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Full Length Research Paper

Screening of Parameters and Production of Amylases using *Aspergillus oryzae* by Submerged Fermentation Process

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Abstract

Aspergillus oryzae MTCC 3107 is a potential fungal source for the production of the industrially important enzymes amylases. In the present investigation, submerged fermentation method is adopted and it involves screening of variable by systematic method as well as by using Plackett-Burman design. Systematic method of screening variables like carbon and nitrogen sources, inoculum age and inoculum levels was carried out and the optimal values obtained are – starch (as carbon source)- 20g/l, peptone (as nitrogen source) – 45g/l, inoculum age – 48h and inoculum level is 2% (v/v). Plackett- Burman design was used for screening of mineral elements and out of seven variables, four were found have significant role in the production of amylases which include $CaSO_4$, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$ and $MnSO_4.H_2O$.

Key words: *Aspergillus oryzae, Amylases, submerged fermentation, Plackett-Burman design.*

Introduction

Industrial use of microbial enzymes has gained importance over the past few decades. Extra cellular enzymes like amylases, produced by microbial sources are being used in industries of food and beverages, textiles, pharmaceuticals and confectionaries (Ibunkun and Akinduila, 1998). Amylases obtained from *Aspergillus sps* have high saccharification efficiency as compared to bacterial enzymes and find significant application in industries (Aquino et al, 2003; Carlsen et al, 1996). However, *Aspergillus oryzae* was reported to be non-toxic and has high productivity of alpha amylases (Abe et al., 1988). The strain *Aspergillus oryzae* MTCC 3107 has been studied by few authors and it is reported for its ability to produce amylases and glucoamylases (Shruthi et al, 2013). Solid state fermentation method was employed and agricultural residues were used which include wheat bran, rice bran and paddy husk (Shruthi et al., 2013; Anshikaa Grover et al, 2013), while Shruthi et al. (2013) have also evaluated the enzyme activity obtained from the strain and confirmed its industrial applicability.

In the present investigation *Aspergillus oryzae* MTCC 3107 was used for amylase production by Submerged fermentation and procedures employed include systematic optimization of few physical and nutritional parameters, followed by screening of mineral sources using Plackett- Burman design. The partial factorial design helps in screening large number of independent variables using minimum number of experimental runs (Naveena et al, 2005; Sayyad et al, 2007).

Materials and Methods

***Microorganism: Aspergillus oryzae* MTCC 3107**, procured from MTCC, Institute of Microbial Technology, Chandigarh, Punjab was used in the present study. The organism is maintained in PDA slants. It is subcultured once in a month and stored at 4^{o}C.

Inoculum preparation for seed culture

Fresh potato infusion was used for the preparation of seed culture (Frank et al, 1993). The fresh potatoes weighing 400 gm were peeled and chopped into small pieces and boiled in 500ml distilled water for 30 minutes. The pieces were mashed filtered and by using cheese cloth. To the filtrate, obtained 20g of dextrose was added and the volume was made to 1000ml. pH was adjusted to 3 using 0.1N NaOH and 0.1 HCl. The above sterilized potato infusion medium was inoculated with 1ml fungal spores and the flasks were incubated at 30^{o}C for 48h at 120 rpm to obtain seed culture.

Production medium

The production was carried out by submerged fermentation and the medium composition is as follows (g/l) (Singh et al., 2009): Soluble starch – 5, yeast extract – 2, potassium dihydrogen phosphate – 1 and Magnesium sulphate – 0.5. The pH was adjusted to 5. After inoculating with 2ml of seed culture the production was carried out at 30^{o}C and 120 rpm in an incubated orbital shaker.

Screening of carbon and nitrogen sources

The basic production medium has starch as carbon source. Keeping rest of the variables constant, the carbon sources screened include- glucose, sucrose, lactose and starch a concentration of 5g/l. The best source from the above was selected and its concentration was then varied as follows (g/l): 5, 10, 15, 20 and 25. Samples were withdrawn every 24 hours to estimate amylase activity. In the same way different organic and inorganic nitrogen sources were selected for study at 2g/l supplementation, which include- Ammonium nitrate, Ammonium sulfate, Peptone, Potassium nitrate, Urea and Yeast extract. The best of them was selected keeping rest of the parameters constant and the selected nitrogen sources concentration was varied between 1.5, 3, 4.5 and 6 g/l.

Effect of inoculum age and inoculums level

The seed culture is the source of inoculum and the inoculum age was investigated between 12h, 24h, 36h, 48h, 60h and 72h. From the optimal value obtained, amount of inoculum level to be added for production medium was optimized. This is by varying the levels between 10 ml to 50ml of seed culture per liter of production medium.

