alcaligenes faecalis* KLU102. RSM was used for optimization and the bacterium grown in the

optimized medium [arabinose (2%), skim milk (4%) and sodium chloride (2%)] yielded L-glutaminase activity of 1.34 IU/mg.

Jesuraj et al. (2017) has reported statistical optimization of L-glutaminase production by mutated strain *Aeromonas veronii* by PBD and CCD. The model was found to be a perfect fit in terms of maximizing enzyme yield, with the productivity improving at every stage to a fourfold output of enzyme (591.11 ±7.97 IU/mL) compared to the native strain (135±3.51 IU/mL).

Conclusion

Statistical optimization has enhanced L-glutaminase production under submerged fermentation by marine *B. subtilis* JK-79 (KC492745) (Kiruthika and Saraswathy, 2014) upto 3.48 fold when compared with the basal SWG medium. Thus, application of PBD and RSM for optimization studies proved to be an effective method for improving the L-glutaminase production. Results of this study revealed that marine *B. subtilis* JK-79 could be a promising source for L-glutaminase production.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors thank the Principal, G. C. T and the Management, K. C. T for providing the necessary facilities and fund to carry out this research work.

REFERENCES

American Cancer Society's Cancer Facts and Figures (2018). https://www.lls.org/http%3A/llsorg.prod.acquia-sites.com/facts-and-statistics/facts-and-statistics-overview/facts-and-statistics.

Chandrasekaran M (1996). Harnessing of marine microorganisms through soild state fermentation. Journal of Scientific and Industrial Research 55:468-471.

Douer D (2008). Is asparginase a critical component in the treatment of acute lymphoblastic leukemia? Best Practice and Research: Clinical Haemotology 21:647-658.

El-Ghonemy DH (2014). Microbial amidases and their industrial applications: A review. Journal of Medical Microbiology and Diagnosis 4(173):1-6.

Hensley CT, Wasti AT, De Berardinis RJ (2013). Glutamine and cancer: cell biology, physiology, and clinical opportunities. Journal of Clinical Investigation 123(9): 3678-84.

Imada A, Igarasi S, Nakahama K, Isono M (1973). Asparginase and glutaminase activities of microorganisms. Journal of General Microbiolgy 76:85-99.

Iyer P, Singhal RS (2008). Production of glutaminase (E.C.3.2.1.5) from *Zygosaccharomyces rouxii*: Statistical optimization using response surface methodology. Bioresource Techology 99:4300-4307.

Iyer P, Singhal RS (2009). Screening and selection of Marine isolate for L-glutaminase production and media optimization using response surface Methodology. Applied Biochemistry and Biotechnology 159:233-250.

Iyer P, Singhal RS (2010a). Glutaminase production using *Zygosaccharomyces rouxii* NRRRL-Y 2547: effect of aeration, agitation regimes and feeding strategies. Chemical Engineering and Technology 33:52-62.

Iyer P, Singhal RS (2010b). Isolation, screening, and selection of an L-glutaminase producer from soil and media optimization using a statistical approach. Biotechnology and Bioprocess Engineering 15:975-983.

Jesuraj SA, Sarker MM, Ming LC, Praya SM, Ravikumar M, Wui WT (2017). Enhancement of the production of $_{L}$-glutaminase, an anticancer enzyme, from *Aeromonas veronii* by adaptive and induced mutation techniques. PLOS ONE 12(8):e0181745.

Keerthi TR, Suresh PV, Sabu A, Kumar SR, Chandrasekaran, M (1999). Extracellular production of L-glutaminase by alkalophilic *Beauveria bassiana*-BTMF S10 isolated from marine sediment. World Journal of Microbiology and Biotechnology 15:751-752.

Kiruthika J, Saraswathy N (2014). Isolation and characterization of a novel L-glutaminase producing marine *Bacillus subtilis* JK-79. Asian Journal of Microbiology Biotechnology and Environmental Science 16(3):601-610.

Krishnakumar S, Alexis Rajan R, Ravikumar S (2011). Extracellular production of L-glutaminase by marine alkalophilic *Streptomyces sp.*-SBU1 isolated from Cape Comorin coast. Indian Journal of Geo-Marine Sciences 40:717-721.

Lowry OH, Rosebrough NN, Farr AL, Randall RY (1951). Protein measurement with the Folin Phenol reagent. Journal of Biological Chemistry 193:265-275.

Montgomery DC (2000). Design and Analysis of Experiments. Fifth Edition, John Wiley and Sons, New York, NY.

Myers RH, Montgomery DC, Anderson-Cook CM (2009). Response surface methodology: process and product optimization using designed experiments. Edition 3, John Wiley & Sons.

Noura El-Ahmady El-Naggar, Sara El-Ewasy M, Nancy El-Shweihy M (2014). Microbial L-asparginase as a potential therapeutic agent for the treatment of Acute Lymphoblastic Leukemia: The Pros and Cons. International Journal of Pharmacology 1:18.

Pandian SR, Deepak V, Sivasubramaniam SD, Nellaiah H, Sundar K (2014). Optimization and purification of anticancer enzyme L-glutaminase from *Alcaligenes faecalis* KLU102. Biologia 69:1644-1651.

Sabu A (2003). Sources, properties and applications of marine therapeutic enzymes. Indian Journal of Biotechnology 11:1211-1225.

Sathish T, Prakasam RS (2010). Enrichment of glutaminase production by *Bacillus subtilis* RSP-GLU in submerged cultivation based on neural network-genetic algorithm approach. Journal of Chemical Technology and Biotechnology 85:50-58.

Sathish T, Prakasam RS (2013). Influence of fermentation process parameters on L-glutaminase production by *Bacillus subtilis* RSP-GLU. International Journal of Pharmaceutical and Chemical Science 1:625-631.

Sivakumar K, Sahu MK, Manivel PR, Kannan L (2006). Optimum conditions for L-glutaminase production by actinomycete strain isolated from estuarine fish, *Chanoschanos* (Forskal, 1775) Indian Journal of Experimental Biology 44:256-258.

Souba WW (1993). Glutamine and cancer. Annals of Surgery 218:715-728.

Suresh Kumar S, Muthuvelayudham R, Viruthagiri T (2013). Production and optimization of L-glutaminase (EC.3.5.1.2) by *Serratia marcescens* using wheat bran under statistical designs. Journal of Chemical, Biological and Physical Sciences 3:2601-2612.

Unissa R, Sudhakar M, Sunil Kumar Reddy A, Naga Sravanthi K (2014). A review on biochemical and therapeutic aspects of glutaminase. International Journal of Pharmaceutucal Science and Research 5:4617-34.

Vellard M (2003). The enzyme as drug: application of enzymes as pharmaceuticals. Current Opinion in Biotechnology 14:444-450.

Vijayan N, Swapna TS, Haridas M Sabu A (2017). 11-Therapeutic enzymes – L-glutaminase. Current developments in Biotechnology and Bioengineering pp. 233-248.