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OPTIMIZATION OF CULTURAL CONDITIONS FOR THE PRODUCTION OF ALPHA AMYLASE BY WILD AND MUTANT STRAIN OF *ASPERGILLUS ORYZAE* IN STIRRED FERMENTER

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Abstract

The present study deals the optimization of cultural conditions for the production of alpha amylase by *Aspergillus oryzae* IIB-30 and its mutant derivative *A. oryzae* EMS-18 in stirred fermenter. The time of fermentation for enzyme production by both wild and mutant strains was studied. It was found that the time required for maximal enzyme production (608 U/ml) in case of mutant strain was reduced to 48 h compared with 64 h by wild strain for maximum enzyme production (335 U/ml). The kinetic depiction of results showed optimal fermentation period for enzyme production to be 64 h and 48 h, respectively. The other cultural conditions such as initial pH (5), aeration rate 1.5 vvm (mutant), dissolved oxygen (15%), inoculum size (10%) and agitation intensity (200 rpm) were optimized for enzyme production.

Introduction

Filamentous fungi are being extensively used due to their ability to produce large number of industrial enzymes. Among the filamentous fungi *Aspergillus oryzae* is widely used for the production of numerous hydrolytic enzymes such as alpha amylase, glucoamylase, proteases etc. Alpha amylase which is an extracellular enzyme randomly cleave α-1,4 glycosidic linkages in starch to produce glucose, maltose or dextrin (Omemum *et al*., 2005; Bhanja *et al*., 2007; Leman *et al*., 2009; Haq *et al.,* 2010). Alpha amylases are gaining more importance because their spectrum of application has widened in many fields such as clinical, medicinal and analytical chemistry. Beside their use in starch saccharification they also find applications in food, baking, brewing, detergent, textile and paper industries (Agger *et al*., 2001; Haq *et al.,* 2005; Shafique *et al*., 2009). With the development of effective techniques large scale production of alpha amylase becomes an attractive business (Zangirolami *et al*., 2002). Fermenters of different working volumes may be used for the large scale alpha amylase production as an industrially important enzyme under controlled conditions. Maximum enzyme production is one of the most important goals in biotechnological processes. By optimizing the cultural conditions such as inoculum size, temperature, pH, agitation, aeration and dissolved oxygen etc. the enzyme production can be enhanced by many fold (Gigras *et al*., 2002).

Enzyme production commences at a low rate during the logarithmic growth phase but reaches its maximum value during the stationary phase towards onset of sporulation. Time course study and agitation determines the efficacy of the batch process and subsequent product formation. The pattern of accumulated reducing sugar after specific incubation time is characteristic to the species (Matrai *et al*., 2000). Alpha amylase production at different agitation rates (100-300) at 30°C were estimated and maximum amount of enzyme was obtained at 150-200 rpm after 72 h. According to Mariani *et al*., (2000), the maximum alpha amylase production was obtained after 120 h in a fermenter (New Brunswick) operating at 300 rpm and airflow of 11/l/min in a limited dissolved oxygen concentration. It was determined that the increase in agitation rate was not

favorable for enzyme production; despite of this an increase was verified in dissolved oxygen. Enzyme production was better with *A. oryzae* NRRL 6270 at 30ºC after 96 h when spore suspension used was 1 x10^7 spores/ml (Francis *et al*., 2002).

Materials and Methods

The chemicals used in this study such as Sodium potassium tartarate, 3,5-dinitro salicylic acid, phenol, Sodium metabisulphate, dihydrogen phosphate, Manganese sulphate, yeast extract, Ferrous sulphate, Magnesium chloride, diammonium sulphate, starch, Ferrous sulphate etc were of analytical grade and obtained from Sigma (USA), BDH (UK), E-Merck (Germany), Acros (Belgium) and Fluka (Switzerland). All other chemicals were of the highest possible purity.

Aspergillus oryzae IIB-30 and its mutant derivative EMS-18 were obtained from institute of Industrial Biotechnology.

