International Journal of Engineering Research & Technology (IJERT)
ISSN: 2278-0181
Vol. 7 Issue 06, June-2018

Medium Optimization for Production of A-Amylase by Bacillus Subtilis and Aspergillus Niger in Submerged Fermentation

M. B. Khadse
Department of Agricultural Engineering
Maharashtra Institute of Technology
Aurangabad, Maharashtra, India

K. P. Babar
Department of Agricultural Engineering
Maharashtra Institute of Technology
Aurangabad, Maharashtra, India

***Abstract*— The present study was carried out to optimize the fermentation medium for production of α-amylase using *Bacillus subtilis* and *Aspergillus niger* strain in submerged fermentation. The effects of incubation period, temperature, p^H, different carbon source, nitrogen source and metal salt of the medium were optimized. The maximum production of enzyme was found at 72 h (3.04 U/ml) in *Bacillus subtilis* and (4.35 U/ml) in *Aspergillus niger*. The optimal temperature and p^H were found to be 35^0c at p^H 7 in *Bacillus subtilis* and 45^0c at p^H 7 in *Aspergillus niger*. Starch (3.04 U/ml) and peptone (3.92 U/ml) was observed to be best carbon and nitrogen source in *Bacillus subtilis* and in *Aspergillus niger* it was found to be starch (3.05 U/ml) and casein (1.36 U/ml) respectively. The effect of different metal salt were investigated and it as found that $MgSO_4$ (3.14U/ml) and (2.96 U/ml) was observed to be best metal salt source in *Bacillus subtilis* and *Aspergillus niger* respectively.**

Keywords*— *Bacillus subtilis, Aspergillus niger, α-amylase

I. INTRODUCTION

In modern times most important products of biology origin are enzymes, because they have numerous applications in various industrial processes[1]. The world market for industrial enzymes is estimated to be 1.6 billion, split between food enzymes (29%), feed enzymes (15%) and general technical enzymes (56%)[2]. Food enzymes are the most widely used and still represent the major share in enzyme market.

Amylase constitute a group of industrial enzymes, which alone covers approximately 30% of the enzyme market[3]. Alpha amylase is a hydrolase enzyme that catalyses the hydrolysis of internal α-1.4-glycosidic linkages in starch to yield products like glucose and maltose. It is a calcium metalloenzyme i.e. it depends on the presence of a metal co factor for its activity[4]. These uses have placed greater stress on search for novel α-amylase for more efficient processes[5]. These enzymes are extensively employed in processed food industry such as baking, brewing, preparation of digestive aids, production of cakes, fruit juices and starch syrups[6].

In recent year, the potential of using severa micro-organisms as biotechnological sources of industrially relevant enzymes has stimulated interest in the exploration of extracellular enzymatic activity[7]. Amylase can be produced by different species of micro-organisms, but for commercial applications amylase is mainly derived from the genus *Bacillus*[8]. Several enzymes of industrial importance have been extracted from the fungai belonging to the genus *Aspergillus*[9].

Fermentation is the technique of biological conversion of complex substrates into simple compounds by various micro-organisms such as bacteria and fungai.Fermentation has been classified into ssf and smf mainly based on the type of substrate used during fermentation. Smf utilizes free flowing liquid subtstrates, such as molasses and broths.The bioactive compounds are secreated into the fermentation broth.An additional advantage of the this technique is that purification of products is easier.Usually production of amylase has been carried out using well defined chemical media by submerged fermentation (smf).[10].

The present study was carried out to optimize the fermentation medium for the production of α-amylase using different micro-organism in submerged fermentation.

II. MATERIAL AND METHOD

A. *Procurement of raw material*

Bacillus subtilis (NCIM 2439) and *Aspergillus niger* (NCIM 1054) these two microbial strain were procured from National Laboratory (NCIM) Pune, Maharashtra, India.These strain was sub cultured on nutrient agar and potato dextrose agar slant respectively and maintained at 4^0c. Various chemicals and instruments used were obtained from Department of Agricultural Engineering Maharashtra Institute of Technology, Aurangabad.