Plackett-Burman design

Plackett-Burman experimental design was used to identify the significance of the ingredients of the mineral solution for optimum production of amylase enzyme. Plackett-Burman is a technique devoted to the screening of controlled experimental factors and the measurement of their responses, according to one or more criteria. According to Plackett and Burman (1946), their factorial design allows estimation of random error variability and test for the statistical significance of the parameter estimates (Mary Anupama et al, 2008). The elements chosen for the study and their concentration range are as follows (mg/l): KH_2PO_4 (0.5 to 1.0), $MgSO_4.7H_2O$ (0.5 to 1.0), $NaH_2PO_4.2H_2O$ (0.5 to 1.0), $MnSO_4.H_2O$ (0.5 to 1.0), $CaSO_4$ (0.5 to 1.0), $FeSO_4.7H_2O$ (0.5 to 1.0), $ZnSO_4.7H_2O$ (0.5 to 1.0). This design has resulted in an output of 10 experimental runs with 7 independent variables. For each variable, a high (+) and low (-) levels were tested. The Plackett-Burman design with variables incorporated is represented table I.

The values of carbon and nitrogen source were set according to the preliminary experimental results. Enzyme production was carried out in the same way as mentioned earlier and mineral solution was added according to their concentrations in different runs in Plackett-Burman design.

Amylase assay

The amylase produced during the submerged fermentation process was estimated using the protocol of Swetha et al, (2007). One unit of amylase is expressed as milligrams of maltose released per ml of production medium per hour at 37^{o}C.

Results and Discussion

Optimization of nutritional requirements

Screening of carbon sources

Among the carbon supplements, starch which is a direct substrate for the enzyme was found to be best inducer for amylase production while presence of glucose produced from either sucrose, lactose or from the direct supplementation repressed amylase production (Esfahanibolandbalaie et al, 2008). Supplementation of starch has resulted in 1200units of amylase while least amount was produced with lactose as carbon source (Figure I) at the end of 72 hours.

Optimization of starch concentration

Among the carbon sources, starch showed maximum amylase production hence the effect of its concentration on amylase production was evaluated. Increase in the concentration of starch has shown an initial increase in the enzyme concentration, while beyond 20 g/l, due to slight gelling of the medium a slight decrease in product yield was observed (figure II). Swetha et al. (2007) have recorded similar observation for starch as carbon source at 20 g/l concentration.

Screening of nitrogen source on amylase production

Of all the nitrogen sources tested peptone showed maximum enzyme production at the end of 72h by *A. oryzae* (figure III). Inorganic supplements proved to have an insignificant effect on amylase production in accordance with Jin et al, (1998), while organic supplements; essentially peptone has resulted in maximum enzyme units.

Optimization of peptone concentration

Increase in the concentration of peptone has shown an initial increase in the enzyme concentration, while beyond 45 g/l not much increase in enzyme levels were observed. The results agree with studies of Anto et al (2006), who confirmed that organic nitrogen supplements contribute to higher enzyme production. Addition of peptone at 40g/l concentration has resulted in 4800 units of amylase activity (figure IV).

Effect of inoculum age and inoculums level

Inoculum age of 48 hours and inoculum level of 2% (v/v) was found to be optimal for amylase production (figure V & VI). Inoculum age is critical as starch present in the seed culture induces enzymes that are required for its hydrolysis, while with increase in time, the product inhibition sets in and results in decrease in potential enzyme production by the mycelium (Haq et al, 2006). Inoculum levels

also play a crucial role. If less of inoculum is added lag time gets extended while if more of fungal mycelium is added the carbon and nitrogen sources would be consumed for biomass production (Sharma et al., 1996).

Plackett- Burman Design

The results obtained at the end of 72h were indicated in table II. These were further analyzed by STATISTICA 6.0 software to find the mineral salts that are significant in the production of amylase. Pereto chart (figure VII) indicates that the salts $MnSO_4.H_2O$, $FeSO_4.7H_2O$, $MgSO_4.7H_2O$, and $CaSO_4$ are significant inducers for amylase production by *Aspergillus oryzae* as compared to the other three salts, $NaH_2PO_4.2H_2O$, $ZnSO_4.7H_2O$ and KH_2PO_4. Only four variables have their p values more than 0.05 indicating their significant role in production.

