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RESEARCH ARTICLE

Screening of Medium Components for The Enhanced Production of *Pseudomonas fluorescens* L-Glutaminase by Plackett Burman Design

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Abstract

In this present investigation medium components influencing the production of L-Glutaminase were screened by using Plackett Burman Design. Total 33 different media components comprising of 11 carbon sources, 11 nitrogen sources and 11 mineral sources were screened for enhanced production of L-Glutaminase in submerged fermentation. The statistical analysis viz. regression coefficient and ANNOVA were used to identify the most significant factors among the selected one. A mathematical model was developed to predict the relative influence of different factors. Among 33 different nutrients Gelatin, Tryptone, Starch potato and KCl were found to be most effective media constituents.

INTRODUCTION

L-Glutaminase (L-Glutamine amidohydrolase E.C 3.5.1.2) is a hydrolytic enzyme that deaminates the L-glutamine to L-glutamic acid and ammonia. L-Glutaminase has an essential role in the metabolism of nitrogen at the cellular level. (Brosnan et al. 1995, Carter and Welboume, 1997, Padma and Simghal, 2007, Riberg et al., 1995. It has proved to be a potent anti-leukemic agent, thereby aiding patients against cancer. The principle behind the use of L-glutaminase as an anti-cancer agent is that it causes selective death of tumour cell by depriving it from L-glutamine and thus prohibits malignancy by nutritionally depriving the tumour cells (Roberts et al., 2001, Zhao et al., 2004). It has therapeutic properties even against the HIV. L-Glutaminase enhances the flavour of fermented foods by increasing their glutamic acid content. Food industry also incorporates L-glutaminase for the purpose of being an excellent food preservative and flavouring agent. Furthermore, it has replaced mono-sodium glutamate, which is considered to be allergic in nature (Sabu, 2000). It also aids in the production of important chemicals like threonine through gamma glutamyltransfer (Tachiki et al., 1998). Thus, due to these characteristics of L-glutaminase it has attracted both commercial as well research sectors to find out different feasible sources for production in large scale.
The present studies focus on the production of this enzyme by using *Pseudomonas fluorescens*. The statistical modeling is used to screen the significant nutritional factors which can enhance the enzyme production. Economic feasibility is one of the most important factors to be considered during enzyme production. A well proven statistical screening ensures better production. Single factor screening at a time is tedious, time consuming and erroneous. Since the interactions between the factors were ignored, hence misinterpretation of results may take place so statistical designing using Plackett Burman Design (PBD) was used to ensure both qualitative and quantitative studies of constituents effecting enzyme production (Plackett and Burman, 1946, Yugandhar et al., 2008, Cavalitto and Mignone, 2007).

MATERIALS AND METHOD

Microorganism

The Isolate of *Pseudomonas fluorecens* (MTCC 103) was used to study the production of L-glutaminase. The culture was maintained at 37ºC on nutrient agar slants for 24 hours and sub-cultured periodically. Parent broth was

prepared by inoculating the colonies in nutrient broth in a 250 ml flask for 18 hours in shake flask. The parent medium was the sterile nutrient broth.

Shake flask fermentation

The freshly grown culture of *Pseudomonas fluorecens* cells was used as inoculum. 1 ml of inoculum was added in the 250 ml flask containing 50 ml of sterile nutrient broth supplemented with 0.1 % (w/v) L-glutamine. The fermentation medium was agitated at 180 RPM at 37 °C for 36 hours in a orbital shaker. The concentration of medium components in each flask was taken according to the Plackett Burman design. To screen the significant nutritional factors, total 33 flasks were prepared with each of 11 carbon sources, 11 nitrogen sources and 11 mineral sources.

Estimation of enzyme activity

L-Glutaminase activity in the fermentation broth was determined by the L-Glutaminase (E.C.3.5.1.2) assay method of Imada *et al.* 1974 by nesslerization. One unit of L-Glutaminase is defined as the amount of enzyme which liberates 1µmole of ammonia in 1 min at 37^{o} C.

Statistical design

The Plackett Burman design was used as the bio-statistical tool to screen various nutritional sources like carbon, nitrogen and mineral salts. Eleven components from various sources i.e. carbon, nitrogen and minerals were taken for the production of L-Glutaminase by submerged fermentation. Plackett Burman design uses a statistical method consisting of orthogonal matrix. Regression analysis is used to analyse the data. Screening of N-1 variable in N experiments is done here. Nutrients are taken at a single level. Their presence or absence determines the positive or negative effect of the constituent in the enzyme production. Based on the published data, empirical concentration was fixed. Preparation of media, the process of fermentation and enzyme assay was done as described in shake flask fermentation. The tables 1, 2 and 3 illustrate the different designs generated for various nitrogen sources, carbon sources and mineral salts respectively.