B. *Methods*

a. Preparation of Inoculum and addition

The inoculum was prepared by the addition of sterile distilled water in to the freshly grown agar slants, from this 0.5ml suspension was inoculated in to 100ml of sterilized fermentation medium and incubated in a shaking incubator at 120rpm[11].

b. Fermentation media

Optimization of α-amylase production by *Bacillus subtilis* and *Aspergillus niger* was carried out by using submerged fermentation. The fermentation medium for *Bacilllus subtilis* was (g/l) 6.0g bacteriological peptone, 0.5g $MgSO_4.7H_2O$, 0.5g KCl, 1.0g starch and fermentation medium for *Aspergillus niger* was (g/l) 0.1g $MgSO_4$, 1.0g $(NH_4)_2SO_4$, 2.0gKH_2PO_4, 7.0g K_2HPO_4, 0.5g Na-citrate and the fermentation medium was maintained at p^H 7[12][13].

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c. Enzyme production

Erlenmeyer flasks (250 ml) containing 100ml of amylase producing fermentation medium were inoculated with 0.5 ml suspension of culture and incubated at 120rpm at 35^0c[14].

d. .Extraction of amylase enzyme from the fermentation medium

Amylase after incubation, the fermentation medium was harvested by centrifuged at 5000 rpm for 20 minutes at 4^0c.The supernantant was collected and the amylase activity estimated[12].

e . Assay of amylase

The amylase activity was determined by incubating 1ml of soluble starch (1%) in 0.1M phosphate buffer (p^H 6.9) are pre incubated at 37^0c for 10 minutes. The supernantant collected from the centrifugation of the production media was used as enzyme source, 0.1-0.5ml of this was added to the reaction mixture. The reaction was terminated by the addition of 1.0ml of 3,5-dinitrosalicylic acid reagent after incubation at 37^0c for 30 minutes. The contents were mixed well and kept in water bath for 10 minutes. Then they were cooled and diluted with 10 ml of distilled water. The colour developed was read at 540nm. One unit of enzyme activity was defined as the amount of enzyme that release 1µ mole of reducing sugar (maltose) per minute under the assay condition[15].

C. Optimization studies for enzyme production

In a sequential order, the various process parameter affecting the enzyme production were optimized for maximal enzyme production for which best amylase activity was observed. The different optimization process parameter are as follows.

a. *Effect of incubation time:-*

The effect of incubation period was optimized by incubating production medium for different incubation peroids(24hr, 48hr, 72hr)[16].

a. *Effect of temperature:-*

The effect of temperature on amylase production was investigated by incubating the fermentation medium at different temperatures (25^0c, 35^0c, 45^0c)[17].

b. *Effect of p^H:-*

The effect of p^H on amylase production was investigated by varying the p^H values set to (6.0,7.0, 8.0)[13].

c. *Effect of carbon sources:-*

The effect of different carbon sources such as starch, maltose and glucose each at 1% concentration was investigated on amylase production[14].

d. *Effect of nitrogen sources:-*

The effect of different nitrogen sources such as peptone, casein, ammonium sulphate each at 1% concentration was investigated on amylase production[14].

e. *Effect of metal salts:-*

The effect of metal salts on α-amylase production was determined by adding different metal salts in the fermentation medium.The metal salts selected for present study are $FeSO_4$, $MgSO_4$, $ZnSO_4$[16].

III. EXPERIMENTS AND RESULTS

The experiments were conducted for "The optimization of culture conditions based on the stepwise modification of the governing parameter such as incubation time, incubation temperature, incubation p^H, carbon source, nitrogen source and metal salt source". Data presented here show that *Bacillus subtilis* and *Aspergillus niger* produces α-amylase. The optimal conditions for α-amylase production have been determined under submerged fermentation conditions.

The results obtained during the present investigation are presented and discussed under suitable heading. The results were discussed in the view of relevant scientific literature available in the country and elsewhere:

A. Incubation time

Effect of different incubation time was determined by incubating production medium for different incubation periods.

Table 1 Effect of different incubation time on amylase production from Bacillus subtilis and Aspergillus niger by submerged fermentation

Sr. No.	Microbial strain used	Time in hours	O.D. at 540nm	Mg of maltose released	Enzyme activity (IU/ml)
1.	Bacillus subtilis	24hr	0.623	1.020	2.82
		48hr	0.679	1.075	2.97
		72hr	0.690	1.100	3.04
2.	Aspergillus niger	24hr	0.024	0.094	0.26
		48hr	0.391	0.738	2.04
		72hr	0.998	1.574	4.35

*O.D.: Optical dentisity

From the above table it is revealed that among the three timings activity tested for amylase production viz; 24, 48 and 72 hours, the optimum incubation period for α-amylase poduction was noted at 72hrs and production found decreased in the rest of the incubation period.