Vegetative inoculum: One hundred milliliter of the fermentation medium was transferred to a 1.0 L conical flask followed by the addition of approximately 20-25 glass beads (2.0 mm dia.). The flask was cotton plugged and sterilized. Four milliliter of the conidial suspension was transferred aseptically to the flask, which was then incubated at 30°C on an orbital shaking incubator (Model: 10X400.XX2.C, SANYO Gallenkamp, PLC, UK) at 200 rpm for 24 h.

Fermenter studies: Scale up studies were carried out in a 7.5 L glass fermenter (Model: Bioflow-110 Fermenter/Bioreactor, USA) with a working volume of 5.0 L. The fermenter glass vessel containing 4.7 L fermentation medium was sterilized in a stainless steel autoclave (Model: KT-40 L, ALP, Japan) for 20 min at 15 lbs/in^2 pressure (121ºC) and cooled at room temperature. Vegetative inoculum was transferred to the vessel through a port at the top plate under aseptic conditions. The incubation temperature was kept at 30°C, while the aeration and agitation rates were maintained at 1.0 L/L/min (vvm) and 200 rpm, respectively throughout the fermentation period. The air, to be supplied was sterilized by passing through membrane filters (0.45 µm pore size). Sterilized solution of 0.1 N HCl/ NaOH was used for pH adjustment. The sterilized silicone oil 10% (v/v) was used to control foam formed during the fermentation process. After incubation, the fermented broth was filtered. The filtrate was used for the estimation of alpha amylase.

Estimation of alpha amylase: The estimation of alpha amylase was carried out according to the method of Rick & Stegbauer (1974). "One unit of activity was that amount of enzyme, which in 10 min liberates reducing group from 1% Lintner's soluble starch corresponding to 1mg of maltose hydrate." The enzyme activity was determined by taking 1 ml of diluted filtrate in a test tube. One milliliter of starch solution (1%) was also added into it. A blank was run parallel by replacing the filtrate with 1 ml of distilled water. After incubation of 10 min at 40°C, the reducing sugar liberated was measured at 546 nm by the DNS method (Miller, 1959) using maltose as a standard.

Estimation of dry cell mass (DCM): Dry cell mass was determined by filtering the culture broth through preweighed Whatman filter paper No. 44. Mycelium was thoroughly washed with tap water and dried in an oven at 105°C for 2 h. The dry cell mass was weighed and calculated as g/l by subtracting the initial weight from the final weight.

Kinetic study: Kinetic parameters for batch fermentation were determined according to the method describe by Pirt (1975) and Lawford and Rouseau (1993). The following parameters of kinetics were studied:-

The value of specific growth rate i.e., μ (h^{-1}) was calculated from plot of ln (x) vs time of fermentation. Product yield co efficient namely Yp/x was determined by the equation:

$$Yp/x=dP/dx.$$

The volumetric rate of product formation Qp (U/l/h) was determined from the maximum slope of enzyme produced vs time of fermentation. The volumetric rate for biomass formation Qx (g cell mass /l/h) was determined from the maximum slope of cell mass formation vs time of fermentation. Specific rate constant for product formation was determined by the equation:

$$q_p = \mu \times Y\ p/x$$

Results and discussion

The fermentation rate of both the wild (IIB-30) and mutant (EMS-18) strains of *A. oryzae* for the alpha amylase production was investigated in stirred fermenter (Fig. 1). The time course aliquots were withdrawn after every 8 h aseptically and subjected to enzyme estimation up to 96 h of fermentation period. It was found that the enzyme production was increased gradually and reached its maximum (335 U/ml) and (608 U/ml) after 64 h for wild and 48 h of fermentation for mutant respectively. The dry cell mass was (18.2) and (19.8), g/l respectively. Rapid decline in enzyme production was in case of wild and mutant strain when incubation period was increased from optimum time period. It may be due to the denaturation or proteolysis of enzyme because of interaction with other compounds in the fermentation medium or may be due to the depletion of the nutrients and formation of other by products in the fermentation medium (Ramesh & Lonsane, 1990; Kirshna & Chandrasekaran, 1996). Effect of different initial pH (4-6.5) of fermentation medium was also investigated in stirred fermenter (Fig. 2). The enzyme production by both cultures was found to be optimum at pH 5. Further increase in the pH reduced the enzyme production, as enzymes are usually very sensitive to minor changes in pH. Any increase or decrease in H^+ ion concentration has significant effect on the growth of mycelium and hence, on the enzyme excretion (Kasim 1983; Stamford *et al*., 2001; Gupta *et al*., 2008).

