FINAL REPORT

LARGE-SCALE PRODUCTION OF α-AMYLASE FROM *ASPERGILLUS ORYZAE*

RG/96/BT/04

DEPARTMENT OF BIOCHEMISTRY,

FACULTY OF MEDICINE,

UNIVERSITY OF JAFFNA,

KOKUVIL.

Revised

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A. IT SHOULD CONTAIN THE FOLLOWING INFORMATION:

1. (a) Contract Number :	RG/96/BT/04
(b) Title of Project:	Large-scale production of α-amylase from *Aspergillus oryzae*
(c) Institution where research was carried out:	Department of Biochemistry, Faculty of Medicine, University of Jaffna, Kokuvil.
(d) Chief Scientific investigator and co-investigator:	Prof. V. Arasaratnam and Prof. K. Balasubramaniam
(e) Date of Award:	April 1996
(f) Date of Completion:	October 1998
(g) Total allocation:	350,000/- (Received only 269,250/-)
(h) Total spent:	269,250/-
(i) No. of Research Assistants / Technical Assistants and period of service:	Februaary 1997 to March 1998
(j) Whether RA has registered for, or obtained, postgraduate degree:	Registered for Ph.D.

2. DESCRIPTION OF RESEARCH CARRIED OUT

2.1 SELECTION OF α-AMYLASE PRODUCING *ASPERGILLUS ORYZAE*

2.1.1 Collection of strains from different sources and purification of fungal strains

Samples (10g) were collected under sterile conditions in sterile bottles from different sources. In total six solid samples were collected and mixed with 25ml sterile normal saline ($9.0gl^{-1}$ NaCl). From the saline infusion, samples were taken and inoculated to potato dextrose agar (PDA) plates. The seventh sample (G) from laboratory environment was collected by keeping the PDA plates opened in the laboratory for 15 minutes. The plates were inoculated at room temperature. From the fungal colonies developed, samples were taken and repeated cultivation was carried at 30°C to obtain pure culture. From each sample the colonies obtained were named as for example the colonies obtained from sample A were labeled as A_1, A_2, A_3, and so on.

2.1.2 Selection of potential α-amylase producers

The selected *Aspergillus oryzae* strains were separately grown on PDA plates. After the development of the colonies (4^{th} day) 100ml iodine reagent (I_2, $2.0gl^{-1}$ and KI, $20.0gl^{-1}$) was added to the PDA plates and the excess was removed after 30 seconds. The diameters of the colour less (halos) areas in starch-iodine plates were measured.

2.1.3 Liquid & solid media

Solid media contained soy meal powder (500g) and mineral solution (500ml). The mineral solution contained (gl^{-1}) $FeSO_4$, 0.062, $MgSO_4$ $7H_2O$, 0.063 and $CuSO_4$ $5H_2O$, 0.01.

The liquid media contained soy meal powder (500g), the above mineral solution (500ml) and 500ml distilled water.

2.1.4 Preparation of inocula

Strains A_3, B_{12} and C_6 were sub cultured once in every two weeks on PDA slants and stored at 4°C. Whenever the spores were required, they were suspended in 1.0% (v/v) Tween – 80. The number of spores was counted using a haemocytometer.

2.1.5 Cultivation of the selected strains in solid and liquid media

Solid medium (100g) was taken in a conical flask sterilized and inoculated with 4day old spores of the three different strains (5 x 10^7 spore g^{-1} medium). Similarly the liquid medium was also inoculated with the 4 days old spores (5 x 10^7 spore ml^{-1} medium). The solid and liquid media were incubated at room temperature. The liquid medium was kept in an orbital shaker (100rpm). The α-amylase produced was estimated.

2.1.6 Extraction of the enzyme from mouldy media

To estimate the enzyme activity produced in the solid state fermentation, the mouldy medium was mixed with of 0.01 M citrate – phosphate buffer (pH 5.1) in 1:5 ratio for 30 min. The enzyme extract was appropriately diluted and used for the enzyme assay.

The spent medium of submerged fermentation was appropriately diluted for enzyme assay.

2.1.7 Determination of α-amylase activity

Activity of α-amylase was determined by measuring reducing sugar with alkaline 3,5 dinitrosalicylic acid (DNS) reagent after 5 min hydrolysis of $10gl^{-1}$ starch at 30°C and pH 5.1.

One unit of α-amylase activity was defined as the amount of enzyme that released 1μmole of reducing sugars from $10gl^{-1}$ starch solution in one minute at 30°C. The

activity of α-amylase in the solid medium is presented as Ug^{-1} DMM (Dry Mouldy Medium).

2.2 EXTRACTION STUDIES

2.2.1 Effect of extraction time

The solid medium was mixed with 0.01M citrate-phosphate buffer (pH 5.1) in 1:5 ratio at room temperature for different period of time from 1 to 60 min at 150rpm. The optimum time for extraction was determined.

2.2.2 Determination of the suitable extractant

The mouldy medium was mixed with different extractants such as distilled water, tap water, $10gl^{-1}$ glycerol, $10gl^{-1}$ NaCl and 0.01M citrate – phosphate buffer (pH 5.0) to optimized extraction time. The mouldy medium to extractant ratio was 1:5.

2.2.3 Determination of optimum mouldy medium to extractant

The mouldy medium to extract ratio (1:3, 1:5, 1:10, 1:15 and 1:20) was varied at the optimized extraction time with the best extractant.

2.2.4 Determination of the optimum pH of the extractant

With most suitable extractant, under optimized extraction time and mouldy medium to extractant ratio, the optimum pH (in the range of 4.0 to 6.5) was determined to extract α-amylase.

2.3 KINETIC STUDIES OF THE FUNGAL α-AMYLASE

2.3.1 Effect of time

Starch ($10gl^{-1}$) in 0.01 M citrate-phosphate buffer (pH 5.1) was mixed with the enzyme extract and incubated at 30°C while mixing. The product formed was

measured as reducing equivalents using dinitrosalicylic acid. From this experiment the reaction time for kinetic studies was fixed.

2.3.2 Optimum pH

Optimum pH for the activity of the α-amylase was studied with the pH values in the range from 3.2 to 7.6 with 0.4 units difference at 30°C using. 0.01M citrate-phosphate buffer at the fixed reaction time.

2.3.3 Effect of temperature

Effect of temperature on the activity of α-amylase was determined at different temperatures from 30 to 80°C with 5°C difference at optimum pH and fixed reaction time.

2.4 OPTIMIZATION OF CULTURE CONDITIONS

2.4.1 Effect of the age of the spore inoculum

To the solid medium spore inocula of the age of 4, 6, 9, 11, 13 and 27 day were added and incubated at 30°C. The size of the spore inoculum added was 5.0×10^7 spores gl^{-1} medium.

2.4.2 Effect of the size of the spore inoculum

To the solid medium, spore inocula of the optimized age with different sizes (4.5×10^5 to 4.5×10^{10} spore gl^{-1} media) were inoculated at 10^1 difference. The inoculated media were incubated at 30°C.

