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Screening and optimization of nutrients for L-asparaginase production by *Bacillus cereus* MNTG-7 in SmF by plackett- burmann design

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The medium components influencing the enzyme production were screened using Plackett-Burman design. A total of 67 nutrients comprising of fifteen each of carbon, nitrogen, mineral/salt and eleven each of inorganic nitrogen sources and amino acid sources were screened for the production of L-asparaginase by SmF. This design involves screening of up to 'n-1' variables in just 'n' number of experiments. Regression coefficients and t-values were calculated by subjecting the experimental data to statistical analysis. Among the 67 nutrients based on their performance in terms of product yield and cost, tapioca starch, L-asparagine, ammonium oxalate, gelatin and $CaCO_3$ were identified as most effective and therefore selected for inclusion in surface methodology studies.

Key words: L-asparaginase, nutritional factors, submerged fermentation, *Bacillus cereus* MNTG-7.

INTRODUCTION

A major potential therapeutic application of enzymes is in the treatment of cancer. Asparagianse has proved to be particularly promising in the treatment of acute lymphocytic leukaemia. Tumour cells are deficient in aspartate-ammonia ligase activity, which restricts their ability to synthesize the normally non-essential amino acid, L-asparagine. Therefore they are forced to extract it from body fluids. The action of Asparaginase does not affect the functioning of normal cells, which are able to synthesize enough for their own requirements, but reduce the free exogenous concentration, and so induce a state of fatal starvation in the susceptible tumour cells (Sabu, A, 2003). A 60% incidence of complete remission has been reported in a study of almost 6,000 cases of acute lymphocytic leukaemia. This enzyme is administered intravenously.

L-sparagine+H_2O L-aspartate+NH_3

Several reports are available on the production of L-asparaginase from bacteria, fungi and animal sources. It is desirable to search for new bacterial isolates producing L-asparaginase with novel properties from as many different sources as possible.

The important application of the L-asparaginase enzyme is in the treatment of acute lymphoblastic leukemia (mainly in children), Hodgkin disease, acute myelocytic leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma treatment, reticulosarbom and melanosarcoma (Stecher et al., 1999; Verma et al., 2007) . The role of L-asparaginase in lymphocytic leukemia cells treatment is based on the fact that these cells are not capable of synthesizing L-asparagine and they rely on the exogenous sources to get hold of L-asparagine (Lee et al., 1989). On the contrary, normal cells are protected from L-asparagine starvation due to their ability to generate this essential amino acid (Duval et al., 2002). The antineoplastic activity is attributed to the depletion of L-asparagine by the action of L-asparaginase (Lee et al., 1989). Though many species producing L-asparaginase are available, only *E. coli* and *Erwinia cartovora* asparaginases are currently in medical use as drugs in the treatment of lymphocytic leukemia, because of high substrate affinity

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(Verma et al., 2007; Schwartz et al., 1966) and rapid clearance of the enzyme from the media of the reaction (Stecher et al., 1999; Broome, 1965).

MATERIALS AND METHODS

Microorganism

The newly isolated bacterial mutant strain of *Bacillus cereus* MNTG-7 that produces L-asparaginase was employed in the present study. The culture was grown on MNTG-7 agar slants at 37°C for 24 h, subcultured at monthly intervals and stored in the refrigerator.

Preparation of spore suspension

Spore suspension was prepared by transferring 5 ml suspension prepared from 24 h old slant culture, into 250 ml Erlenmeyer flasks containing 45 ml of sterile inoculum medium. The composition of inoculum medium is (g/L): Na_2HPO_4. $2H_2$ 0, 6; KH_2PO_4, 3; NaCl 0.5; 1 M $MgSO_4.7H_20$, 2; 0.1M $CaCl_2$ $2H_20$, 1 ml; 20% Glucose stock solution, 10 ml (pH 7.0).

Shake flask fermentation

Five ml of inoculum (10% v/v) was added to 45 ml of production medium in 250 ml Erlenmeyer flask. The flasks were incubated at 28°C on rotary incubator shaker for 24 h (120 rpm). At the end of fermentation, 5 ml broth was collected and centrifuged at 4000 rpm for 10 min. and assayed for L-asparaginase activity. The composition of production medium is (g/L): Na_2HPO_4 .$2H_20$, 6; KH_2PO_4, 3; NaCl 0.5; L-asparagine, 5; 1M $MgSO_4.7H_20$, 2; 0.1M $CaCl_2$ $2H_20$, 1 ml; 20% Glucose stock solution, 10 ml (pH 7.0).

Protein estimation

The protein content was determined according to a modified Lowry's method (Ohnishi ST, 1978).

