Indian Journal of Science and Technology Vol.2 No. 12 (Dec. 2009) ISSN: 0974- 6846

Application of latin square design for the evaluation and screening of supplementary nitrogen source for L-asparaginase production by *Aspergillus terreus* MTCC 1782

G. Baskar and S.Renganathan[1]
Department of Biotechnology, St. Joseph's College of Engineering, Chennai-600 119, India.
[1]Department of Chemical Engineering, Alagappa College of Technology, Anna University, Chennai - 600 025, India.
basg2004@gmail.com; rengsah@rediffmail.com

Abstract: The effect of ammonium chloride, urea and sodium nitrate as supplementary nitrogen source on the production of extracellular L-asparaginase using *Aspergillus terreus* MTCC 1782 was investigated using 5-level Latin square design. The statistical reliability and significance of the variables was studied by performing ANOVA for experimental L-asparaginase activity using Dataplot software. Among the supplementary nitrogen sources studied, ammonium chloride was found to be the best for maximum L-asparaginase activity (confidence level of 96.61%) with less biomass formation (confidence level of 21.93%). The maximum L-asparaginase activity obtained was of 26.47 IU/mL by *A. terreus* MTCC 1782 using groundnut oil cake powder as natural substrate in submerged fermentation.

Keywords: *Aspergillus terreus,* L-asparaginase, latin square design.

Introduction

L-asparaginase (L-asparagine amino hydrolase, E.C.3.5.1.1) catalyzes the hydrolysis of L-asparagine into L-aspartic acid and ammonia. This has been a clinically acceptable anti tumor agent for the effective treatment of acute lymphoblastic leukemia and lymphosarcoma. L-asparagine is an essential amino acid for the growth of tumor cells, whereas the growth of normal cells is not dependent on its requirement as it can be synthesized in amounts sufficient for their metabolic needs using their own enzyme L-asparagine synthetase. The presence of L-asparaginase deprives the tumor cells of an important growth factor and they fail to survive (Broome, 1961; Broome, 1965; Berenbaum *et al*., 1970). Recombinant L-asparaginase of *A. niger* and *A. oryzae* used in processing of starchy food products. It converts the amino acid asparagine to aspartic acid then reduces acrylamide formation during processing of high starch food products (FAO/WHO, 2001; Pedreschi *et al*., 2008). The demand for this enzyme is expected to increase several fold in coming years due to its potential industrial application as food processing aid besides clinical applications.

L-asparaginase produced by several bacterial sources leads to adverse side effects in human trials. Therefore, there is a search for the other sour ces for L-asparaginase production with less adverse effects. It has been observed that eukaryotic microorganisms like yeast and filamentous fungi are potential sources for L-asparaginase production. These studies suggest L-asparaginase production by filamentous fungi is under nitrogen regulation (Sarquis *et al*., 2004). The *A. nidulans* (Shaffer *et al*., 1988), *A. terreus* (Ali *et al*., 1994; Sarquis *et al.,* 2004) using synthetic substrates and *A. niger* (Mishra, 2006) using agro-wastes from three leguminous crops were reported to produce L-asparaginase which was not toxic and appeared to have myelosuppressive and immunosuppressive activity. Mesophilic fungus *Cylindrocarpon obtusisporum* MB-10 was also reported to produce intracellular L-asparaginase (Raha *et al*., 1990), which was very specific for L-asparagine and did not hydrolyse D-asparagine or L-glutamine. However, only few researchers studied the production of L-asparaginase using naturally available cheaper substrate. It also has been observed from our preliminary study that *A. terreus* MTCC 1782 was found to be a potential fungal source for L-asparaginase and used for further investigation in this study.

Statistical experimental designs have been used in several steps of optimization strategy and it is better acknowledged than traditional one variable at a time method (Kwak *et al*., 2006; Zheng *et al*., 2008). Statistical experimental design such as Latin Square Design (LSD) minimizes the error in determining the effect of parameters, which allows simultaneous, systematic, and efficient variation of all parameters than classical method. LSD was first used in agricultural research to adjust for fertility differences in two physical directions (Box *et al*., 1978; Torbjorn *et al*., 1998).