2.4.3 Effect of paddy husk

To the solid medium, wet paddy husk was mixed in the former to later in the ratio of 3:7. The medium was inoculated with 5×10^7 spore g^{-1} medium and incubated at 30°C. The wet paddy husk was prepared by soaking the paddy husk (460g) is water

and suction drying before mixing. The control medium was prepared without the paddy husk.

2.4.3 **Effect of different ratios of soy meal powder to rice bran**

To the soy meal powder, rice bran was mixed in different ratios (5:0, 4:1, 3:2, 2:3, 1:4 and 0:5). Then the total sugar and protein concentrations were estimated and carbohydrate to protein ratio was maintained at the same level by adding purified waxy maize starch (Stardex, Sweden). (Table 1) The media were inoculated with optimized amount of spore inoculum with optimum age and incubated at 30°C.

2.4.4 **Effect of different ratios of soy meal powder to wheat bran**

The above experiment was repeated by replacing rice bran with wheat bran. (Table 2).

3. RESULTS OBTAINED

3.1 SELECTION OF α-AMYLASE PRODUCING *ASPERGILLUS ORYZAE*

3.1.1 Collection and purification of fungal strains

Seven samples were collected for the isolation of α-amylase producing *Aspergillus oryzae*. Here PDA medium was used because it is the more general medium for the fungi growth. From the different samples, it was possible to isolate different number of pure fungal colonies (Table 3) and each was considered as obtained from different strains. From the colony morphology and sporulating nature, it was found that samples obtained from parboiled paddy husk (Sample E), kitchen waste (Sample F) and laboratory environment (Sample G) contained only *Aspergillus niger* strains and not *Aspergillus oryzae*. Among the samples obtained from rice flour (Sample A), rice (Sample B), corn flour (Sample C) and raw paddy husk (Sample D) 25, 31.3, 16.7 and 25% of the strains respectively were of *Aspergillus oryzae*. Thus from a total of 42 fungal strains isolated from different samples 21.4% of them were *Aspergillus oryzae*. That is, strains A_3, A_6, B_1, B_4, B_8, B_9, B_{12}, C_6 and D_2 were *Aspergillus oryzae*. From these nine *Aspergillus oryzae* strains potential α-amylase producers were selected in the following experiments.

3.1.2 Selection of potential α-amylase producers

When the fungal strains are grown on PDA plates, the amylase producers consume starch around the colonies. To identify the potential amylase producers, to the plates, iodine reagent was added after taking the replica of the fungal colonies. The diameters of the clear zones around the colonies are directly proportional to the ability of amylase production. Thus larger the diameter of the clear zone higher is the potential of amylase producer.

Among the *Aspergillus oryzae* strains, those obtained from rice flour (A_3), rice (B_{12}) and corn flour (C_6) formed the colourless areas with the diameters of 5.0, 9.0 and

6.0mm respectively (Table 4). Among the three strains, *Aspergillus oryzae* B_{12} produced the largest diameter. However the strains A_3, B_{12} and C_6 were considered as the most useful strains to produce α-amylase and selected for further studies.

3.1.3 Comparison of the α-amylase production in solid and liquid media by the selected three strains

To select the best α-amylase producer and to select the appropriate method of fermentation this experiment was carried out. The α-amylase produced by the three organisms in solid state fermentation and submerged fermentation are given in Table 5. All three fungal strains produced maximum α-amylase activity in the solid medium and the production of the enzyme was faster in the solid medium than in the liquid medium. The utilization of nutrients was better in solid medium than in liquid medium. Among the three organisms, the performance of *Aspergillus oryzae* B_{12} was the best both in liquid and solid medium. It produced almost double the amount of α-amylase than *Aspergillus oryzae* strains A_3 and C_6. Hence this was selected for further studies. In submerged liquid cultures, filamentous fungi generally produce smaller quantities of secretary enzymes than they do in surface cultivation. As the nutrients were better utilized in solid state fermentation than in submerged fermentation, it was decided to carry out solid state fermentation. Further due to simplicity of the cultivation equipment and the lowest expense for operation, application of surface cultivation method, the traditional method is more appropriate for the less privileged areas. As there is no continuous electricity supply to Jaffna peninsula, it was decided to follow the solid state fermentation method.

3.2 OPTIMUM CONDITIONS FOR THE EXTRACTION OF α-AMYLASE FROM MOULDY MEDIUM

The effect of time on the extraction of α-amylase from mouldy medium was studied. Enzyme extraction was increased up to 30 min and there after no significant increase in enzyme extraction was observed (Table 6). Here α-amylase was extracted from mouldy medium using 0.01M citrate-phosphate buffer (pH 5.1) as extractant. The

mouldy medium to extractant ratio was 1:5. Therefore for the following studies the extraction time was fixed as 30 minutes.

When distilled water, tap water, $10gl^{-1}$ glycerol, $10gl^{-1}$ NaCl at 0.01M citrate-phosphate buffer (pH 5.1) were used as extractants, the enzyme was best extracted by 0.01M citrate-phosphate buffer (pH 5.1) at 30 min (Table 7). Hence this buffer was selected for the following studies.

The mouldy medium to 0.01M citrate-phosphate buffer (pH 5.1) ratio (1:3, 1:5, 1:10, 1:15 and 1:20) was varied and α-amylase was extracted for 30minutes. The maximum enzyme activity was extracted in the ratio of 1:15 (Table 8). However the dilution of the enzyme was very high at this ratio and hence mouldy medium to extractant ratio of 1:8 was selected. Because at this point the enzyme extraction was 92.6% of the maximum amount of enzyme activity that can be obtained with the ratio of 1:15.

When the pH of the 0.01M citrate-phosphate buffer was changed from 4.0 to 6.5, maximum enzyme was extracted at pH 4.5 (Table 9). Hence in the following studies to extract the enzyme 0.01M citrate-phosphate buffer (pH 4.5) was used in the mouldy medium to extractant ratio of 1:8. The extraction time was fixed to 30 min.

3.3 KINETIC STUDIES OF THE FUNGAL α-AMYLASE

In the initial stages of these studies, the reaction conditions to determine the enzyme activity was randomly fixed based on the properties of the other fungal enzymes. After optimizing the extraction conditions the kinetic properties of the crude enzyme were studied.

When the enzyme activity was measured with time the first order kinetics was maintained up to 6 min and hence the reaction time was fixed as 5 min.

When the pH was increased from 3.2, the activity was increased and reached maximum at pH 5.2 (Figure 1). Hence the pH of 0.01M citrate-phosphate buffer was fixed as 5.2 for enzyme assay.

When the reaction temperature for the enzyme activity was increased from 30°C to 80°C in 0.01M citrate-phosphate buffer (pH 5.2) and the reaction time was fixed for 5 min, the maximum activity was obtained at 55°C (Figure 2).