Enzyme assay

L-asparaginase was assayed colorimetrically (Sinha et al., 1991) . A standard curve was prepared with ammonium sulfate. One L-asparaginase unit (IU) is defined as that amount of enzyme which liberates 1 µ mole of ammonia per min under the optimal assay conditions (Imada 1973).

STATISTICAL DESIGN

Screening of nutrients using plackett-burman design

Fifteen each of carbon, nitrogen and mineral/salt and eleven each of inorganic nitrogen sources and amino acids were selected for the production of L-asparaginase using *Bacillus cereus* MNTG-7 and screened using Plackett -Burman design. In this design, generally a multiple of four, i.e. 4, 8, 12, 16, 20…4n experiments are required to screen 3, 7, 11, 15, 19…4n-1, components respectively, where 'n' is a multiple of 4 (Plackett-Burman, 1944). In this design, the columns represent the combinations and rows represent the variables (nutrients). The ingredients are taken at two levels (lower and higher) . Lower level in the design is represented as '-' and higher level as '+'. The contribution of an ingredient towards the growth of the organism or yield of the enzymes is determined based on the t-value (main effect) calculated from the experimental result (Ramana Murthy, 1994; Adriane and Lacis, 1991). The value (main effect) of an ingredient is calculated as follows:

t-value or main effect of an ingredient X = (average of sum of the enzyme activities where the ingredients is '+') – (average of sum of the enzyme activities where the ingredient is '-')

The nutrients are ranked based on their t-values. The nutrient with highest t-value is considered to be the best and ranked one (Akhnazarova and Kafarov 1982).

RESULTS

The medium components influencing the enzyme production were screened using Plackett-Burman design. In this step, a total of 67 nutrients comprising of fifteen each of carbon, nitrogen, mineral/salt and eleven each of inorganic nitrogen sources and amino acid sources were screened using Plackett-Burman design for the production of L-asparaginase by SmF. The use of PBD, a statistical methodology in the form of an orthogonal matrix, allows screening of up to 'n-1' variables in just 'n' experiments.

In this step, nutrients were taken only at one level, i.e., either the nutrient is present or absent to evaluate the positive or negative effect of the nutrient on enzyme production. The concentrations of each nutrient were fixed empirically, based on the literature survey and our own experience. The list of nutrients with respective concentrations is given in Tables 1 - 5.

The procedures for the preparation of medium, conduct of fermentation and enzyme assay were done as described earlier.

DISCUSSION

The principle source of L-asparaginase for clinical trials is the bacterium *E. coli* (Adamson and Fabro, 1968). Although production and purification techniques have been developed, they generally provide a quantity of enzyme sufficient for only limited trials. To overcome this constraint, production of Lasparaginase by other bacterium can be explored. Also medium factors for enhanced production of L-asparaginase can be investigated by statistical modeling.

There is little information available regarding statistical optimization of the medium factors for L-asparaginase production. Screening and selection of the optimum concentration of medium components are very important to determine the overall economic feasibility of the production process. The reach of optimized fermentation conditions, particularly associated to physical and chemical parameters, is of primary and great importance for the development of any process, due to their impact upon its economics and practicability.

The diversity of combinatory interactions among medium

Table 1. Plackett-Burman design for the screening of fifteen carbon sources (mg/ml) N = 16.

Dextrose	Lactose	Dextrin white	Starch potato	Starch soluble	Starch maize	Starch tapioca	Starch pharma	Maltose	Mannitol	Xylose	Fructose	Galactose	Sucrose	Cellulose	Yield (IU/ml)
1	1	1	1	0	1	0	1	1	0	0	1	0	0	0	37.5
1	1	1	0	1	0	1	1	0	0	1	0	0	0	1	22.5
1	1	0	1	0	1	1	0	0	1	0	0	0	1	1	42.5
1	0	1	0	1	1	0	0	1	0	0	0	1	1	1	33.75
0	1	0	1	1	0	0	1	0	0	0	1	1	1	1	26.25
1	0	1	1	0	0	1	0	0	0	1	1	1	1	0	43.75
0	1	1	0	0	1	0	0	0	1	1	1	1	0	1	36.25
1	1	0	0	1	0	0	0	1	1	1	1	0	1	0	31.25
1	0	0	1	0	0	0	1	1	1	1	0	1	0	1	31.25
0	0	1	0	0	0	1	1	1	1	0	1	0	1	1	97.5
0	1	0	0	0	1	1	1	1	0	1	0	1	1	0	41.25
1	0	0	0	1	1	1	1	0	1	0	1	1	0	0	42.5
0	0	0	1	1	1	1	0	1	0	1	1	0	0	1	36.25
0	0	1	1	1	1	0	1	0	1	1	0	0	1	0	31.25
0	1	1	1	1	0	1	0	1	1	0	0	1	0	0	33.75
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	30.25

Table 2. Plackett-Burman design for the screening of fifteen organic nitrogen sources (mg/ml) N = 16.