The cultures were inoculated in different type of media compositions (as described in the above mentioned tables) and fermentation was carried out. After that centrifugation at 10,000 RPM for 10 minutes at 4^{0}C was done, this was followed by determination of enzyme activity by nesslerization.

RESULTS AND DISCUSSION

Table 1: Design for various nitrogen sources (concentration=1g/L)

Trial No	A	B	C	D	E	F	G	H	I	J	K
1.	0	0	1	0	1	1	0	1	1	1	0
2.	0	0	0	1	0	1	1	0	1	1	1
3.	1	0	0	0	1	0	1	1	0	1	1
4.	1	0	1	1	1	0	0	0	1	0	1
5.	1	1	1	0	0	0	1	0	1	1	0
6.	1	1	0	0	0	1	0	1	1	0	1
7.	0	0	0	0	0	0	0	0	0	0	0
8.	1	0	1	1	0	1	1	1	0	0	0
9.	0	1	0	1	1	0	1	1	1	0	0
10.	1	1	0	1	1	1	0	0	0	1	0
11.	0	1	1	1	0	0	0	1	0	1	1
12.	0	1	1	0	1	1	1	0	0	0	1

A:Tryptone, **B**; Yeast Extract, **C**: Casein, **D**: Meat Extract, **E**: Malt Extract, **F**: Beef extract, **G**: Urea, **H**: Peptone, **I**: Gelatin, **J**: Soybean, **K**: Glycine

Table 2: Design for various carbon sources (concentarion= 0.20 g/L)

Trial No.	A	B	C	D	E	F	G	H	I	J	K
1.	0.20	0.20	0.00	0.00	0.00	0.20	0.00	0.20	0.20	0.00	0.20
2.	0.20	0.00	0.00	0.00	0.20	0.00	0.20	0.20	0.00	0.20	0.20
3.	0.00	0.20	0.20	0.20	0.00	0.00	0.00	0.20	0.00	0.20	0.20
4.	0.20	0.00	0.20	0.20	0.00	0.20	0.20	0.00	0.00	0.00	0.00
5.	0.00	0.00	0.20	0.00	0.20	0.20	0.00	0.20	0.20	0.20	0.00
6.	0.00	0.20	0.00	0.20	0.20	0.00	0.20	0.00	0.20	0.00	0.00
7.	0.20	0.20	0.00	0.20	0.20	0.20	0.00	0.00	0.00	0.20	0.00
8.	0.00	0.20	0.20	0.00	0.20	0.20	0.20	0.00	0.00	0.00	0.20
9.	0.00	0.00	0.00	0.20	0.00	0.20	0.20	0.20	0.20	0.20	0.20
10.	0.20	0.20	0.20	0.00	0.00	0.00	0.20	0.00	0.20	0.20	0.00
11.	0.20	0.00	0.00	0.00	0.20	0.00	0.00	0.20	0.00	0.00	0.00
12.	0.20	0.00	0.20	0.20	0.20	0.00	0.00	0.00	0.20	0.00	0.20

A:Glucose, **B**:Lactose, **C**:Starch Potato, **D**:Starch Soluble, **E**:Maltose, **F**:Mannitol, **G**:Sucrose, **H**:xylose, **I**:Galactose, **J**:Mannose, **K**:Fructose

Table 3: Design for various Mineral Salts (concentarion= 0.20g/L)