A general requirement for a bioreactor is the provision of aeration system that can maintain a high dissolved oxygen level for aerobic fermentation. In this connection rate of agitation and different volume of air supply was studied for the enzyme production in stirred fermenter (Fig. 6). The enzyme production was increased as the agitation intensity was increased and found to be maximal at 200 rpm. Change in the rate of agitation resulted reduction in enzyme production. Probably higher stirring speed above 200 rpm resulted in mechanical and oxidative stress, excessive foaming, disruption and physiological disturbance of cells, while lower stirring speed seemed to limit oxygen levels along with the lacking of homogeneous suspension of the fermentation medium and breaking of the clumps of cells. The enzyme production increased with the increase of aeration and reached maximum at 1.0 vvm (wild) & 1.5 vvm (mutant). The anaerobic

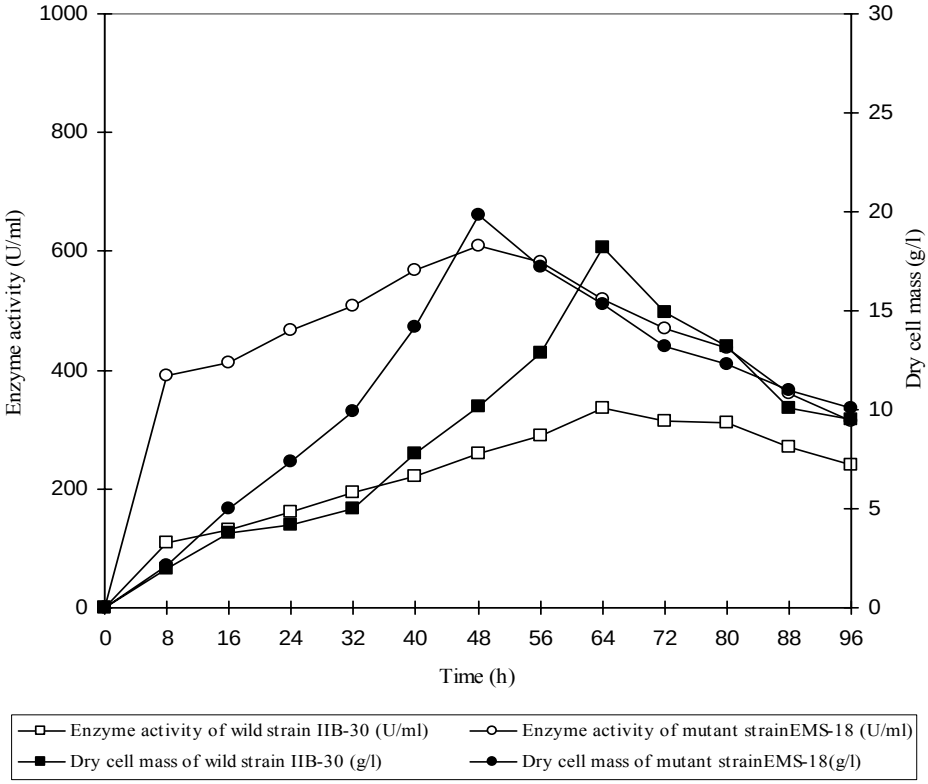

Fig. 1. Comparison of alpha amylase production by wild (IIB-30) and mutant strain of *A. oryzae* (EMS-18) in stirred fermenter*
* Incubation temperature 30°C, pH 5.0, agitation rate 160 rpm, aeration 1vvm.