Thus the enzyme activity was determined in 0.01M citrate-phosphate buffer (pH 5.2) at 55°C and the reaction time was fixed for 5 min.

3.4 OPTIMIZATION OF CULTURE CONDITIONS

3.4.1 Effect of the age of the spore inoculum

When the spores of different ages (4, 6, 9, 11, 13 and 27 day) were used as inocula, the highest α-amylase activity (901.3 Ug^{-1}DMM) was obtained with the 4 days old spores (Figure 3). With the increase in the age of the spores the α-amylase production was decreased. This could be due to the decreased germination of the spores with their age. When the age of the spores was increased from 4 to 6 days, the α-amylase production was decreased from 901.3 to 625.3 Ug^{-1} DMM. Therefore 4days old spores were selected for this studies.

3.4.2 Effect of the size of the spore inocula

When the 4 days old spores of different sizes were inoculated to solid medium, the highest amount of α-amylase was produced by 4.5 x 10^8 spore g^{-1} medium (1430.5 Ug^{-1} DMM, Figure 4). With the 4.5 x 10^7 and 4.5 x 10^9 spore g^{-1}medium, the amounts of enzyme activity produced was 86.7 and 84.2% of that obtained with 4.5 x 10^8 spore g^{-1} medium. Thus 4.5 x 10^8 spore g^{-1} medium was selected as the optimum inoculum size.

3.4.3 Effect of paddy husk

When the solid medium was mixed with paddy husk (3:7 ratio), the α-amylase activity obtained at 114h was 593. 2 Ug^{-1} DMM. While in the control medium, the activity obtained at 114h was 1490.0 Ug^{-1} DMM. Thus the incorporation of paddy husk has reduced the α-amylase production.

The solid medium was mixed with paddy husk (solid medium: paddy husk ratio of 3:7) to enable more air circulation so that the fungal growth can be enhanced, leading to a better enzyme production. However the observation showed that the enzyme production with the medium incorporated with paddy husk was less than that in the solid medium (Control) (Table 10). Hence it was decided to avoid paddy husk in the following experiments.

3.4.4 Effect of different ratios of soy meal powder to rice bran

The soy meal powder was only used as the solid medium along with the mineral solution. The amylase production may be improved by changing the medium composition. Hence the soy meal powder to rice bran ratio was varied. The total protein and carbohydrate contents of soy meal powder, rice bran and waxy maize starch are given in Table 11. Based on these data, the protein to carbohydrate ratio in all the media was maintained as 1: 3.3 by adding waxy maize starch to different amounts of soy meal powder and rice bran, containing media. Maximum α-amylase activity was obtained in the medium containing soy meal powder and rice bran in the ratio of 1: 4 (2220 Ug^{-1} DMM) at 96h (Figure 5). This was about 1.53 times higher than just using the soy meal alone with waxy maize starch. Thus it is better to incorporate rice bran with soy meal and this increased amount of enzyme production could be due to the B vitamins present in the rice bran. When rice bran was used alone, 236.6 Ug^{-1} DMM was obtained. This was only 11% of the amount of α-amylase activity obtained with the soy meal to rice bran mixture with the ratio of 1:4: Thus for the improved α-amylase production not only the protein to carbohydrate ratio is important but also the quality of protein should be considered. Thus addition

of rice bran to soy meal powder has tremendously improved the α-amylase production.

3.4.5 **Effect of different ratios of soy meal powder to wheat bran**

In this experiment too the protein to starch ratio was maintained as 1:2.3. When the soy meal powder was mixed with wheat bran in different ratios, maximum α-amylase activity (2179.8 ug^{-1} DMM) (Figure 6) was obtained at 48h. Hence the soy meal powder to wheat bran ratio which gave the highest α-amylase activity was 1:4. This observation was very much similar to that obtained with soy meal powder rice bran mixture, in which case alone the optimum ratio between soy meal powder and rice bran was 1:4 6220.ug^{-1} DMM). The activities obtained were also very much closer to each other. The result obtained with soy meal - rice bran mixture in the ratio of 1:4 was 1.02 times of that obtained with soy meal wheat bran mixture in the ratio of 1:4. Thus the wheat bran and rice bran must be providing the nutrients for α-amylase production similarly. However when wheat bran was used alone, significant amount of α-amylase activity (1240.3 ug^{-1} DMM) produced. This was 5.25 times higher than that obtained with rice bran alone. This is because the ratios of protein to starch in wheat bran and rice bran were 1:2:3 and 1:3:3. As the protein content of rice bran is less, the enzyme production is reduced. However the rice bran with soy meal powder and waxy maize starch behave similar to wheat bran at soy meal to bran ratio of 1:4. Thus in Sri Lanka even though the wheat bran is not available, for α-amylase production rice bran is a good substitute.

4. CONCLUSIONS DRAWN AND RECOMMENDATIONS, IF ANY, FOR IMPLEMENTATION

Among the different *Aspergillus oryzae* strains isolated B12 was the best α-amylase producer. Enzyme was best extracted in 0.01M citrate- phosphate buffer at pH 4.5 in the mouldy medium to buffer ratio of 1.8. Fermentation time for α-amylase production by *A. oryzae* B_{12} has been shortened from 114 to 48h by using the solid medium, containing soy meal flour to wheat bran or rice bran in the ratio of 1.4 inoculated with 4 days old spore inoculum of 4.5×10^8 spores g^{-1} medium density.

Recommendations

Storage stability

Storage stability of α-amylase in unextracted form (Dry mouldy bran) and extracted form need to be studied. The moisture content and other physical conditions to store the mouldy bran will be studied and the comparison between the stability of unextracted and extracted form need to be studied. The storage stability of the α-amylase at different pH values temperatures needs to be determinedwith time.

Purification

Removal of the spores and purification of α-amylase needs further investigation. Purification of α-amylase using alginate beads is being studied.

Scaling up

After optimizing the conditions, production and purification of α-amylase in large scale and production of maltose from starch by *A. oryzae* α-amylase has to be investigated.

5. (A) CITATION OF PERIODICALS REPORTING WORK DONE UNDER THIS CONTRACT, GIVING AUTHOR, TITLE, JOURNAL, VOLUME AND PAGE NUMBER.

(B) OTHER RELEVANT LITERATURE REFERENCE.

1. Ueno, S., Miyama, M., Izumiya, M. and Kusaka, I. (1987). Sdecretary enzyme production and condition of Aspergillus oryzae in submerged liquid culture. ***Appl. Microbiol. Biotechnol.*** **26 :** 273 – 276.

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6. AN EXPLANATIONS OF SIGNIFICANT DEPARTURE FROM THE LEVEL OF ACTIVITY FOR SEEN BY THE CONTRACT

Although it was anticipated to produce α-amylase from *Aspergillus oryzae* 22788, we had to deviate from the original plan, as the organism was a low α-amylase producer. The isolation and screening of a good α-amylase producing *Aspergillus oryzae* has consumed considerable amount of time. However the project could have been completed by scaling up of the process and stabilizing the enzyme, if the assistance would have been extended.