Identifying a low cost substrate is important to develop an economically viable bioprocess for any product. Groundnut oil cake is used as animal feed, was obtained as waste after extraction oil form groundnut. In the present work the groundnut oil cake powder (particle size of 80/120 mesh) was used as alternate substrate to synthetic L-proline. LSD was used to evaluate and compare the effect of urea, ammonium chloride and sodium nitrate as independent supplementary nitrogen source on production of extracellular L-asparaginase by *A. terreus* MTCC 1782 in submerged fermentation using groundnut oil cake powder as substrate.

Materials and methods

Fungal strain

The filamentous fungi *A. terreus* MTCC 1782 was obtained from Institute of Microbial Technology, Chandigarh, India. The spores and mycelial fragments of this fungus was cultivated in Czapek agar slants at 37°C for 4 days, stored at 4°C and periodically subcultured.

Indian Journal of Science and Technology Vol.2 No. 12 (Dec. 2009) ISSN: 0974- 6846

Ground nut oil cake powder

The ground nut oil cake used in this work was purchased from local market (Chennai, India), dried overnight at 65°C to remove moisture if any, powdered and sieved. The ground nut oil cake powder passed through 80 and retained by 120 meshes was used as natural substrate for production of L-asparaginase.

Inoculum culture

Inoculum culture was cultivated in modified Czapek agar slants with the following ingredients in g. Solution-A: L-asparagine, 1.0; $NaNO_3$, 4.0; KCl, 1.0; $MgSO_4.7H_2O$, 0.052; $FeSO_4.7H_2O$, 0.02; were dissolved in 100 mL of distilled water and stored in refrigerator. Solution-B: K_2HPO_4 2.0; was dissolved in 100 mL of distilled water and stored in refrigerator. Solution-C: $ZnSO_4$. 7 H_2O 1.0; was dissolved in 100 mL of distilled water. Solution-D: 0.5 gram of $CuSO_4$. H_2O 0.5; was dissolved in 100 mL of distilled water. For one litre of modified Czapek-Dox was prepared using 50 mL of solution-A, 50 mL of solution-B, 1 mL of solution-C, solution-D, 30 g of glucose and 20 g of agar mixed in 900 mL distilled water. The inoculum culture slants were incubated at 37°C for 4 days. Inoculum culture as conidial suspensions was prepared with concentrations of 10^7 to 10^8 conidia per mL.

Production and isolation of crude enzyme

Based on LSD experiment design, 100 mL of liquid Czapek-Dox media modified with different supplementary nitrogen source (Sodium nitrate, Urea and Ammonium chloride), 2% groundnut oil cake powder, 1% L-asparagine, 0.2% glucose, 0.152% K_2HPO_4, 0.052% KCl, 0.052% $MgSO_4.7H_2O$, traces of $ZnSO_4.7H_2O$ and $FeSO_4.7H_2O$ was prepared in 250 mL in Erlenmeyer flask. Erlenmeyer flask were inoculated with 1 mL of conidial suspension and submitted to orbital shaking at 160 rpm, 30°C and pH 6.2 for 4 days. Then culture suspension was filtered through whattman-2 filter paper and cell-free filtrate was used as crude enzyme solution for estimation of L-asparaginase activity.

Assay of L-asparaginase activity

L-asparaginase activity of the crude enzyme solution was determined by Nesslarization, the most commonly used method for estimation of L-asparaginase activity. The quantity of ammonia formed during the hydrolysis of 0.04 M L-asparagine was estimated using Nessler's Reagent in spectrophotometric analysis at 480 nm. One unit (IU) of L-asparaginase activity is defined as the amount of enzyme which liberates 1 µmole of ammonia per minute under the standard assay conditions (Wriston & Yellin, 2001).