Trial No.	A	B	C	D	E	F	G	H	I	J	K
1.	0.20	0.20	0.00	0.20	0.20	0.20	0.00	0.00	0.00	0.20	0.00
2.	0.20	0.20	0.20	0.00	0.00	0.00	0.20	0.00	0.20	0.20	0.00
3.	0.20	0.00	0.20	0.20	0.20	0.00	0.00	0.00	0.20	0.00	0.20
4.	0.00	0.00	0.20	0.00	0.20	0.20	0.00	0.20	0.20	0.20	0.00
5.	0.20	0.00	0.00	0.00	0.20	0.00	0.20	0.20	0.00	0.20	0.20
6.	0.00	0.20	0.20	0.20	0.00	0.00	0.00	0.20	0.00	0.20	0.20
7.	0.00	0.20	0.00	0.20	0.20	0.00	0.20	0.20	0.20	0.00	0.00
8.	0.00	0.20	0.20	0.00	0.20	0.20	0.20	0.00	0.00	0.00	0.20
9.	0.20	0.00	0.20	0.20	0.00	0.20	0.20	0.20	0.00	0.00	0.00
10.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
11.	0.20	0.20	0.00	0.00	0.00	0.20	0.00	0.20	0.20	0.00	0.20
12.	0.00	0.00	0.00	0.20	0.00	0.20	0.20	0.00	0.20	0.20	0.20

A:$Na_2HPO_4.2H_20$, **B**:KH_2PO4, **C**:NaCl, **D**:$MgSO_4.H_2O$, **E**:$CaCl_2.2H_2O$, **F**:K_2HPO_4, **G**:NaH_2PO_4, **H**:$FeSO_4.7H_2O$, **I**:$ZnSO_4.7H_2O$, **J**:$MgCl_2$, **K**:KCl

The resultant activity for all the designs (designs described in Tables 1, 2 and 3 respectively) are shown in Tables 4, 5 and 6 respectively after determination of enzyme activity by nesslerization.

Table 4: Enzyme activities of different trial conditions from design of various nitrogen sources

Trial No.	IU/ml
1	39.5
2	42
3	24.25
4	24
5	4
6	3.5
7	10.12
8	8
9	46.5
10	25.375
11	11.5

12	52

Table 5: Enzyme activities of different trial conditions from design of various carbon sources

Trial No.	**IU/ml**
1	45.0
2	39.2
3	43.6
4	47.0
5	39.9
6	42.1
7	41.0
8	43.0
9	43.2
10	44.0
11	42.4
12	39.8

Table 6: Enzyme activities of different trial conditions from design of various Mineral salts

Trial No.	**IU/ml**
1	48.0
2	10.0
3	35.6
4	45.0
5	14.0
6	42.0
7	60.0
8	37.0
9	20.7
10	44.9
11	12.0
12	56.0

The enzyme activity of all the runs were taken individually and then the result was analyzed. From the analysis of the result it was found that the gelatin ave the maximum contribution of 25.4% followed by tryptone (16.56%).Urea and yeast extract gave an appreciable contribution of 14.29% and13.17% respectively (Fig-1).

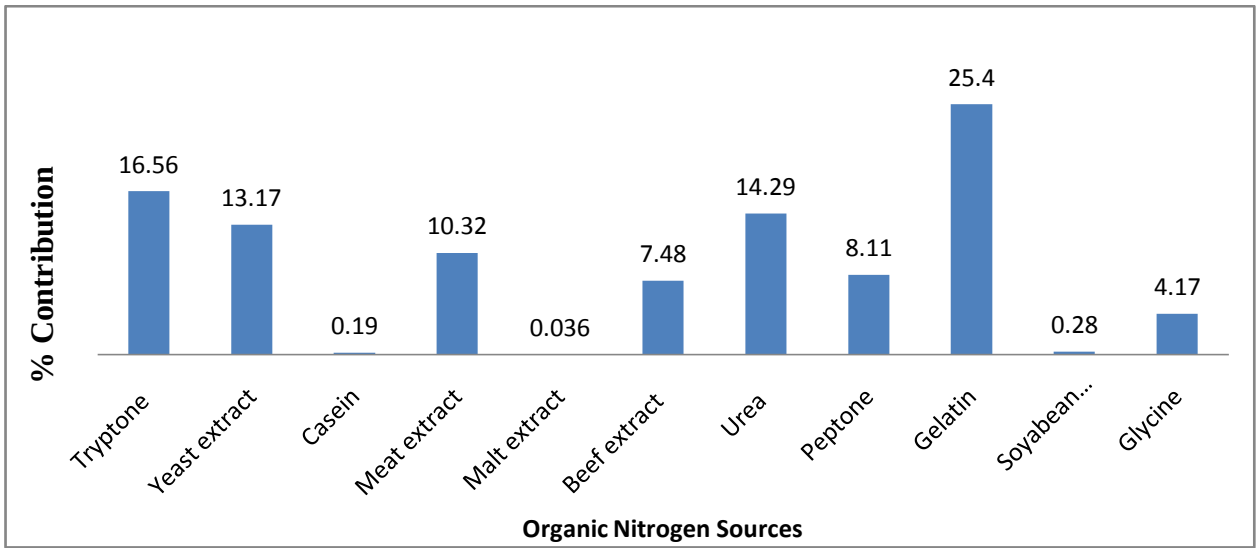

Figure 1: Contribution of various organic nitrogen sources.