Table 1: Preparation of solid medium containing different ratios of soy meal powder to rice bran.

Constituents	**Soy meal to rice bran ratio**					
	5:0	**4:1**	**3:2**	**2:3**	**1:4**	**0:5**
Soy meal powder (g)	203.5	188.8	160.1	126.4	77.4	-
Rice bran (g)	-	47.2	106.7	189.5	309.8	500
Waxy maize starch (g)	296.5	264.0	233.2	184.1	112.8	-
Mineral solution (ml)	500	500	500	500	500	500

The media were taken in 5-liter conical flasks.

Mineral solution contained (gl^{-1}) $FeSO_4$; 0.062, $MgSO_4.7H_2O$, 0.063 and $CuSO_4.5H_2O$, 0.01.

Starch contents of soy meal powder, rice bran and waxy maize starch were 30.0, 34.0 and 86.3% respectively.

Substrate to mineral solution ratio was 1.0 : 1.0.

The protein to carbohydrate ratio was 1:3.3.

Table 2: Preparation of solid medium containing different ratios of soy meal powder to wheat bran.

Constituents	Soy meal to wheat bran ratio					
	5:0	**4:1**	**3:2**	**2:3**	**1:4**	**0:5**
Soy meal powder (g)	260.6	231.1	193.5	145.9	84.6	-
Wheat bran (g)	-	57.8	129.0	218..9	338.5	500
Waxy maize starch (g)	239.4	211.1	177.5	135.2	76.9	-
Mineral solution (ml)	500	500	500	500	500	500

The media were taken in 5-liter conical flasks.

Mineral solution contained (gl^{-1}) $FeSO_4$; 0.062, $MgSO_4.7H_2O$, 0.063 and $CuSO_4.5H_2O$, 0.01.

Starch contents of soy meal powder, wheat bran and waxy maize starch were 30.0, 34.0 and 86.3% respectively.

Substrate to mineral solution ratio was 1.0 : 1.0.

The protein to carbohydrate ratio was 1:2.3.

Table 3: Number of *Aspergillus oryzae* colonies present among the fungal colonies obtained from different sources. The fungal samples were grown in PDA plates at 30°C.

Source	Sample	Fungal colonies obtained (No.)	*Aspergillus oryzae* colonies (No.)
A	Rice flour	8	2
B	Rice	16	5
C	Corn flour	6	1
D	Raw paddy husk	4	1
E	Parboiled paddy husk	1	Nil
F	Kitchen waste	5	Nil
G	Laboratory environment	2	Nil

Table 4: The diameter of the colourless (halos) zones obtained around the *Aspergillus oryzae* colonies after the addition of iodine solution.

Sample	*Aspergillus oryzae* strain	Colourless zone diameter (mm)
A	A_3	5.0
	A_6	2.0
B	B_1	3.0
	B_4	2.0
	B_8	0.5
	B_9	2.0
	B_{12}	9.0
C	C_6	6.0
D	D_2	1.0

Table 5: α-Amylase produced by the three *Aspergillus oryzae* strains grown in solid state and submerged cultures.

Aspergillus oryzae strain	Amylase activity		Relative activity (%)	
	Solid fermentation (Ug^{-1} DMM)	Submerged fermentation (Uml^{-1})	Solid fermentation	Submerged fermentation
A_3	215.4 (96h)	28.3 (114h)	51.5	49.2
B_{12}	417.9 (96h)	57.5 (114h)	100	100
C_6	264.2 (96h)	38.2 (114h)	63.2	66.4

DMM* – Dry Mouldy Medium.

The time at which maximum α-amylase activity obtained is given in brackets.

Table 6: Effect of time on the extraction of α-amylase from *Aspergillus oryzae* mouldy medium using 0.01M citrate-phosphate buffer (pH 5.1) as the extractractant with the ratio of the former to the later as 1:5.

Time (min)	Relative activity (%)
1	50.0
5	66.7
10	75.0
15	79.2
20	83.2
25	91.5
30	100.0
35	100.0
40	100.0
45	100.0
50	100.0
60	100.0

$$\text{Relative activity (\%)} = \frac{\text{Activity obtained under a condition}}{\text{Maximum activity obtained at that set of reactions}} \times 100$$

Table 7: Effect of different extractants on the extraction of α-amylase from *Aspergillus oryzae* mouldy medium at 30min. The mouldy medium to extractant ratio of 1:5 was used.

Extractant	Relative activity (%)
Distilled water	65.2
Tap water	07.2
Glycerol ($10gl^{-1}$)	90.5
NaCl ($10gl^{-1}$)	70.1
0.01M Citrate-phosphate buffer (pH 5.1)	100

Table 8 : Effect of mouldy medium to 0.01M citrate phosphate buffer (pH 5.1) ratio on the extraction of α-amylase from *Aspergillus oryzae* mouldy medium. The extraction time was 30 min.

Mouldy medium to extractant ratio	**Relative activity (%)**
1:3	54.5
1:5	65.4
1:8	92.6
1:10	96.6
1:15	100
1:20	100

Table 9: Effect of pH on the extraction of a-amylase from mouldy medium of *Aspergillus oryzae* B12. Mouldy medium to extractant (0.01M citrate-phosphate buffer) ratio was 1:8.Extraction time was 30min.

pH	Relative activity (%)
4.0	92.5
4.5	100.0
5.0	90.2
5.5	84.3
6.0	75.2
6.5	70.3

Table 10: Effect of incorporation of paddy husk into the solid medium the production of α-amylase by *Aspergillus oryzae* B_{12}.

Condition	Activity (Ug^{-1} DMM)
Test	593.2 (114h)
Control	1490.0 (114h)

Table 11: The protein and carbohydrate contents of soy meal powder, rice bran and waxy maize starch.

	Protein (%)	**Carbohydrate (%)**
Soy meal powder	47.2	30.0
Rice bran	10.3	34.0
Waxy maize starch	00.0	08.3
Wheat bran	15.2	34.0