Strain *Bacillus subtilis* showed the maximum activity of 3.04U/ml while starain *Aspergillus niger* showed the maximum activity of 4.35U/ml with the temperature of incubation period was kept at 35^0c and p^H 7. Nearly similar observations were obtained by[12][13].

B. Incubation temperature

Effect of different incubation temperature was determined by incubating production medium for different incubation temperature.

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Table 2 Effect of Different Incubation Temperature On Amylase Production From Bacillus Subtilis And Aspergillus Niger By Submerged Fermentation

Sr. No.	Microbial strain used	Temperature in (^{0}c)	O.D. at 540nm	Mg of maltose released	Enzyme activity (IU/ml)
1.	Bacillus subtilis	25^{0}c	0.291	0.550	1.52
		35^{0}c	1.810	2.363	6.53
		45^{0}c	0.865	1.321	3.65
2.	Aspergillus niger	25^{0}c	0.491	0.839	2.32
		35^{0}c	0.631	1.111	3.07
		45^{0}c	0.923	1.495	4.13

*O.D.: Optical dentisity

Temparature is one of the important physical factor infuencing enzyme production. The production medium incubated at different temperature i.e. 25^{0}c, 35^{0}c and 45^{0}c.

From the above table it is revealed that strain *Bacillus subtilis* when icubated at different temperature it was found that incubation temperature of 35^{0}c was found to be optimum for maximum amylase activity i.e. 6.53U/ml and for strain *Aspergillus niger* it was found to be 4.13U/ml at 45^{0}c with the production medium was kept for 72 hrs at 7 p^H.

C. Incubation p^H

Effect of different incubation p^H was determined by incubating production medium for different incubation p^H.

Table 3 Effect of different incubation ph on amylase production from Bacillus subtilis and Aspergillus niger by submerged fermentation

Sr. No.	Microbial strain used	P^H	O.D. at 540nm	Mg of maltose released	Enzyme activity (IU/ml)
1.	Bacillus subtilis	6	1.021	1.524	4.21
		7	2.310	2.750	7.62
		8	1.561	2.143	5.92
2.	Aspergillus niger	6	0.381	0.684	1.89
		7	0.601	0.973	2.69
		8	0.176	0.405	1.12

*O.D.: Optical dentisity

Amylase production indicates greater influence by p^H. The production medium incubated at different p^H i.e. 6, 7 and 8. When p^H is altered below or above the optimum activity appear to be decreased or becomes denatured. Different ogranism have different p^H optima.

From the above table it is revealed that strain *Bacillus subtilis* when icubated at different p^H it was found that incubation P^H of 7 was found to be optimum for maximum amylase activity i.e. 7.62 U/ml with temperature of production medium was kept at 35^{0}c for 72 hrs. and for strain *Aspergillus niger* maximum amylase activity was found to be 2.69 U/ml at optimum p^H 7 with the production medium was kept for 72 hrs at 45^{0}c.

D. Carbon source

Effect of different carbon source (1% concentration) was determined by incubating production medium for different carbon sources.

Table 4 Effect of Different Carbon Source on Amylase Production From Bacillus Subtilis And Aspergillus Niger By Submerged Fermentation

Sr. No.	Microbial strain used	Carbon source	O.D. at 540nm	Mg of maltose released	Enzyme activity (IU/ml)
1.	Bacillus subtilis	Glucose	0.443	0.756	2.09
		Maltose	0.568	0.923	2.55
		Starch	0.691	1.100	3.04
2.	Aspergillus niger	Glucose	0.326	0.604	1.67
		Maltoe	0.622	1.071	2.96
		Starch	0.554	1.104	3.05

*O.D.: Optical dentisity

Different carbon sources like glucose, maltose and starch were used at 1% level in the current study. The result revealed that the different requirements of carbon source for different organisms.