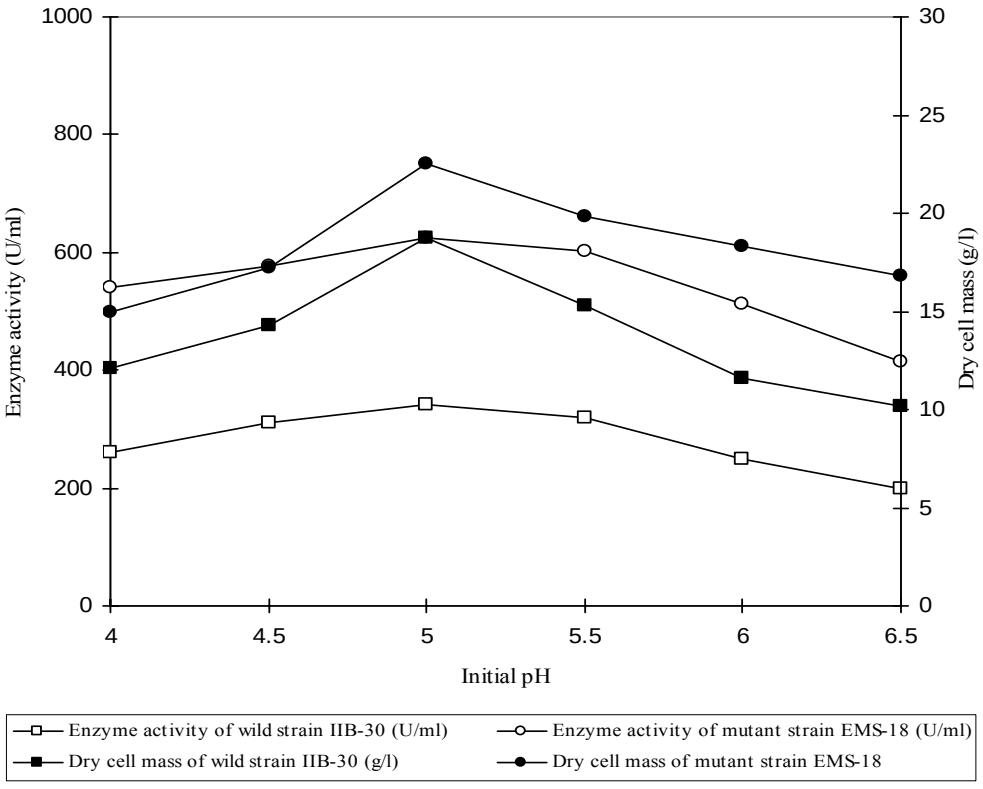

Fig. 2. Effect of initial pH of media on the alpha amylase production by *A. oryzae* IIB-30 and its mutant derivative *A. oryzae* EMS-18 *
*Incubation time 48 h, temperature 30°C, agitation intensity 160 rpm, aeration 1vvm