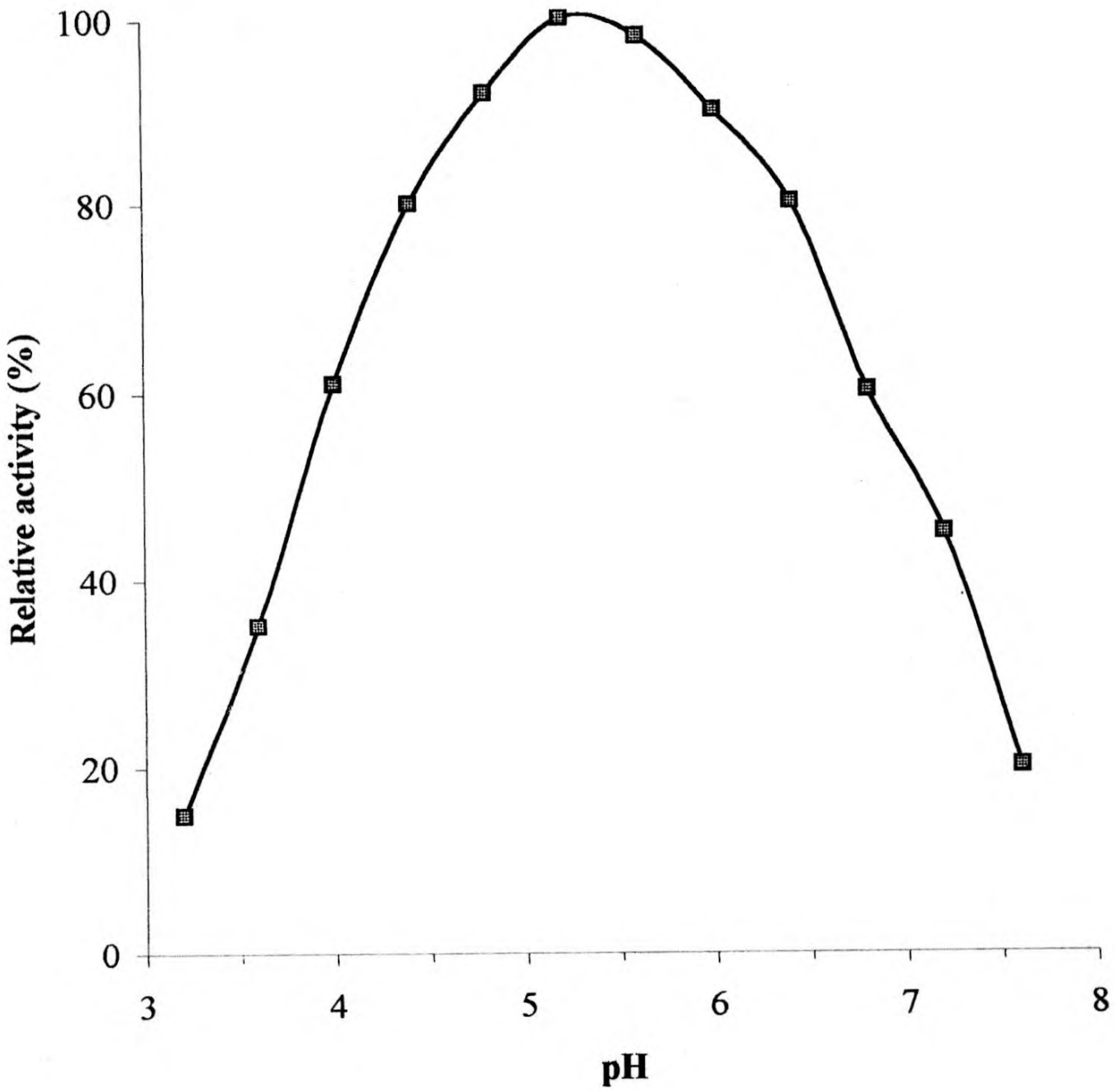

Figure 1: Effect of pH on the activity of α-amylase produced by *Aspergillus oryzae* B12 at 30°C and 5 min.

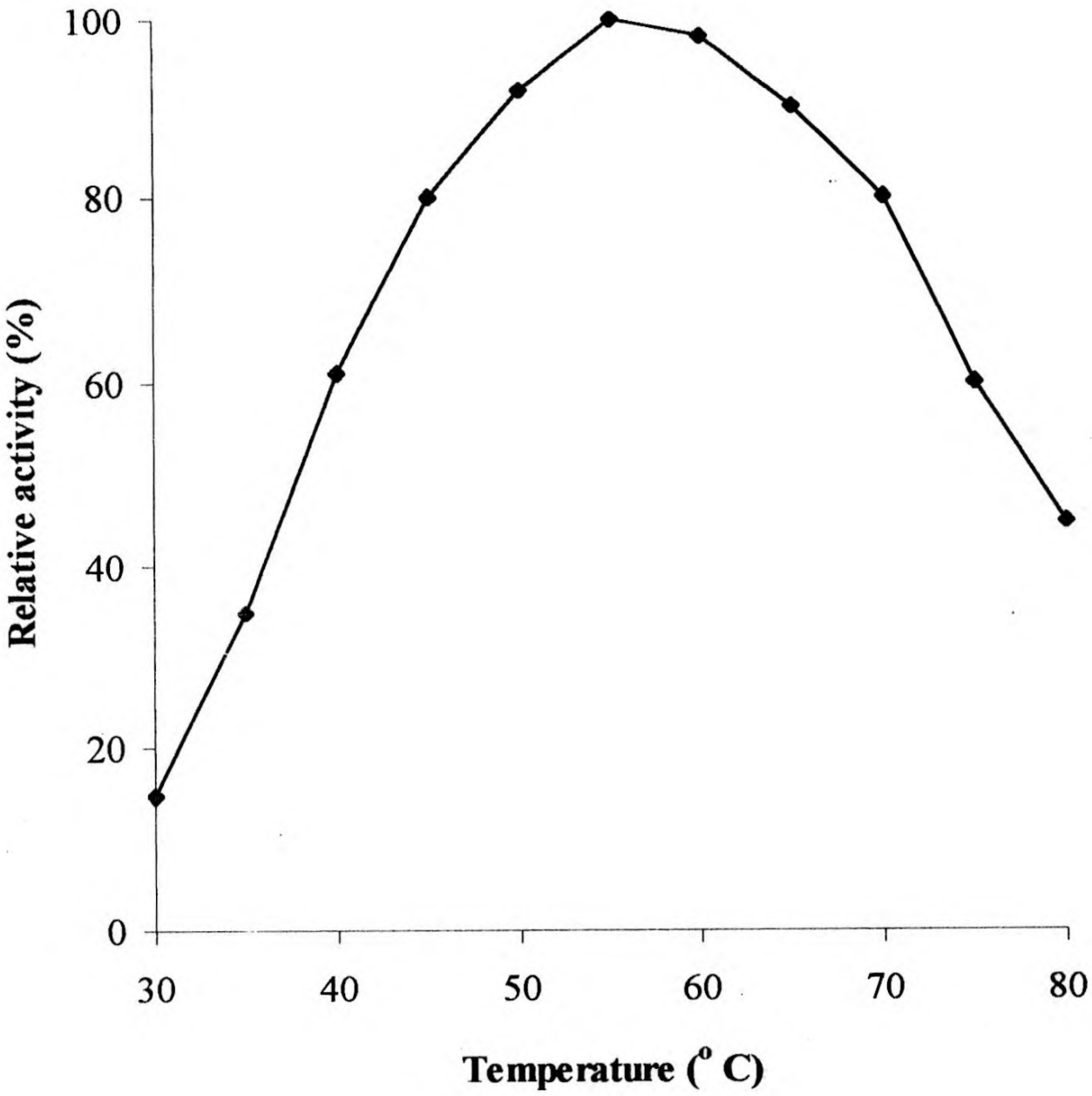

Figure 2: Effect of temperature on the activity of α-amylase produced from *Aspergillus oryzae* B12 at pH 5.2 and 5 min.