Soya bean meal	Tryptone	Gelatin	Yeast extract	Soya peptone	Yeast nitrogen base	Casein	Meat extract	Peptone (HM*)	Casamino acids	Malt extract	Beef extract	Urea	Albumin	Peptone (Q**)	Yield (IU/ml)
1	1	1	1	0	1	0	1	1	0	0	1	0	0	0	31.25
1	1	1	0	1	0	1	1	0	0	1	0	0	0	1	31.25
1	1	0	1	0	1	1	0	0	1	0	0	0	1	1	18.75
1	0	1	0	1	1	0	0	1	0	0	0	1	1	1	21.25
0	1	0	1	1	0	0	1	0	0	0	1	1	1	1	21.25
1	0	1	1	0	0	1	0	0	0	1	1	1	1	0	48.75
0	1	1	0	0	1	0	0	0	1	1	1	1	0	1	20.00
1	1	0	0	1	0	0	0	1	1	1	1	0	1	0	23.75
1	0	0	1	0	0	0	1	1	1	1	0	1	0	1	21.25
0	0	1	0	0	0	1	1	1	1	0	1	0	1	1	26.25
0	1	0	0	0	1	1	1	1	0	1	0	1	1	0	23.75
1	0	0	0	1	1	1	1	0	1	0	1	1	0	0	21.25
0	0	0	1	1	1	1	0	1	0	1	1	0	0	1	32.25
0	0	1	1	1	1	0	1	0	1	1	0	0	1	0	31.25
0	1	1	1	1	0	1	0	1	1	0	0	1	0	0	38.25
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	30.25

Peptone (HM*): Hi-Media. Peptone (Q**): Qualigen.

Table 3. Plackett-Burman design for the screening of fifteen mineral salts (0.05 mg/ml) N = 16.

$CaCl_2$	$CaCO_3$	K_2HPO_4	KH_2PO_4	$MnCl_2$	NaCl	KCl	Na_2HPO_4	Na_2SO_4	$MgCl_2$	$MnSO_4$	$NaHPO_4$	$MgSO_4$	Fe $(NH_4)SO_4$	$ZnSO_4$	Yield (IU/ml)
0.05	0.05	0.05	0.05	0	0.05	0	0.05	0.05	0	0	0.05	0	0	0	36.25
0.05	0.05	0.05	0	0.05	0	0.05	0.05	0	0	0.05	0	0	0	0.01	31.25
0.05	0.05	0	0.05	0	0.05	0.05	0	0	0.05	0	0	0	0.05	0.01	21.25
0.05	0	0.05	0	0.05	0.05	0	0	0.05	0	0	0	0.05	0.05	0.01	18.75
0	0.05	0	0.05	0.05	0	0	0.05	0	0	0	0.05	0.05	0.05	0.01	26.25
0.05	0	0.05	0.05	0	0	0.05	0	0	0	0.05	0.05	0.05	0.05	0	26.25
0	0.05	0.05	0	0	0.05	0	0	0	0.05	0.05	0.05	0.05	0	0.01	32.5
0.05	0.05	0	0	0.05	0	0	0	0.05	0.05	0.05	0.05	0	0.05	0	25.00
0.05	0	0	0.05	0	0	0	0.05	0.05	0.05	0.05	0	0.05	0	0.01	25.00
0	0	0.05	0	0	0	0.05	0.05	0.05	0.05	0	0.05	0	0.05	0.01	25.00
0	0.05	0	0	0	0.05	0.05	0.05	0.05	0	0.05	0	0.05	0.05	0	21.25
0.05	0	0	0	0.05	0.05	0.05	0.05	0	0.05	0	0.05	0.05	0	0	23.75
0	0	0	0.05	0.05	0.05	0.05	0	0.05	0	0.05	0.05	0	0	0.01	22.75
0	0	0.05	0.05	0.05	0.05	0	0.05	0	0.05	0.05	0	0	0.05	0	21.25
0	0.05	0.05	0.05	0.05	0	0.05	0	0.05	0.05	0	0	0.05	0	0	26.65
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	30.25

Table 4. PBD for the screening of eleven inorganic nitrogen sources (0.4 mg/ml) N = 12.