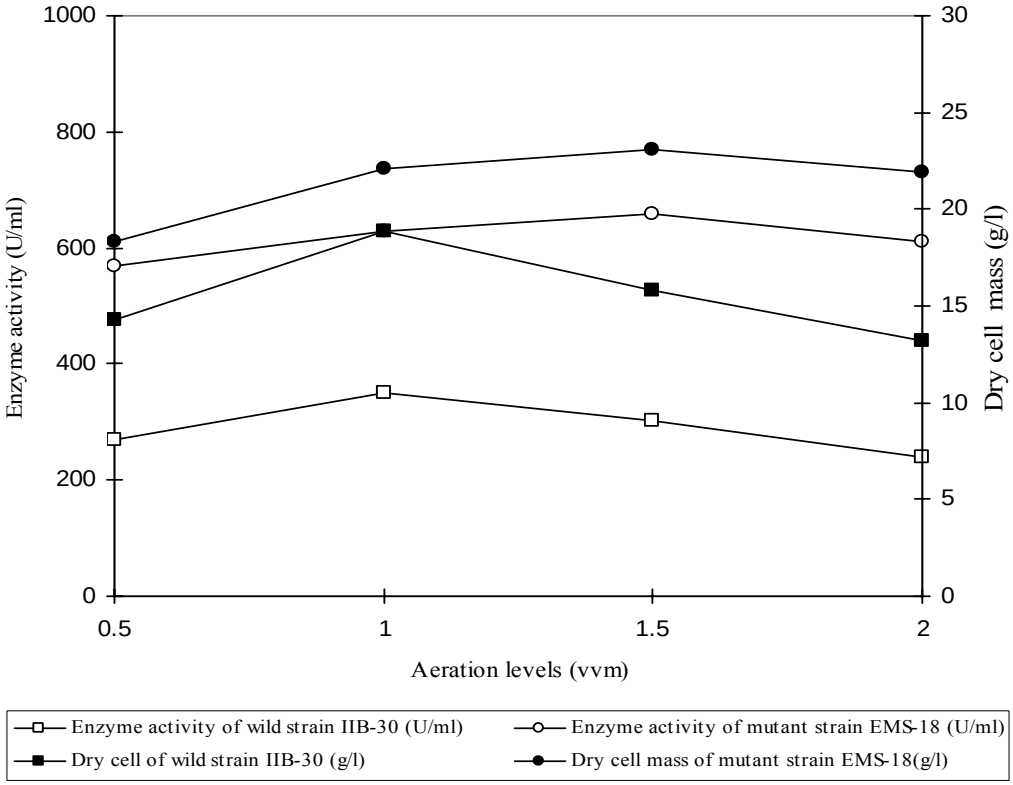

Fig. 3. Effect of different aeration levels on the alpha amylase production by *A. oryzae* IIB-30 and its mutant derivative *A. oryzae* EMS-18 *
*Incubation time 48 h, incubation temperature 30°C, agitation intensity 160 rpm

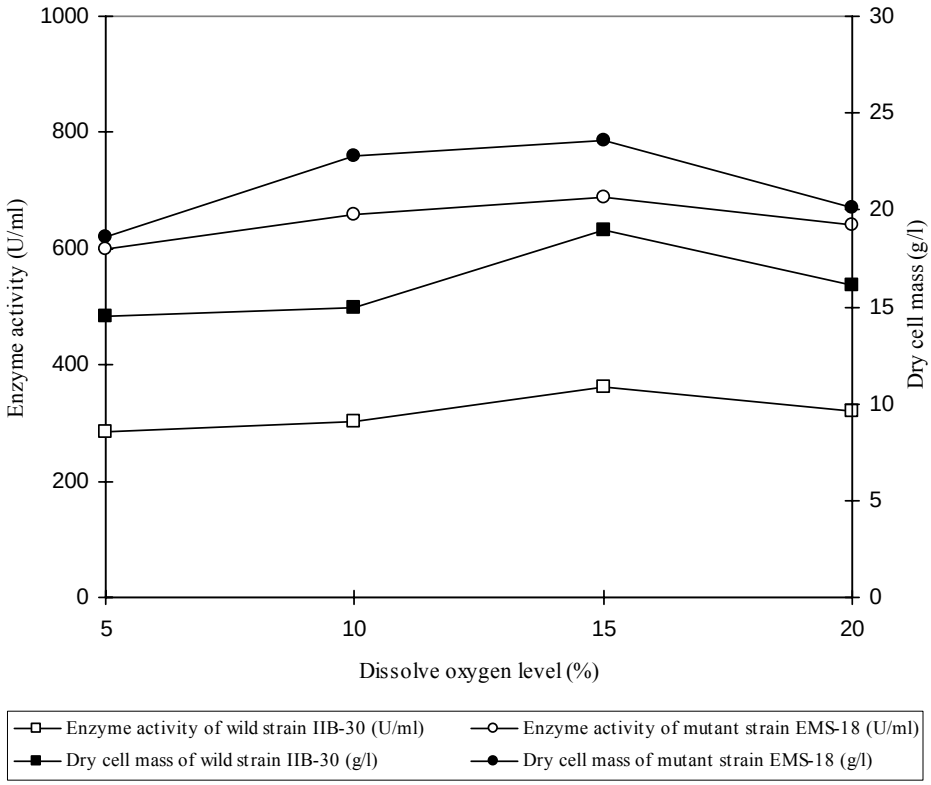

Fig. 4. Effect of different level of dissolved oxygen on the alpha amylase production by *A. oryzae* IIB-30 and its mutant derivative *A. oryzae* EMS-18 *
*Incubation time 48 h, initial pH 5.0, incubation temperature 30°C, agitation intensity 160 rpm, aeration 2.0 vvm.