From the above table it is revealed that strain *Bacillus subtilis* when incubated at different carbon sources it was found that starch was found to be optimum carbon source for maximum amylase activity i.e. 3.04 U/ml with temperature of production medium was kept at 35^{0}c for 72 hr and p^H 7 and for strain *Aspergillus niger*, optimum carbon source was found to be starch for maximum amylase activity i.e. 3.05 U/ml with the production medium was kept at 45^{0}c for 72 hrs at p^{H}7.

E. Nitrogen source

Effect of different nitrogen source (1% concentration) was determined by incubating production medium for different nitrogen sources.

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Table 5 Effect of different nitrogen source on amylase production from Bacillus subtilis and Aspergillus niger by submerged fermentation

Sr. No.	Microbial strain used	Nitrogen source	O.D. at 540nm	Mg of maltose released	Enzyme activity (IU/ml)
1.	Bacillus subtilis	Peptone	0.931	1.419	3.92
		Casein	0.691	1.107	3.06
		Ammonium sulphate	0.584	0.933	2.58
2.	Aspergillus niger	Peptone	0.010	0.054	0.15
		Casein	0.241	0.492	1.36
		Ammonium sulphate	0.125	0.333	0.92

*O.D.: Optical dentisity

Different nitrogen sources like peptone, casein and ammonium sulphate were used at 1% level in the current study.

From the above table it is revealed that strain *Bacillus subtilis* when incubated at different nitrogen sources it was found that peptone was found to be optimum nitogen source for maximum amylase activity i.e. 3.92 U/ml with temperature of production medium was kept at 35^0c for 72 hr and p^H 7 and for strain *Aspergillus niger*, optimum nitrogen source was found to be casein for maximum amylase activity i.e. 1.36 U/ml with the production medium was kept at 45^0c for 72 hrs at p^H7.

F. Metal salt

Effect of different metal salt (0.1% concentration) was determined by incubating production medium for different mteal salt sources.

Table 6 Effect of Different Metal Salt Source on Amylase Production From Bacillus Subtilis And Aspergillus Niger By Submerged Fermentation

Sr. No.	Microbial strain used	Carbon source	O.D. at 540nm	Mg of maltose released	Enzyme activity (IU/ml)
1.	Bacillus subtilis	$MgSO_4$	0.721	1.136	3.14
		$ZnSO_4$	0.00	0.00	No growth found
		$FeSO_4$	0.00	0.00	No growth found
2.	Aspergillus niger	$MgSO_4$	0.621	1.071	2.96
		$ZnSO_4$	0.00	0.00	No growth found
		$FeSO_4$	0.001	0.00	No groth found

*O.D.: Optical dentisity

Different metal salt sources like $MgSO_4$, $ZnSO_4$ and $FeSO_4$ were used at 0.1% level in the current study. Supplementation of salts certain ions provided good growth of bacteria and fungai and thereby better enzyme production.

From the above table it is revealed that strain *Bacillus subtilis* when incubated at different metal salt source it was found that $MgSO_4$ was found to be optimum metal salt source for maximum amylase activity i.e. 3.14 U/ml with temperature of production medium was kept at 35^0c for 72 hr and p^H 7 and used starch and peptone as a best optimum carbon and nitrogen source respectively.

For strain *Aspergillus niger*, optimum metal salt source was found to be $MgSO_4$ for maximum amylase activity i.e.2.96 U/ml with the production medium was kept at 45^0c for 72 hrs at p^H7 and used starch and casien as a best optimum carbon and nitrogen source respectively.

IV. CONCLUSION

Thus in the light of scientific data of the present investigation it may be concluded that bacterial species and fungal species can be a good source for the production of a very important enzyme amylase being used industrially. The amylases were produced by optimizing the various parameter and it was revealed that in *Bacillus subtilis*, maximum amylase activity was found to be 3.14U/ml with optimum time, temperature, p^H,carbon , nitrogen and metal source was found to be 72hr, 35^0c, p^H 7 ,starch ,peptone and $MgSO_4$ respectively. While in *Aspergillus niger*, maximum activity was found to be 2.96 U/ml with optimum time, temperature, p^H, carbon, nitrogen and metal salt was 72hr, 45^0c, p^H 7, starch casein and $MgSO_4$ respectively.

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