Comparison of supplementary nitrogen sources by latin square design

The statistical experimental designs are used to evaluate, screen and optimize the carbon, nitrogen sources and other medium and operating conditions. In particular the LSD is used to find the best source by evaluating and comparing the effect of various carbon or nitrogen sources (Kwak *et al*., 2006; Zheng *et al*., 2008). The effect of independent variables on response follows a linear model and is given by equation 1. The residual standard deviation reflects the effect of variables, smaller the residual standard deviation of a variable, more the effect on response.

Table 1. Experimental variables in coded and actual units

Experimental variables in actual unit	Coded unit (Level)				
	1	2	3	4	5
Sodium nitrate (X_1) (%, w/v)	0	0.3	0.6	0.9	1.2
Urea (X_2) (%, w/v)	0	0.4	0.8	1.2	1.6
Ammonium chloride (X_3) (%, w/v)	0	0.4	0.8	1.2	1.6

Table 2. Latin square experimental design with L-asparaginase activity and cell mass

Std. Run	Variables in coded unit			L-asparaginase activity (IU/mL)	Cell mass (mg/mL)
	X_1	X_2	X_3		
1	1	1	1	22.55	7.60
2	2	1	2	14.82	29.00
3	3	1	3	13.12	34.00
4	4	1	4	12.32	11.11
5	5	1	5	26.45	9.70
6	3	2	1	6.51	9.20
7	4	2	2	14.50	12.70
8	5	2	3	15.25	8.90
9	1	2	4	28.32	5.31
10	2	2	5	20.05	9.80
11	5	3	1	4.74	7.90
12	1	3	2	11.99	7.40
13	2	3	3	19.89	3.73
14	3	3	4	22.39	12.00
15	4	3	5	21.06	12.30
16	2	4	1	15.19	2.85
17	3	4	2	14.72	2.76
18	4	4	3	27.83	14.40
19	5	4	4	22.55	16.60
20	1	4	5	35.46	6.65
21	2	5	1	25.59	13.50
22	5	5	2	19.30	3.62
23	1	5	3	21.54	8.100
24	2	5	4	24.21	11.50
25	3	5	5	29.33	5.50

$$Y = \mu + RX_1 + CX_2 + TX_3 \quad (1)$$

Where Y denotes any observation for which, X_1 and X_2 are blocking factors and X_3 is the primary factor. µ denotes the general location parameter, R denotes the residual standard deviation for X_1, C denotes the residual standard deviation for X_2 and T denotes the residual standard deviation for X_3 (Box *et al*., 1978; Torbjorn *et al*., 1998).

In the present investigation three supplementary nitrogen sources such as urea, sodium nitrate and

Indian Journal of Science and Technology Vol.2 No. 12 (Dec. 2009) ISSN: 0974- 6846

ammonium chloride were explored at 5-level to study their independent effect on L-asparaginase production by *A. terreus* MTCC 1782. Groundnut oil cake powder was used as substrate in shake culture fermentation. Table 1 gives the coded and actual values of the variables. 5-level LSD for three variables was developed using Data plot software (Standard Engineering Division, NIST, Gaithersburg, MD 20899-8980) is given in Table 2. Experiments were conducted for L-asparaginase production by *A. terreus* MTCC 1782 as mentioned in production and isolation step. The analysis of variance (ANOVA) is applied to find the significance of the factors by providing estimates of grand mean and factor effects.

Table 3. ANOVA on LSD for L-asparaginase activity

Factor	Degree of freedom (DF)	Sum of squares (SS)	Mean square (MS)	F-value	Confidence level (%)
Sodium nitrate	4	148.108	37.027	1.162	62.46
Urea	4	274.921	68.730	2.156	86.38
Ammonium chloride	4	475.985	118.996	3.733	96.61
Residual	12	382.532	31.877		
Total	24	1281.546	53.397		

Table 4. ANOVA on LSD for cell mass

Factor	Degree of freedom (DF)	Sum of squares (SS)	Mean square (MS)	F-value	Confidence level (%)
Sodium nitrate	4	121.342	30.335	0.525	28.04
Urea	4	365.985	91.496	1.583	75.84
Ammonium chloride	4	100.633	25.158	0.435	21.93
Residual	12	693.409	57.784		
Total	24	1281.371	53.390		