The goodness of the chosen model was determined by regression analysis. A regression model with R^2 value close to 1 indicates that the model chosen is reliable. The regression coefficient values obtained for the Plackett- Burman design is 0.99173 indicating its best fit by 99%. The t- values and p- values were also represented in table III. The variable whose p value is less than 0.05 and whose t-values are high are $MnSO_4.H_2O$, $FeSO_4.7H_2O$, $MgSO_4.7H_2O$, and $CaSO_4$.

Systematic optimization studies have revealed the optimal conditions to be maintained to get maximum production, while the essential minerals were supplemented at their zero values and a final batch study was carried out whose composition is as follows: Starch -20 g/l, Peptone -45 g/l, $CaSO_4$, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$ and $MnSO_4.H_2O$ at 0.75mg/l. Experiments were conducted in triplicate and the amylase yield obtained at the end of 72h was 4850 units. The yield can further be enhanced if concentrations of salt supplements were optimized.

Table 1. Levels of the variables incorporated in Plackett-Burman design

S. No	KH_2PO_4 (mg/l)	$MgSO_4.7H_2O$ (mg/l)	$NaH_2PO_4.2H_2O$ (mg/l)	$MnSO_4.H_2O$ (mg/l)	$CaSO_4$ (mg/l)	$FeSO_4.7H_2O$ (mg/l)	$ZnSO_4.7H_2O$ (mg/l)
1	+1	-1	+1	-1	+1	-1	-1
2	+1	+1	+1	+1	+1	+1	+1
3	-1	-1	-1	+1	+1	+1	-1
4	0	0	0	0	0	0	0
5	+1	+1	-1	+1	-1	-1	-1
6	-1	+1	+1	-1	-1	+1	-1
7	-1	-1	+1	+1	-1	-1	+1
8	+1	-1	-1	-1	-1	+1	+1
9	0	0	0	0	0	0	0
10	-1	+1	-1	-1	+1	-1	+1

Table 2. Units of amylase produced by Plackett- Burman Design

S. No	KH_2PO_4 (mg/l)	$MgSO_4.7H_2O$ (mg/l)	$NaH_2PO_4.2H_2O$ (mg/l)	$MnSO_4.H_2O$ (mg/l)	$CaSO_4$ (mg/l)	$FeSO_4.7H_2O$ (mg/l)	$ZnSO_4.7H_2O$ (mg/l)	Amylase Units
1	1.000000	0.500000	1.000000	0.500000	1.000000	0.500000	0.500000	1040
2	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1040
3	0.500000	0.500000	0.500000	1.000000	1.000000	1.000000	0.500000	1040
4	0.750000	0.750000	0.750000	0.750000	0.750000	0.750000	0.750000	1040
5	1.000000	1.000000	0.500000	1.000000	0.500000	0.500000	0.500000	800
6	0.500000	1.000000	1.000000	0.500000	0.500000	1.000000	0.500000	1360
7	0.500000	0.500000	1.000000	1.000000	0.500000	0.500000	1.000000	200
8	1.000000	0.500000	0.500000	0.500000	0.500000	1.000000	1.000000	1200
9	0.750000	0.750000	0.750000	0.750000	0.750000	0.750000	0.750000	920
10	0.500000	1.000000	0.500000	0.500000	1.000000	0.500000	1.000000	1360