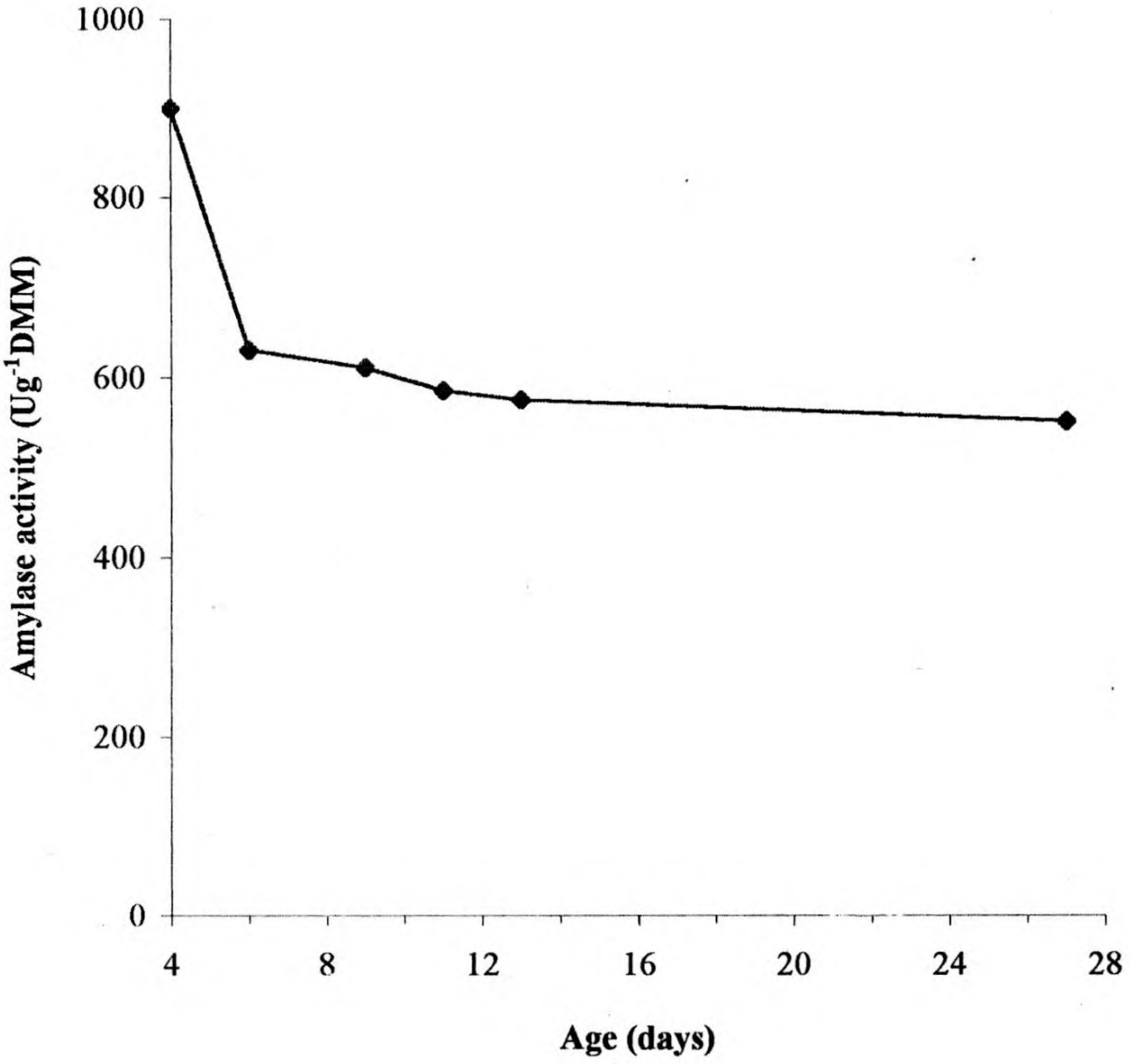

Figure 3: Effect of age of the *Aspergillus oryzae* B12 spore inoculum on the production of α-amylase in solid medium.