Results and discussion

The experimental L-asparaginase activity and cell mass were analyzed to study and evaluate the independent effect of urea, ammonium chloride and sodium nitrate as supplementary nitrogen source on L-asparaginase synthesis by *A. terreus* MTCC 1782 using groundnut oil cake powder using Data plot Software. ANOVA (a formal F-test) in Table 3 gives the F-value and confidence level of independent variables. Higher the F-value higher will be the confidence level (Zheng *et al.*, 2008). It was observed that ammonium chloride has higher influence (confidence level of 96.61%) on L-asparaginase production than urea (confidence level of 86.38 %) and sodium nitrate (confidence level of 62.46%). ANOVA in Table 4 shows that ammonium chloride has lesser influence (confidence level of 21.93%) on growth of *A. terreus* than urea (confidence level of 75.84%) and sodium nitrate (confidence level of 28.04%). Ammonium chloride was found to be the best supplementary nitrogen source for L-asparaginse production with less cell mass formation.

The mean, effect and residual standard deviation for L-asparaginase production and cell mass formation in Table 5 and 6 gives the independent effect of supplementary nitrogen sources at different concentration. Urea and ammonium chloride have lesser effect on L-asparaginase production at their low to middle level (concentration) and increases the L-asparaginase production (23.99 and 26.47 IU/mL respectively) at their higher level. Sodium nitrate has lesser effect (17.66 IU/mL) at its higher level, higher effect (23.97 IU/mL) at its lower level. The mean L-asparaginase activity of 23.97 and 23.15 IU/mL were obtained for modified Czapek-Dox media supplemented with urea and sodium nitrate respectively was comparatively low when ammonium chloride was used at high level. Urea has high influence (18.28 mg/mL) on growth at its low level (effect > 0) and the least effect (minimum of 8.44 mg/mL) at its middle and higher level (effect < 0) with low and constant standard deviation among the variables and their levels. Ammonium chloride and sodium nitrate have the least influence on cell mass formation (8.21 and 7.01 mg/mL respectively) at their low level and higher level and they give higher growth at their middle level (13.83 and 12.69 mg/mL respectively). Among the supplementary nitrogen sources studied at different concentrations, modified Czapek-Dox media with groundnut oil cake and supplemented with ammonium chloride gives maximum L-asparaginase production (26.47 IU/mL) with less cell mass formation (8.79 mg/mL).

Table 5. Effect of supplementary nitrogen source on L-asparaginase activity

Factor	Level	Mean	Effect	SD (Effect)
Sodium nitrate	1	23.97	4.385	2.258
	2	18.83	-0.755	2.258
	3	17.21	-2.376	2.258
	4	20.26	0.674	2.258
	5	17.66	-1.927	2.258
Urea	1	17.85	-1.736	2.258
	2	16.92	-2.664	2.258
	3	16.02	-3.571	2.258
	4	23.15	3.564	2.258
	5	23.99	4.406	2.258
Ammonium chloride	1	14.92	-4.669	2.258
	2	15.06	-4.519	2.258
	3	19.53	-0.062	2.258
	4	21.95	2.369	2.258
	5	26.47	6.881	2.258

The linear model represents the effect of independent variables on L-asparaginase activity and cell mass were given in equation 2 and 3, and the Residual Standard Deviation (RSD) of model parameters were given in Table 7. Lesser the RSD higher will be the significance of the variable for maximization problem (Box *et al.*, 1978) and vice versa for minimization.

$$Y_{activity} = 7.307 + 6.346X_3 \quad (2)$$

$$Y_{biomass} = 7.306 + 7.683X_3 \quad (3)$$

The RSD of ammonium chloride (X_3) was low (6.346)

Indian Journal of Science and Technology Vol.2 No. 12 (Dec. 2009) ISSN: 0974- 6846

for L-asparaginase production and high (7.683) for cell mass formation. Ammonium chloride was validated as the best supplementary nitrogen source for maximum L-asparaginase production with less cell mass formation by *A. terreus* MTCC 1782 using Czapek-Dox media modified by groundnut oil cake as substrate.