Similarly starch potato (24.36%) gave the maximum contribution as a carbon source for production of enzyme L-Glutaminase. Lactose with contribution of 21.36% was the second best carbon source. Mannose was the third best. Maltose and mannitol had almost same contribution. Starch soluble and galactose are not appreciable choice for L-Glutaminase production because they participated only 0.21% and 0.56% to the enzyme production (Fig-2).

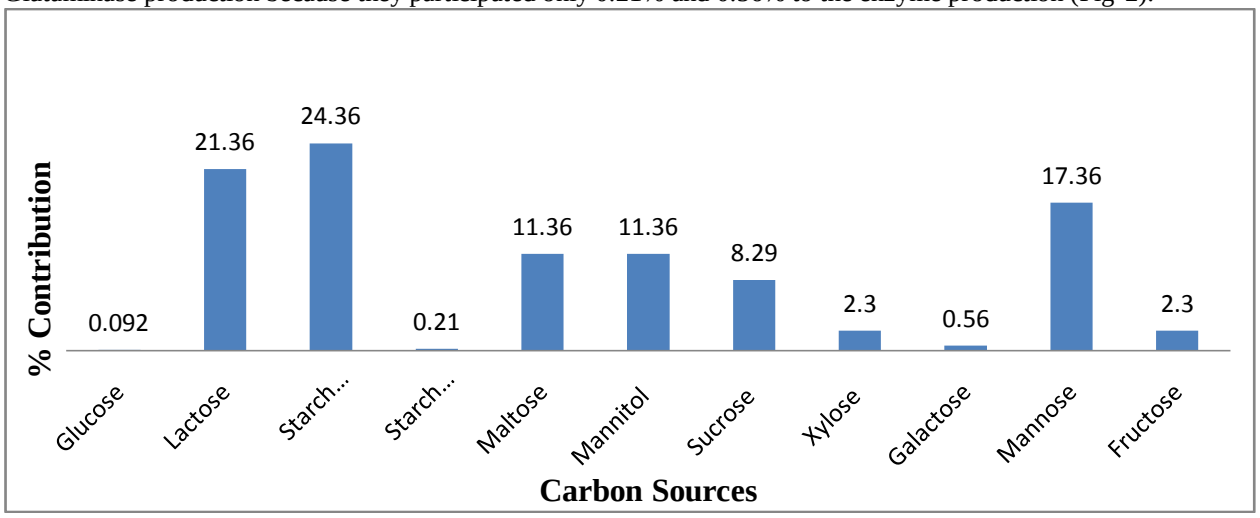

Figure 2: Contribution of various organic carbon sources.

Mineral source being an equally important factor was also screened. KCl was found as an excellent source as it contributed 22.14% during enzyme production. $MgSO_4.H_2O$ was second best with a contribution of 17.82% (Fig-3).