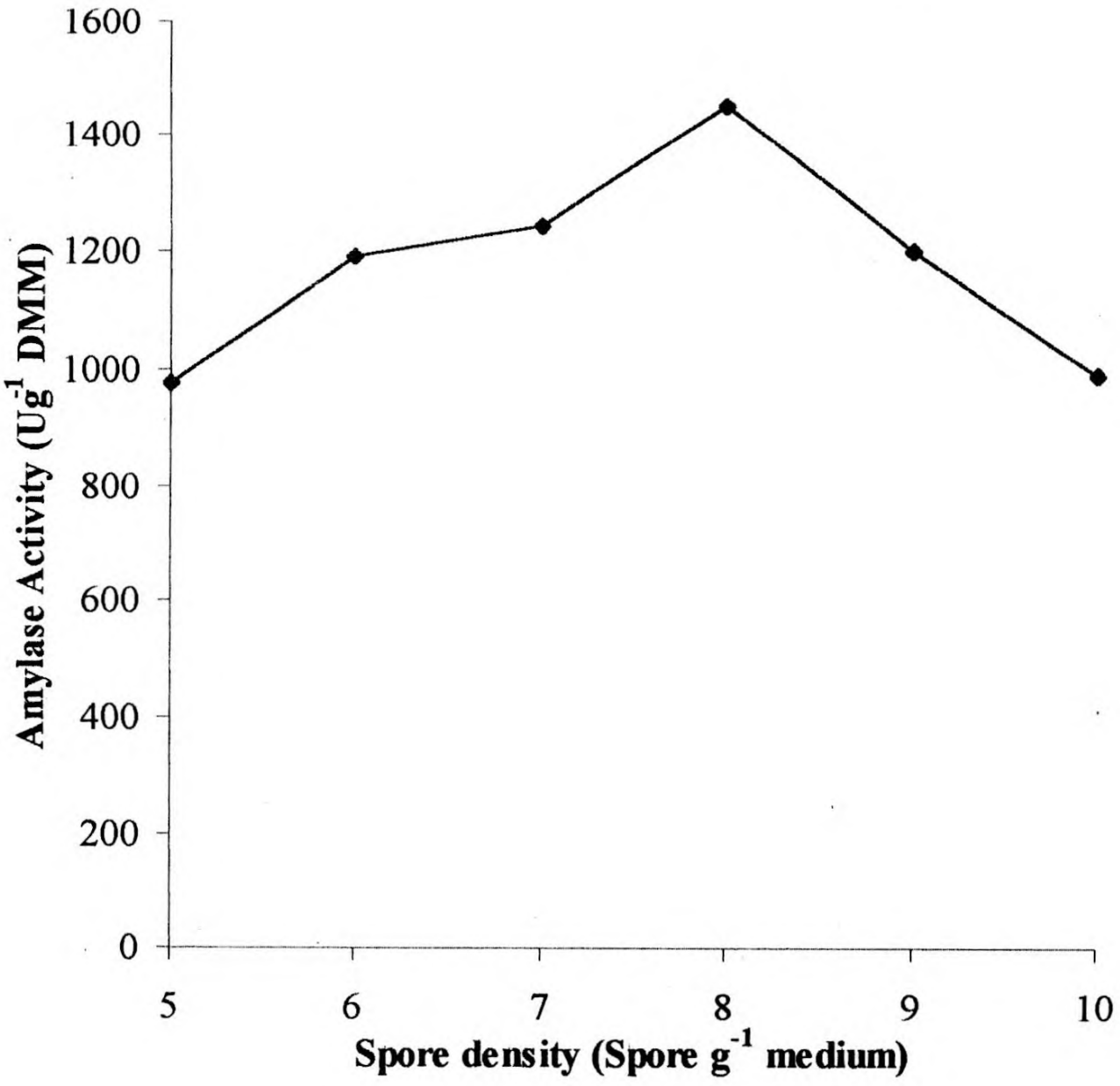

Figure 4: Effect of different sizes of the 4 days old *Aspergillus oryzae* B12 spore inoculum on the production of α-amylase in solid medium. The spore density was from 4.5×10^5 to 4.5×10^{10}.

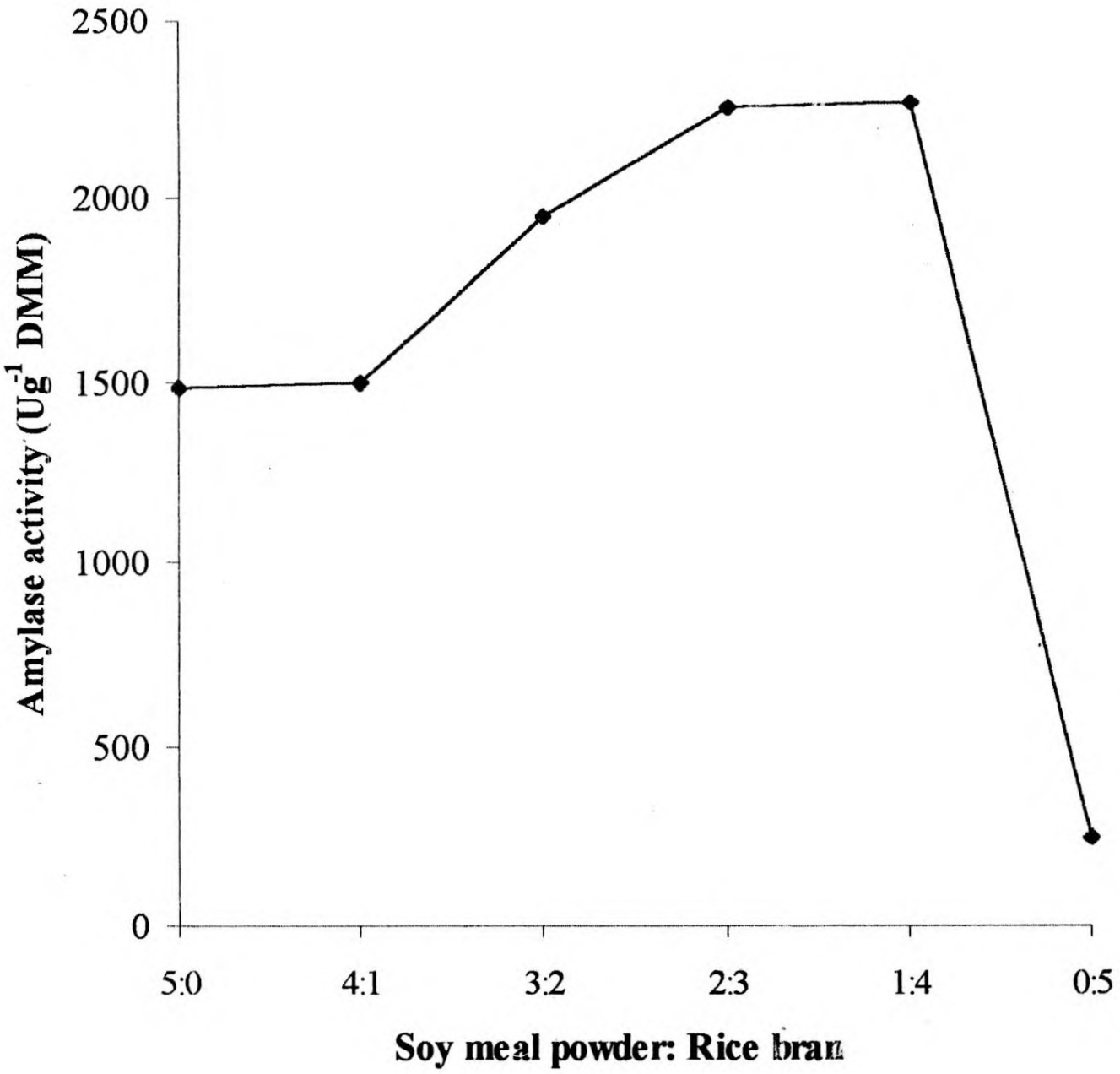

Figure 5: Effect of different ratios of soy meal powder to rice bran on α- amylase production by *Aspergillus oryzae* B12 in solid medium inoculated with 4 day old 4.5 x 10^8 spore g^{-1} medium.