The Dex-Mean plot is a Graphical ANOVA, used to evaluate the effect of independent variables on response. Fig.1 and 2 shows the effect of supplementary nitrogen sources (Sodium nitrate - X_1, Urea - X_2, Ammonium chloride - X_3) on L-asparaginase production and cell mass formation. Ammonium chloride was found to be primary supplementary nitrogen source for maximum L-asparaginase production (Fig.1) with low cell mass formation (Fig.2) by *A. terreus* MTCC 1782 using Czapek-Dox media modified with groundnut oil cake powder. The residual was plotted against normal distribution and forms an approximate linear line for both L-asparaginase production (Fig.3) and cell mass formation (Fig. 4), which indicates that the model was well fitted with the experimental results. As the residuals from the fitted model are normally distributed, all the major assumptions of the model have been validated.

Table 6. Effect of supplementary nitrogen source on cell mass formation

Factor	Level	Mean	Effect	SD (Effect)
Sodium nitrate	1	7.01	-3.633	3.041
	2	11.37	0.731	3.041
	3	12.69	2.046	3.041
	4	12.80	2.156	3.041
	5	9.34	-1.301	3.041
Urea	1	18.28	7.636	3.041
	2	9.18	-1.463	3.041
	3	8.66	-1.979	3.041
	4	8.65	-1.993	3.041
	5	8.44	-2.201	3.041
Ammonium chloride	1	8.21	-2.435	3.041
	2	11.09	0.451	3.041
	3	13.83	3.181	3.041
	4	11.31	0.658	3.041
	5	8.79	-1.855	3.041

Table 7. RSD of model parameters for L-asparaginase activity and cell mass

Model Parameter	Residual standard deviation (RSD)	
	L-asparaginase activity	Cell mass formation
Constant (μ)	7.307	7.306
Sodium nitrate (X_1)	7.528	7.615
Urea (X_2)	7.094	6.765
Ammonium chloride (X_3)	6.346	7.683
Constant and all factors	5.646	7.602

Conclusion

LSD was successfully applied to find the best supplementary nitrogen source for L-asparaginase production by *A. terreus* MTCC 1782 using groundnut oil cake power as low-cost substrate. It was found that LSD is an effective statistical tool to evaluate and compare the various carbon or nitrogen sources and find the best for maximum L-asparaginase production. Among the supplementary nitrogen sources studied, ammonium chloride was found to be the best for the production of L-asparaginase by *A. terreus* MTCC 1782 using a a low-cost substrate groundnut oil cake.