Figure I: Effect of Carbon Sources on Amylase Production by *Aspergillus oryzae*

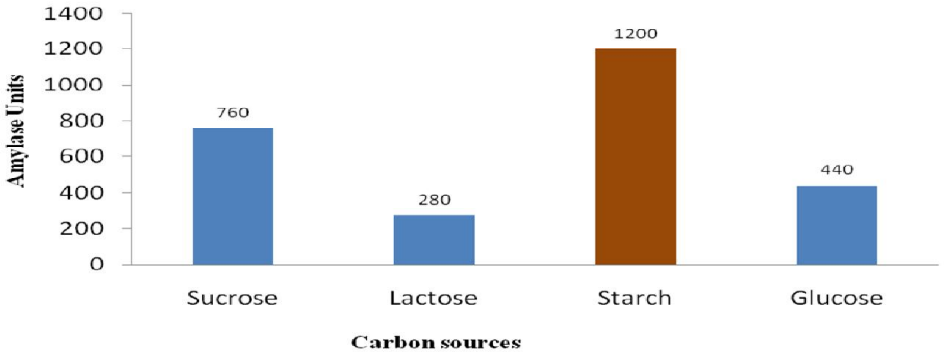


Figure II: Effect of Starch concentration on Amylase Production

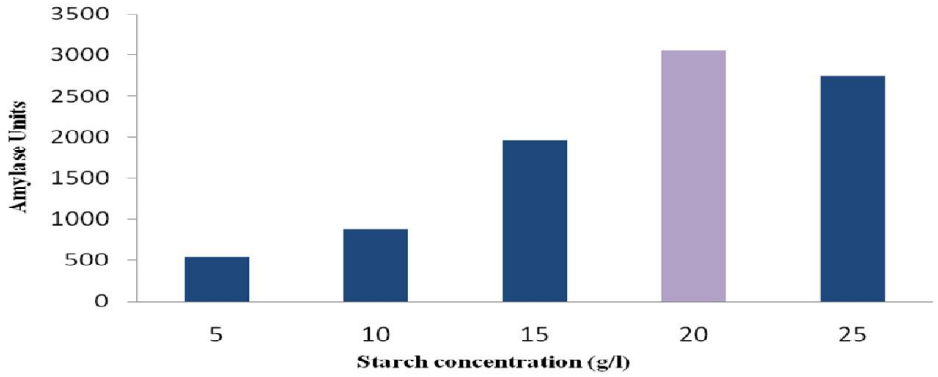


Figure III: Effect of Nitrogen Sources on Amylase Production by *Aspergillus oryzae*

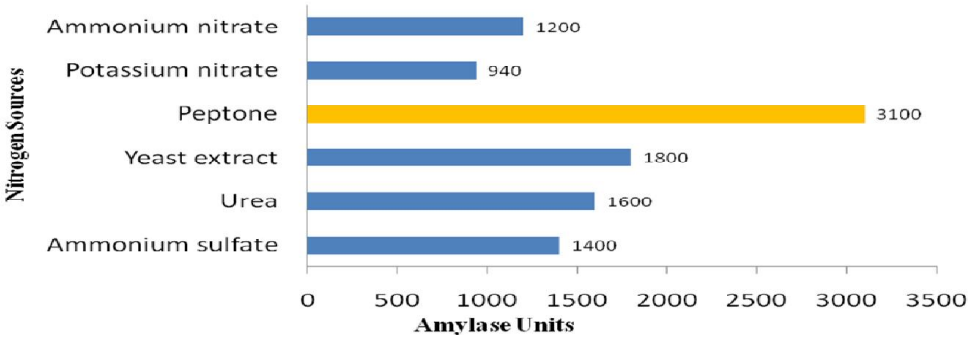


Figure IV: Effect of Peptone on Amylase Production by *Aspergillus oryzae*

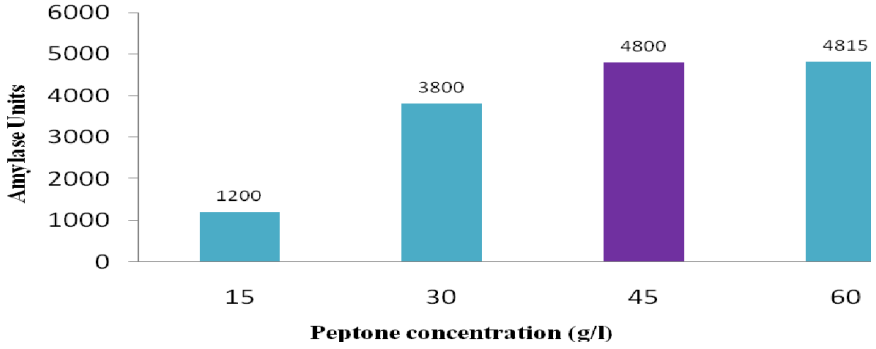


Figure V: Effect of Inoculum Volume on Amylase Production by *Aspergillus oryzae*