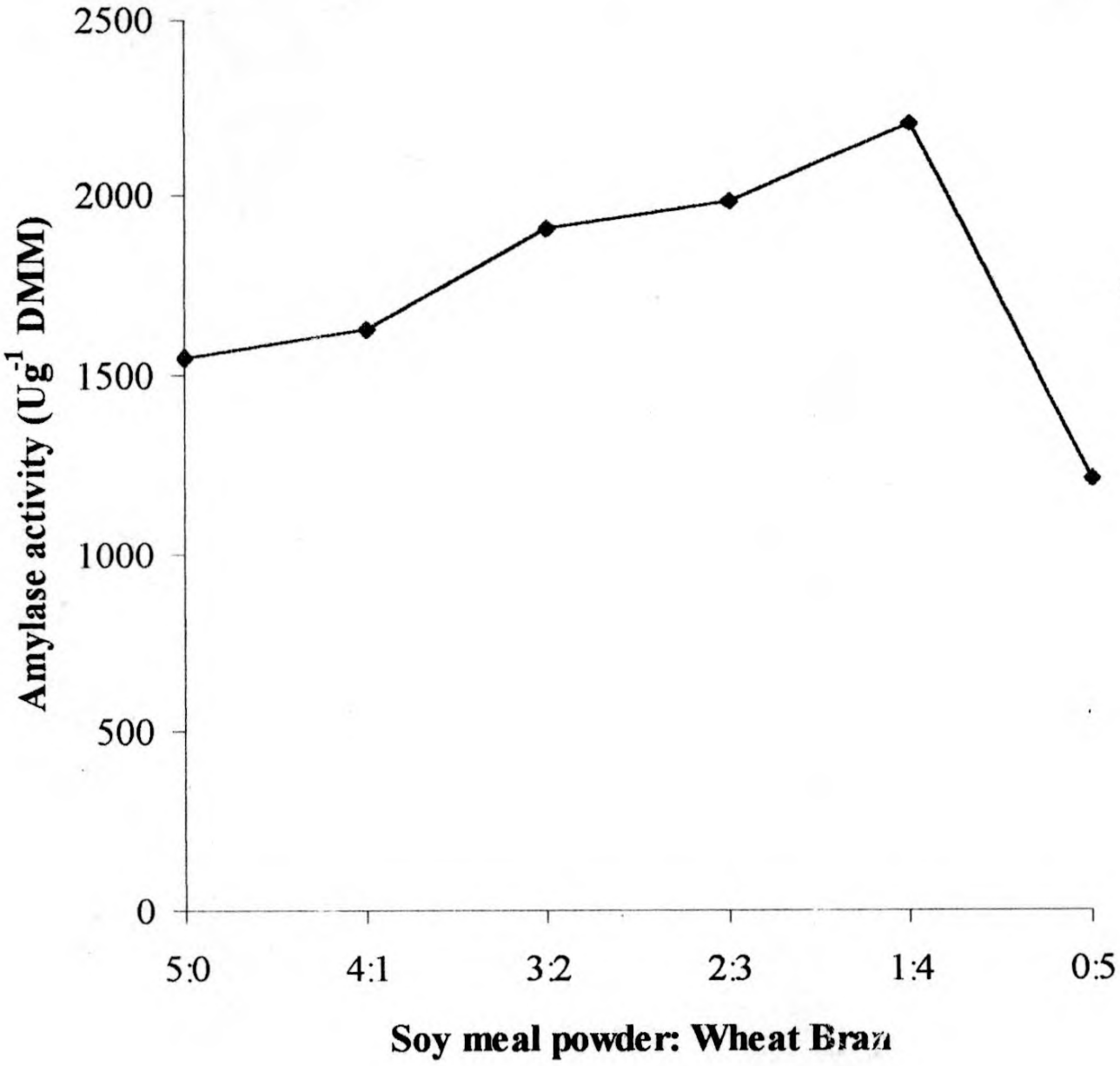

Figure 6: Effect of different ratios of soy meal powder to wheat bran on α-amylase production by *Aspergillus oryzae* B12 in solid medium inoculated with 4 day old 4.5 x 10^8 spore g^{-1} medium.

B. SUMMARY

Title:	Large-scale production of α-amylase from *Aspergillus oryzae*
Research institute:	University of Jaffna
Chief Scientific Investigator:	Prof. V. Arasaratnam
Period of contract:	2nd April 1996 to 17th October 1998
(Dates of award and completion)	

Scientific Background and scope / objectives of the project

As the strain *A. oryzae* 22788 produced very low α-amylase activity, it was decided to isolate a good α-amylase producing. *A. oryzae* strain from natural sources like rice. Despite the progress in submerged cultures, surface cultivation, which was a common method in traditional fermentation processes, has shown little progress and significant expansion of application. However taking into consideration, the simplicity of the cultivation equipment and lower expense for operation, more application of this traditional method with advanced knowledge is being expected by developing country like Sri Lanka. Hence solid surface cultivation was selected to produce α-amylase from *A. oryzae*. Cheap carbon and nitrogen sources such as rice bran, wheat bran, soy meal powder can be utilized for large scale production of α-amylase.

Experimental method

Different *A. oryzae* strains were isolated from rice medium and *A. oryzae* B_{12} was he best among the strains. It was maintained by serial transfer fortnightly on a Potato Dextrose Agar (PDA) medium.

Studies were conducted in rice bran, soy meal powder and wheat bran as the initial raw material. Effect of different rice bran/wheat bran to soy meal powder were studied. The medium was inoculated with spores with concentration of 10^7 spores g^{-1} medium.

Under the optimized ratio of rice bran to soy meal powder, effect of different age and size of the spore inoculum were studied.

Effect of different extractants, extraction time, extractant pH and mouldy medium to extractant ratio were studied.

Results obtained

Enzyme extraction was increased up to 30 min and there after no significant increase in activity was observed when the enzyme from mouldy medium was extracted using distilled water as extractant (Table 7). Enzyme was best extracted in 0.01M citrate-phosphate buffer (pH 5.2) in the mouldy medium to buffer ratio of 1 : 8 (Table 7 and 8). The optimum pH for extraction was 4.5 (Table 9).

The results on effect of different age and size of the spores on α-amylase production are given in Figures 3 & 4.

Maximum α-amylase activity obtained at 96h of cultivation of different ratios of soy meal powder to rice bran (5:0, 4:1, 3:2, 2:3, 1:4 and 0:5) on enzyme production are given in Figure 5. The substitution of rice bran with wheat bran at the same ratios as rice bran led to a reduction in time from 96h to 48h and maximum activities obtained are given in Figure 6.

Conclusions

Enzyme was best extracted in 0.01M citrate-phosphate buffer (0.01M) at pH 4.5, in the mouldy medium to buffer ratio of 1:8. Fermentation time for α-amylase production by *A. oryzae* B_{12} has been shortened from 114h to 48h by using a medium containing soy meal powder to wheat bran in the ratio of 1:4.

C. If the contractor either publishes the results of research under this contract in a recognized journal, or presents such results in the form of a paper to a recognized scientific gathering, copies of the relevant reprint, preprint or manuscript may be accepted in lieu of the corresponding parts of a regular report, provided that the information is sufficiently complete.

1. Senthuran, V., Arasaratnam, V. and Balasubramaniam, K. (2000). Optimization of fermentation conditions for α-amylase production from *Aspergillus oryzae* B_{12} ***Proceedings of the Jaffna Science Association.*** **8(1)** : 11.

2. Senthuran, V., Arasaratnam, V. and Balasubramaniam, K. (2000). α-amylase extraction from mouldy medium and its characterization. ***Proceedings of Jaffna Science Association.*** **8(1)** : 14.

3. Carthikesu, S., Arasaratnam, V., and Balasubramaniam, K. (1995). Preliminary studies on the production and characterization of α-amylase from *Aspergillus oryzae*. ***Proceedings of the 4th Annual Sessions of the Jaffna Science Association***. p.19.

D. A thesis for a post-graduate degree arising out of the project, will not be considered as the final report. But its acceptance for the award of a higher degree could be communicated by appending a note in the report. A copy of the thesis should be submitted for deposition in the Library.

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