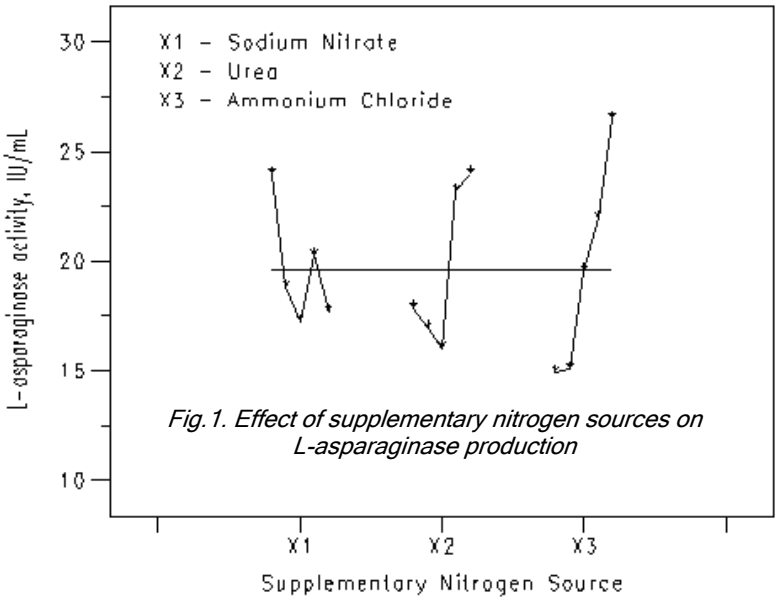

Fig.1. Effect of supplementary nitrogen sources on L-asparaginase production

References

1. Ali SS, Rai V, Soni K, Kulshrestha P and Lai SK (1994) A fungal L-asparaginase with potential antitumor activity. *Ind. J. Microbiol*. 34, 73-76.
2. Berenbaum MC, Ginsburg H and Gilbert DM (1970) Effects of L-asparaginase on lymphocyte target cell reactions *In vitro*. *Nature*. 227, 1147-1148.
3. Box GEP, Hunder WG and Hunder SJ (1978) Statistics for experiments, John Wiley & Sons Inc., New York.
4. Broome JD (1961) Evidence that the L-asparaginase activity of Guinea pig serum is responsible for its antilymphoma effects. *Nature.* 171, 1114-1115.
5. Broome JD (1965) Antilymphoma activity of L-asparaginase *in vivo*: Clearance rates of enzyme preparations from Guinea pig serum and yeast in relation to their effect on tumor growth. *J. Natl. Cancer Inst*. 35, 967-974.
6. Joint FAO/WHO Expert Committee on Food Additives (JECFA) Compendium of Food Additive Specifications (2001) General specifications and considerations for enzyme preparations used in food processing, *FAO Food & Nutrition.* 52(9), 37-39.
7. Kwak KO, Jung SJ, Chumg SY, Kang CM, Huh YI and Bae S O (2006) Optimization of culture conditions for CO_2 fixation by chemoautotrophic

Indian Journal of Science and Technology Vol.2 No. 12 (Dec. 2009) ISSN: 0974- 6846

microorganism strain YN-1 using factorial design. Biochem. Eng. J. 31, 1-7.

8. Mishra A (2006) Production of L-asparaginase, an anticancer agent, from Aspergillus niger using agricultural waste in solid state fermentation. *Appl. Biochem. Biotechnol*. 135, 33-42.
9. Pedreschi F, Kaack K and Granby K (2008) The effect of Asparaginase on acrylamide formation in

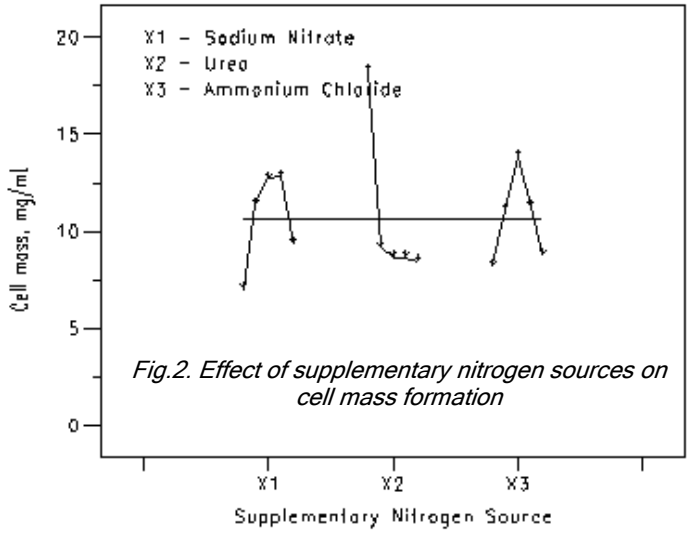

Fig.2. Effect of supplementary nitrogen sources on cell mass formation

French fries. *Food Chem*. 109, 386-392.