Ing. No. of expts.	$NH_4H_2PO_4$	$(NH_4)_2HPO_4$	NH_4Cl	NH_4NO_3	Amm. oxalate	Amm. acetate	Amm. sulphate	$NaNO_3$	KNO_3	Ferric amm. sulfate	Amm. Hydroxide	Yield (IU/ml)
1	0.4	0.4	0	0.4	0.4	0.4	0	0	0	0.4	0	32.25
2	0.4	0	0.4	0.4	0.4	0	0	0	0.4	0	0.4	27.05
3	0	0.4	0.4	0.4	0	0	0	0.4	0	0.4	0.4	27.05
4	0.4	0.4	0.4	0	0	0	0.4	0	0.4	0.4	0	26.25
5	0.4	0.4	0	0	0	0.4	0	0.4	0.4	0	0.4	21.25
6	0.4	0	0	0	0.4	0	0.4	0.4	0	0.4	0.4	37.25
7	0	0	0	0.4	0	0.4	0.4	0	0.4	0.4	0.4	31.25
8	0	0	0.4	0	0.4	0.4	0	0.4	0.4	0.4	0	26.25
9	0	0.4	0	0.4	0.4	0	0.4	0.4	0.4	0	0	27.05
10	0.4	0	0.4	0.4	0	0.4	0.4	0.4	0	0	0	31.05
11	0	0.4	0.4	0	0.4	0.4	0.4	0	0	0	0.4	32.25
12	0	0	0	0	0	0	0	0	0	0	0	30.25

Table 5. PBD for the screening of eleven different amino acids (0.1 /ml) N = 12.

Ing. No.	D-Alanine	L-Cystine	L-Alanine	L-Cysteine	L-Lysine	L-Asparagine	D-Arginine	L-Arginine	L-Histidine	L-Glutamic acid	L-Tryptophan	Yield (IU/ml)
1	1	1	0	1	1	1	0	0	0	1	0	21.25
2	1	0	1	1	1	0	0	0	1	0	1	24.25
3	0	1	1	1	0	0	0	1	0	1	1	22.25
4	1	1	1	0	0	0	1	0	1	1	0	23.75
5	1	1	0	0	0	1	0	1	1	0	1	31.25
6	1	0	0	0	1	0	1	1	0	1	1	56.75
7	0	0	0	1	0	1	1	0	1	1	1	36.25
8	0	0	1	0	1	1	0	1	1	1	0	34.25
9	0	1	0	1	1	0	1	1	1	0	0	32.25
10	1	0	1	1	0	1	1	1	0	0	0	39.25
11	0	1	1	0	1	1	1	0	0	0	1	32.25
12	0	0	0	0	0	0	0	0	0	0	0	30.25

medium components, metabolism of cells and the large number of chemical requirements for processing metabolic products, do not allow satisfactory detailed modeling. Single variable optimization methods are not only tedious, but can also lead to misinterpretation of results, especially taking into account that the interaction between different factors is overlooked (Abdel-Fattah et al., 2005). Statistical experimental designs have been used for many decades and can be adopted on several steps of an optimization strategy, such as for screening experiments or searching for the optimal conditions of a targeted response (Kim et al., 2005; Lee and Gilmore, 2005; Nawani and Kapadnis, 2005; Senthilkumar et al., 2005; Wang and Lu, 2005).

No defined medium has been established for the optimum production of L-asparaginase from different microbial sources. Each organism has its own special conditions for maximum enzyme production. A statistical approach has been employed in the present study for which a Plackett-Burman design is used for identifying significant variables influencing L-asparaginase production by *Bacillus cereus* MNTG-7. The levels of the significant variables were further optimized using response surface methodology.

The most effective nutrient from each category was identified based on the result obtained after subjecting the data to statistical analysis. The nutrients were ranked on the basis of regression coefficient values or t-values obtained from the analysis of the experimental results. The nutrient with the highest regression co-efficient or t-value was considered as effective in L-asparaginase production.

The results of enzyme productivity are shown in Tables 1 - 5. They indicate that in each of the five categories, there was a significant increase in the yield of L-asparaginase when compared to the control. Among the carbon sources, tapioca starch has the highest contribution (15.42%) with positive coefficient (6.390), where as fructose, mannitol, maltose and sucrose showed larger positive effects on the yields and the other carbon sources was insignificant which are shown in Table 6. Tapioca starch was found to be the best carbon source among 15 carbon sources.

Among the organic nitrogen sources, gelatin has the highest contribution (20.30%) with positive coefficient of 3.50. Hence gelatin is found to be the best nitrogen source among 15 (Table 7).