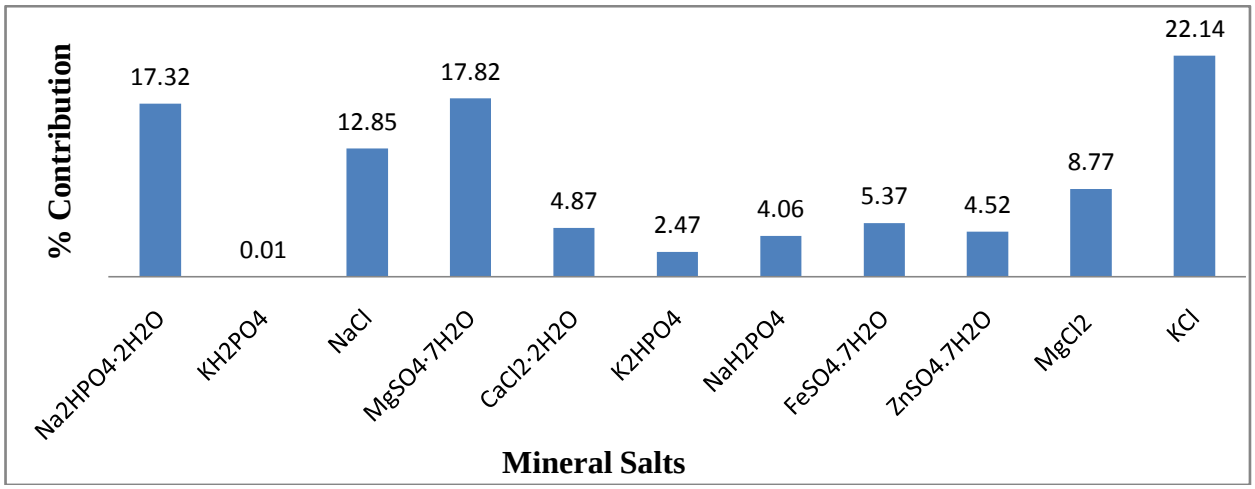

Figure 3: Contribution of various organic carbon sources.

The percentage contribution data of various factors were used to design the mathematical equation to formulate the simultaneous effect of selected factors. The mathematical equations obtained for different statistical models regarding nitrogen sources, carbon sources and mineral salts are given below respectively:

NITROGEN SOURCE

$$R1 = 8.1667 + 13.458 \times \text{Tryptone} + 12.000 \times \text{Yeast Extract} + 10.625 \times \text{Meat Extract} + 9.04 \times \text{Breaf Extract} + 12.5000 \times \text{Urea} + 9.416 \times \text{Peptone} - 16.666 \times \text{Gelatin} + 6.75000 \times \text{Glycine}$$

CARBON SOURCE

$$R1 = 447.0 - 5.08333 \times \text{Lactose} - 5.41667 \times \text{Starch Potato} - 3.75000 \times \text{Maltose} - 3.75000 \times \text{Mannitol} - 3.1667 \times \text{Sucrose} + 1.66667 \times \text{Xylose} - 4.58333 \times \text{Mannose} + 1.6667 \times \text{Fructose}$$

MINERAL SOURCE

$$R1 = 20.53333 + 66.8333\text{Na2HPO4.2H20} + 58.833 \times \text{NaCl} + 69.8333 \times \text{MgSO4.7H2O} + 36.50000 \times \text{CaCl2.2H2O} - 26.00 \times \text{K2HPO4} - 33.333 \times \text{NaH2PO4} + 38.333 \times \text{FeSO4.7H2O} - 35.1666 \times \text{ZnSO4.7H2O} + 49.000 \times \text{MgCl2} - 77.8333\text{KCl}$$

The ANOVA analysis of nitrogen source (Table 7) gives P value less than 0.005 which means that the design was validated. Gelatin gives the best result as its P value is minimum i.e. 0.0012 and F value maximum i.e. 149.35. Tryptone is the second best nitrogen source with P Value 0.0022 and F value 97.38. Followed by urea, yeast extract and meat extract as the best nitrogen source.

Table 7: ANOVA analysis for different nitrogen sources

SOURCE	SUM OF SQUARES	DEGREE OF FREEDOM	MEAN SQUARE	F VALUE	P VALUE
Model	3264.10	8	408.01	73.12	0.0024
A-Tryptone	543.38	1	543.38	97.38	0.0022
B-Yeast extract	432.00	1	432.00	77.42	0.0031
D-Meat extract	338.67	1	338.67	60.70	0.0044
F-Beef extract	245.26	1	245.26	43.95	0.0070
G-Urea	468.75	1	468.75	84.01	0.0027
H-peptone	266.02	1	266.02	47.68	0.0062
J-Gelatin	833.33	1	833.33	149.35	0.0012
L-Glycine	136.69	1	136.69	24.50	0.0158
Residual	16.74	3	5.58		
COR Total	3280.84	11			
Standard deviation			2.36		

R squared	0.9949
Mean	24.23
Adj R Squared	0.9813
Pred Squared	0.9184
Adeq precision	23.871
C.V%	9.75
PRESS	267.83

The coefficient value (Table 8) again validates the authenticity of the result as the coefficient value for gelatin is 16.66 and F value is 73.12.

Table 8: Coefficient Values for different nitrogen sources

Source	**Coefficient**
Tryptone	13.458
Yeast extract	12.00
Meat extract	10.625
Beef extract	9.04
Urea	12.500
Peptone	9.416
Gelatin	16.666
Glycine	6.75

Similarly the ANOVA analysis of carbon sources (Table 9) states that Starch potato is the best carbon source for the production of enzyme since its P value is less than 0.005 followed by mannose and lactose 0.0045 and 0.0053 respectively. Rest of the carbon sources like maltose, sucrose and fructose were not that good as a carbon source.