10. Raha SK, Roy SK, Dey SK and Chakrabarty SL (1990) Purification and properties of an L-asparaginase from Cylindrocarpon obtusisporum MB-10. *Biochem. Int*. 2, 987-1000.
11. Sarquis MIM, Oliviera EMM, Santos AS and Da-Costa GL (2004) Production of L-asparaginase by filamentous Fungi. *Mem. Inst. Oswaldo. Cruz*. 99, 489-492.
12. Shaffer PM, Arst HN Jr, Estberg L, Fernando L, Tran Ly and Sitter M (1988) An asparaginase of

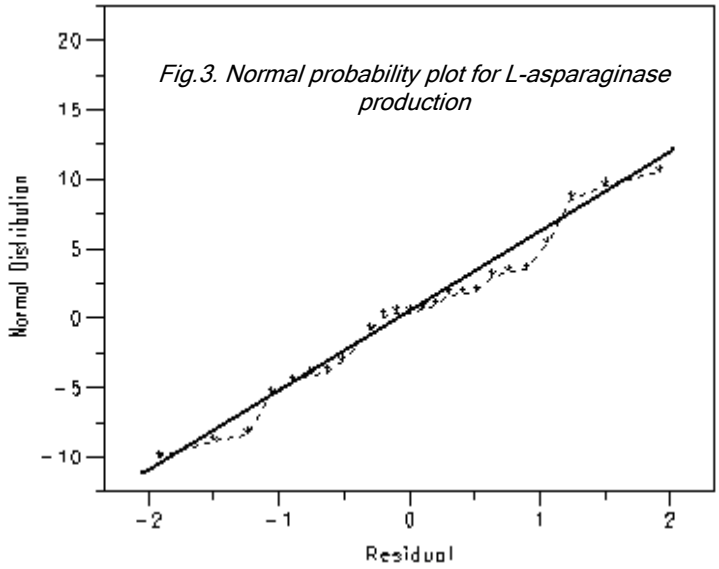

Fig.3. Normal probability plot for L-asparaginase production

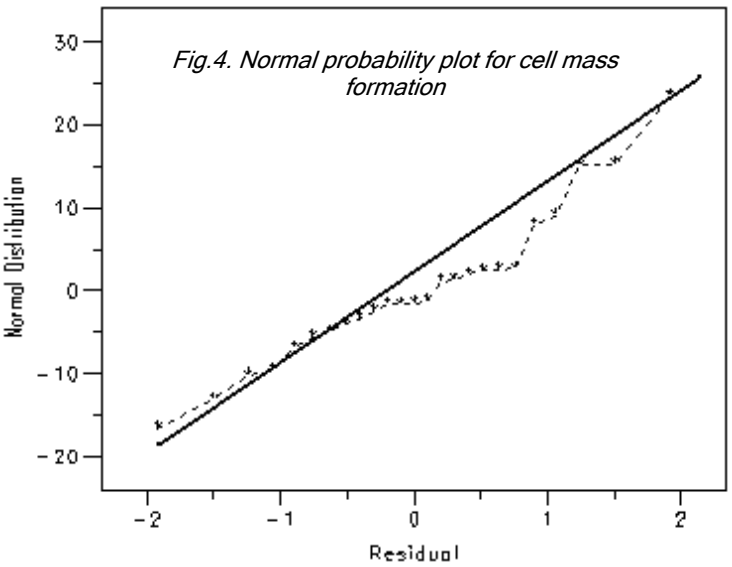

Fig.4. Normal probability plot for cell mass formation

Aspergillus nidulans is subject to oxygen repression in addition to nitrogen metabolite repression. *Mol. Gen. Genet*. 212, 337-341.

13. Torbjorn L, Elisabeth S, Lisbeth A, Bernt T, Asa N, Jarle P and Rolf B (1998) Experimental design and optimization. *Chemometr. Intell. Lab*. 42, 3-40.
14. Wriston JC Jr and Yellin TO (2001) L-asparaginase - a review. *Adv. Enz*. 39, 185-248.
15. Zheng ZM, Hu QL, Hao J, Xu F, Guo NN, Sun Y and Liu DH (2008) Statistical optimization of culture conditions for 1,3-propanediol by *Klebsiella pneumoniae* AC 15 via Central Composite Design. *Bioresour. Technol*. 99, 1052-1056.