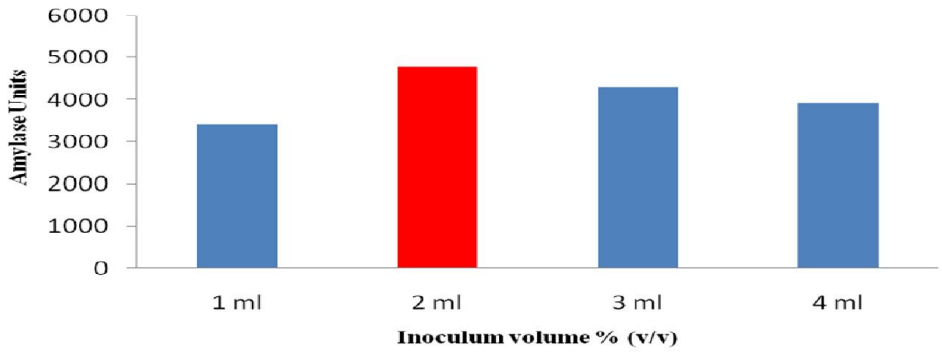
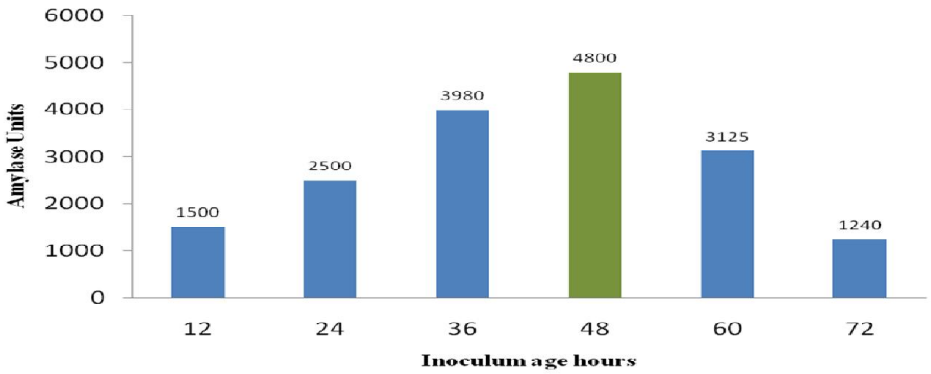


Figure VI: Effect of Inoculum Age on Amylase Production by *Aspergillus oryzae*



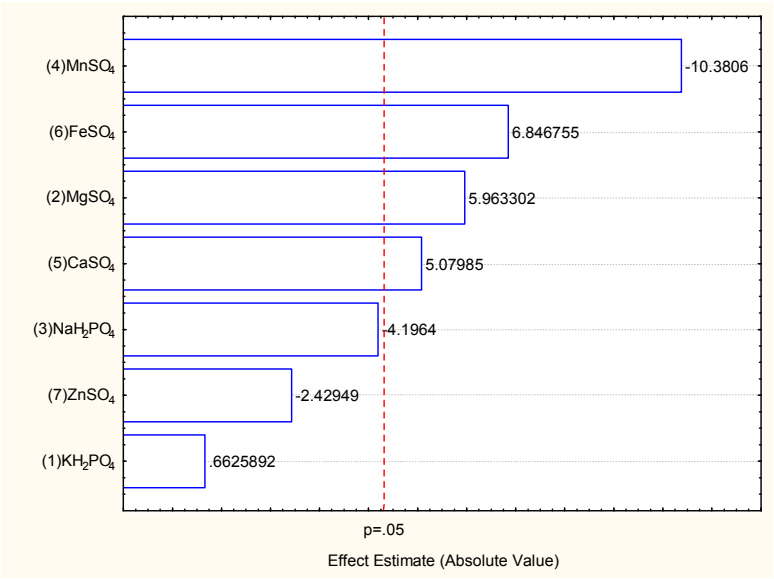


Figure VII: Pereto chart of standardized effects for Plackett- Burman Design

Conclusion

Aspergillus oryzae MTCC 3107 is a potential organism for the production of amylases. Systematic optimization studies have revealed that soluble starch is a good carbon source as compared to simple sugars as it induces genes for the synthesis of amylases. Peptone a complex protein supplementation was found to be good nitrogen source. The optimal values of parameters under study are- starch 20(g/l), peptone 45(g/l), Inoculum age- 48h and inoculum level of 2% (v/v). The salts to be supplemented as obtained from Plackett-Burman design are $CaSO_4$, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$ and $MnSO_4.H_2O$.

Table3. Statistical analysis of Plackett–Burman design results showing estimated effect, regression coefficient and corresponding t - values, P- values and standard error for each variable

	Effect	Coeff.	Std.Err.	t(2)	P
Mean/Interc.	1000.000	1000.000	20.24846	49.3865	0.000410
(1)KH_2PO_4	30.000	15.000	45.27693	0.6626	0.575736
(2)$MgSO_4.H_2O$	270.000	135.000	45.27693	5.9633	0.026988
(3)$NaH_2PO_4.2H_2O$	-190.000	-95.000	45.27693	-4.1964	0.052366
(4)$MnSO_4.H_2O$	-470.000	-235.000	45.27693	-10.3806	0.009153
(5)$CaSO_4$	230.000	115.000	45.27693	5.0799	0.036636
(6)$FeSO_4.7H_2O$	310.000	155.000	45.27693	6.8468	0.020673
(7)$ZnSO_4.7.H_2O$	-110.000	-55.000	45.27693	-2.4295	0.135758

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