Among the different salts, $CaCO_3$ has the highest contribution (14.1%) with positive coefficient Hence $CaCO_3$ was found to be the best one among the 15 minerals (Tables 1- 8). Among the inorganic nitrogen sources, ammonium oxalate has the highest contribution (19.702%) with positive coefficient of 1.75 whereas ammonium sulphate with contribution of 10.05% and with a positive coefficient of 1.25 was considered as the best (Table 9). Among the amino acids L- asparagine has the highest contribution (26.2%) with positive coefficient of 4.75. Hence L-asparagine was selected as the best among 11 amino acids (Table 10).

Table 6. Effect of carbon sources on L-asparaginase production.

S. no.	Carbon source	Coefficient	SS%
1	Dextrose	-2.98	3.36
2	Lactose	-4.7	8.35
3	Dextrin white	3.42	4.42
4	Potato starch	-3.30	4.11
5	Starch soluble	-6.42	15.58
6	Starch maize	-0.95	0.34
7	Tapioca starch	6.39	15.42
8	Pharma starch	2.64	2.63
9	Maltose	4.20	6.67
10	Mannitol	4.67	8.24
11	Xylose	-4.39	7.28
12	Fructose	5.30	10.60
13	Galactose	-2.52	2.39
14	Sucrose	4.83	8.80
15	Cellulose	2.17	1.78

Table 7. Effect of organic/complex nitrogen sources on L-asparaginase production.

S. no.	Organic/complex nitrogen source	Coefficient	SS%
1	Soyabean meal	-0.36	0.22
2	Tryptone	-1.52	3.80
3	Gelatin	3.50	20.30
4	Yeast extract	2.82	13.40
5	Soya peptone	0.02	0.0004
6	Yeast nitrogen base	-2.6	11.10
7	Casein	2.52	10.60
8	Meat extract	-1.60	4.33
9	Peptone (H.M)	-0.30	0.14
10	Casaminoacids	-2.45	10.50
11	Malt extract	1.49	3.68
12	Beef extract	0.55	0.50
13	Urea	-0.58	0.56
14	Albumin	-0.67	0.75
15	Peptone (Q)	-3.52	20.64

Plackett-Burman design offers a good and fast screening procedure and mathematically computes the significance of a large number of factors in each experiment, which is time saving and maintains convincing control on each component.

The optimization of the medium components showing the highest regression coefficients viz., Tapioca starch, L-asparagine, ammonium oxalate, gelatin and $CaCO_3$ was

Table 8. Effect of minerals /salts on L-asparaginase production.

S. no.	Mineral/salt	Coefficient	SS%
1	$CaCl_2$	0.10	0.04
2	$CaCO_3$	1.71	14.10
3	K_2HPO_4	1.40	9.50
4	KH_2PO_4	-0.13	0.08
5	$MnCl_2$	-1.40	9.20
6	Na Cl	-1.11	6.00
7	KCl	-1.07	5.50
8	Na_2HPO_4	0.41	0.82
9	Na_2SO_4	-0.76	2.80
10	$MgCl_2$	-0.79	3.00
11	$MnSO_4$	-0.18	0.15
12	Na_2HPO_4	1.38	9.20
13	$MgSO_4$	-0.79	3.00
14	$Fe(NH_4)SO_4$	-2.71	35.40
15	$ZnSO_4$	-0.49	1.18

Table 9. Effect of inorganic nitrogen sources on L-asparaginase production.

S. no.	Inorganic nitrogen source	Coefficient	SS%
1	$NH_4H_2PO_4$	0.0833	0.0447
2	$(NH_4)_2HPO_4$	-1.4167	12.914
3	NH_4Cl	-0.7833	3.9484
4	NH_4NO_3	0.1833	0.2163
5	Amm. sulphate	1.25	10.0542
6	Amm. acetate	-0.05	0.0161
7	Amm. oxalate	1.75	19.7062
8	$NaNO_3$	-0.7833	3.9484
9	KNO_3	-2.5833	42.9424
10	Ferric amm. sulfate	0.95	5.8073
11	Amm. Hydroxide	0.25	0.4022

Table 10. Effect of amino acids on L-asparaginase production.

S. no.	Amino acid	Coefficient	SS%
1	D-Alanine	0.75	0.6549
2	L-Cystine	-4.8333	27.197
3	L-Alanine	-2.6667	8.2788
4	L-Cysteine	-2.75	8.8043
5	L-Lysine	1.5	2.6195
6	D-Arginine	0.4167	0.2021
7	L-Asparagine	4.75	26.2673
8	L-Arginine	4.0	18.6272
9	L-Histidine	-1.6667	3.2339
10	L-Glutamic acid	0.4167	0.2021
11	L-Tryptophan	1.8333	3.913

carried out using the response surface methodology (RSM).

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