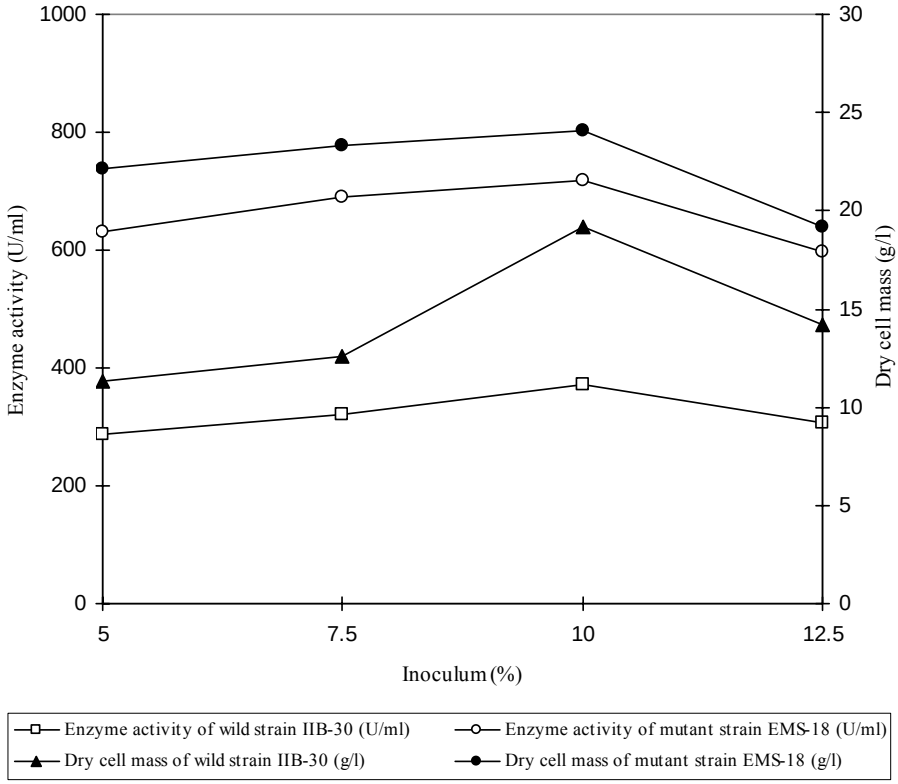

Fig. 5. Effect of different inoculum size on the alpha amylase production by *A. oryzae* IIB-30 and its mutant derivative *A. oryzae* EMS-18 *
*Incubation time 48 h, incubation temperature 30°C, agitation intensity 160 rpm, initial pH 5.0