Table 9: ANOVA analysis for different carbon sources

SOURCE	**SUM OF SQUARES**	**DEGREE OF FREEDOM**	**MEAN SQUARE**	**F VALUE**	**P VALUE**
Model	57.55	8	7.19	42.59	0.0053
B –Lactose	12.40	1	12.40	73.44	0.0053
C-Starch potato	14.08	1	14.08	83.39	0.0028
E-Maltose	6.75	1	6.75	39.97	0.0080
F-Mannitol	6.75	1	6.75	39.97	0.0080
G-Sucrose	4.81	1	4.81	28.50	0.0128
H-Xylose	1.33	1	1.33	7.89	0.0673
K-Mannose	10.08	1	10.08	59.70	0.0045
L-Fructose	1.33	1	1.33	7.89	0.0673
Residual	0.51	3			
COR total	58.06	11			
Standard deviation			0.41		
R squared			0.9913		
Mean			42.52		
Adj R Squared			0.9680		
Pred Squared			0.8604		
Adeq precision			21.916		
C.V%			0.97		

The coefficient values (Table 10) of starch potato, lactose and mannose of 5.41667, 5.08333 and 4.58333 respectively further proves them to be a good carbon source.

Table 10: Coefficient Values for different carbon sources

Source	**Coefficient**
Lactose	5.08333
Starch potato	5.41667
Maltose	3.75000

Mannitol	3.75000
Sucrose	3.1667
Xylose	1.66667
Mannose	4.58333
Fructose	1.66667

After the ANOVA analysis of mineral salts (Table 11), the best mineral salt came out to be KCl because it had the minimum P value and maximum F value followed by $MgSO_4.7H_2O$ & $Na_2HPO_4.2H_20$.

Table 11: ANOVA analysis for different mineral salts

SOURCE	SUM OF SQUARES	DEGREE OF FREEDOM	MEAN SQUARE	F VALUE	P VALUE
Model	3283.27	10	328.33	984.98	0.0248
$Na_2HPO_4.2H_20$	568.56	1	568.56	1705.69	0.0154
NaCl	415.36	1	415.36	1246.09	0.0180
$MgSO_4.7H_2O$	585.20	1	585.20	1755.61	0.0152
$CaCl_2.2H_2O$	159.87	1	159.87	479.61	0.0290
K_2HPO_4	81.12	1	81.12	243.36	0.0408
NaH_2PO_4	133.33	1	133.33	400.00	0.0318
$FeSO_4.7H_2O$	176.33	1	176.33	529.00	0.0277
$ZnSO_4.7H_2O$	148.40	1	148.40	445.21	0.0301
$MgCl_2$	288.12	1	288.12	864.36	0.0261
KCl	726.96	1	726.96	2180.89	0.0136
Residual	0.33	1	0.33		
Cor Total	3283.61	11			
Standard deviation			0.58		
R squared			0.9999		
Mean			35.43		
Adj R Squared			0.9909		
Pred Squared			0.9854		
Adeq precision			91.056		
C.V%			1.63		
PRESS			48.00		

The coefficient value (from Table 12) of KCl is the best which comes out to be 77.8. Hence KCl was chosen as the best mineral source for production of L-Glutaminase enzyme.

Table 12: Coefficient Values of different mineral salts

Minerals	Coefficient
$Na_2HPO4.2H2O$	66.833
NaCl	58.833
MgSO4.7H2O	69.83333
CaCl2.2H2O	36.50000
K2HPO4	26.00
NaH2PO4	33.333
FeSO4.7H2O	38.333
ZnSO4.7H2O	35.16666
MgCl2	49.0000
KCl	77.8333

CONCLUSION

Based on the statistical analysis of various carbon, nitrogen and mineral sources, the best sources were chosen for the production of enzyme L-Glutaminase. Gelatin and tryptone were the best nitrogen sources, Starch potato was the

best carbon source and KCl was the best mineral source. The screened sources can be taken as a reference for production of L-Glutaminase from *Pseudomonas fluorescens*. Further optimization can be done based on the above results. Plackett Burman computes the screening speedily and accurately. Mathematical significance of various factors validates its uses on commercial level.

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