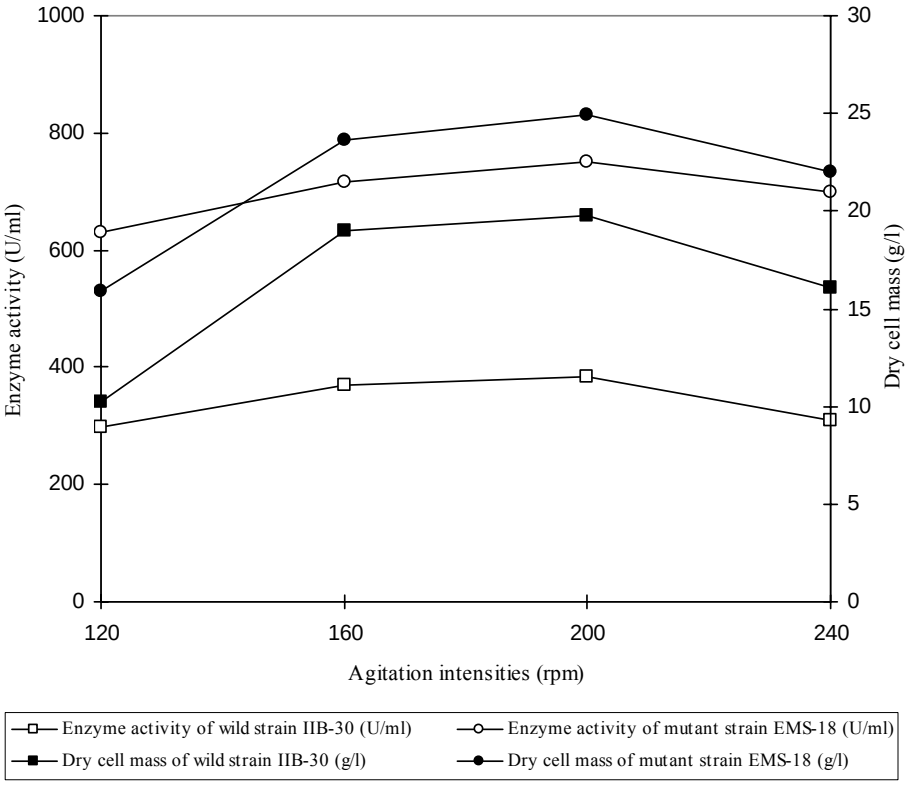

Fig. 6. Effect of different agitation intensity on the alpha amylase production by *A. oryzae* IIB-30 and its mutant derivative *A. oryzae* EMS-18 *
*Incubation time 48 h, incubation temperature 30°C, pH 5.0.

condition available to microorganism greatly disturbed the physiology and metabolism of organism because of this at low level of air supply the productivity of enzyme was greatly inhibited. In addition other toxic by products were produced in the fermentation medium with little titer of enzyme activity, while higher concentration rates have some detrimental effects on the growth of microorganism and subsequently enzyme production in the bioprocess (Ionita *et al*., 2001). Effect of different levels (5-20%) of dissolved oxygen on alpha amylase production by *A. oryzae* was investigated (Fig. 4). Dissolved oxygen at the level of 15% gave the maximum enzyme production by wild (362U/ml) and mutant (687U/ml) strains. The dry cell mass was 19 and 23.6 g/l, respectively. Beyond this level, a decrease in enzyme production was recorded.

The size of inoculum has direct effect on the growth of organism and enzyme production as reported by Allan *et al*., (1996) and Shafique *et al*., (2009). Different inoculum sizes 5-12.5%, v/v (Fig. 5) were tested for enzyme production in fermenter. Of all the inoculum size tested, 10% of inoculum containing 2.6×10^6 conidia/ml was found to be optimum for the enzyme production in fermenter. As the inoculum size was further increased, the enzyme production was decreased. It was due to the fact that over growth of *A. oryzae* produced anaerobic conditions during the fermentation and it consumed majority of substrate for growth and metabolic processes, hence enzyme production was reduced. As the inoculum size was decreased, the enzyme production was also decreased. The reason might be inadequate amount of mycelia produced at low level of conidia which in due course decreased enzyme production.

Evaluation of kinetic parameters $Y_{p/x,}$ $Q_{p,}$ Q_x revealed that production yield by wild and mutant strains was optimum under the following conditions incubation time 64 h (wild) 48 h (mutant), pH 5.0, agitation 200 rpm, dissolve oxygen 15% , aeration 1.0 vvm (wild) 1.5 vvm (mutant), inoculum size 10% and 30°C (Tables 1,2,3,4,5 and 6).

Table 1. Kinetic evaluation of rate of fermentation for the alpha amylase production by *A. oryzae* IIB-30 and its mutant derivatives in stirred fermenter.

Kinetic parameters	Wild	Mutant
μ	0.2	0.22
$Y_{p/x}$	55000	185714
Q_p	5583	10133
Q_x	0.30	0.33
q_p	11000	40857

Kinetic parameters: $Y_{p/x}$= Enzyme produced/g cell mass formation, Q_p = Enzyme produced/l/h, Q_x= g cell mass formation/l/h, q_p=U/g/h, $\mu(h^{-1})_{max}$ = Specific growth rate.

Table 2. Kinetic evaluation of different pH values of media for the alpha amylase production by *A. oryzae* IIB-30 and its mutant derivative in stirred fermenter.

pH	4		4.5		5.0		5.5		6.0		6.5	
Kinetic parametes	Wild	Mutant	Wild	Mutant	Wild	Mutant	Wild	Mutant	Wild	Mutant	Wild	Mutant
$Y_{p/x}$	20915	28032	21487	30353	21678	36066	21551	33604	19607	27822	18288	24642
Q_p	4333	9016	5166	9633	5700	10433	53333	10016	4166	8550	3333	6900
Q_x	0.20	0.25	0.23	0.28	0.31	0.37	0.25	0.33	0.19	0.30	0.17	0.28

Kinetic parameters: $Y_{p/x}$= Enzyme produced/g cell mass formation, Q_p= Enzyme produced/l/h, Q_x= g cell mass formation/l/h

Table 3. Kinetic evaluation of different aeration levels for the alpha amylase production by *A. oryzae* IIB-30 and its mutant derivative in stirred fermenter.

Aeration (vvm)	0.5		1.0		1.5		2.0	
Kinetic parameters	Wild	Mutant	Wild	Mutant	Wild	Mutant	Wild	Mutant
$Y_{p/x}$	18518	27899	19050	28571	18881	310928	18181	28416
Q_p	4500	9483	5833	10466	5016	11000	4000	10183
Q_x	0.23	0.31	0.31	0.36	0.26	0.38	0.22	0.35

Kinetic parameters: $Y_{p/x}$ = Enzyme produced/g cell mass formation, Q_p= Enzyme produced/l/h, Q_x= g cell mass formation/l

Table 4. Kinetic evaluation of different levels of dissolved oxygen for the alpha amylase production by *A. oryzae* IIB-30 and its mutant derivative in stirred fermenter.

Dissolve oxygen (%)	5.0		10		15		20	
Kinetic parameters	Wild	Mutant	Wild	Mutant	Wild	Mutant	Wild	Mutant
$Y_{p/x}$	19052	28903	19937	31840	20133	32258	19655	29110
Q_p	4750	10000	5033	10983	6033	11450	5350	10666
Q_x	0.24	0.31	0.25	0.38	0.31	0.39	0.26	0.33

Kinetic parameters: $Y_{p/x}$= Enzyme produced/g cell mass formation, Q_p= Enzyme produced/l/h, Q_x= g cell mass formation/l/h

Table 5. Kinetic evaluation of different inoculum sizes for the alpha amylase production by *A. oryzae* IIB-30 and its mutant derivative in stirred fermenter.

Inoculum (%)	5.0		7.5		10		12.5	
Kinetic parameters	Wild	Mutant	Wild	Mutant	Wild	Mutant	Wild	Mutant
$Y_{p/x}$	19375	29570	25396	29792	25486	31093	21549	28506
Q_p	4800	10500	5333	11483	6200	11966	5100	9950
Q_x	0.18	0.36	0.21	0.38	0.32	0.40	0.23	0.32

Kinetic parameters: $Y_{p/x}$= Enzyme produced/g cell mass formation, Q_p= Enzyme produced/l/h, Q_x= g cell mass formation/l/h

Table 6. Kinetic evaluation of different agitation speeds for the alpha amylase production by *A. oryzae* IIB-30 and its mutant derivative in stirred fermenter

Agitation intensity	120 rpm		160rpm		200 rpm		240 rpm	
Kinetic parameters	Wild	Mutant	Wild	Mutant	Wild	Mutant	Wild	Mutant
$Y_{p/x}$	19254	30120	19473	31818	29215	39622	19444	30338
Q_p	4966	10500	6166	11933	6416	12500	5166	11666
Q_x	0.17	0.26	0.31	0.39	0.33	0.41	0.26	0.36

Kinetic parameters: $Y_{p/x}$= Enzyme produced/g cell mass formation, Q_p= Enzyme produced/l/h, Q_x= g cell mass formation/l/h

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