J.Natn.Sci.Foundation Sri Lanka 2009 37 (2):93-98

RESEARCH ARTICLE

Isolation of raw starch hydrolysing fungi and purification of α-amylase from *Geotrichum candidum* CMSS06

S.N.T. De Silva, D.P.S.T.G. Attanayaka*, S.F. Nirosha and A.M.W.S. Aththanayaka
Department of Biotechnology, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka, Makandura, Gonawila (NWP)

Revised: 01 September 2008; Accepted: 17 October 2008

Abstract: Three starch degrading fungal species were isolated by exposing raw cassava and potato substrates to air and soil solutions. These three fungi were identified as *Aspergillus* spp., *Mucor* spp. and *Geotrichum candidum* by slide culture method. The optimum culture conditions for the production of extra-cellular α-amylase from these three species were determined by measuring the α-amylase activity, protein content and the pH of the culture media at 12 h intervals.

The highest raw starch hydrolysing α-amylase activity (6540 mU/mL) was shown by the species *G.candidum* 72 h after incubation. The culture supernatant containing the enzyme activity was concentrated by 50% ammonium sulphate precipitation and two peaks were identified to contain the high α-amylase activity following Dowex-cation exchange column chromatography. The two fractions collected from these two activity peaks had the specific activity of 136.3 mU/mL and 394.7 mU/ml for hydrolysing raw starch (RSHI) and soluble starch (SSI) respectively. The purification fold and recovery of the enzyme were 3.5 and 2 in RSHI and 11.2 and 10.9 in SSI. Further the maximum activity of the enzyme in RSHI fraction was detected at 40 º C and pH 7.

Keywords: Cassava, fungi, *Geotrichum candidum,* raw starch, α-amylase

INTRODUCTION

Amylases are an important group of enzymes used in starch based industries. They digest glycosidic linkages in starch to give rise to diverse products including dextrin, and progressively smaller polymers composed of glucose units[1]. α-amylases (α-1,4-D-glucan glucanohydrolases, EC 3.2.1.1) which are endogluconases, act upon polymeric molecules of carbohydrates at internal α-1-4 glucan bonds in polysaccharides containing three or more α-1-4 linkages which results in a mixture of maltose and glucose[2].

In the face of the rising fossil fuel prices, starch derived energy has now become the global focus of attention. In spite of the growing concern on food security, variety of agricultural products are now being used as renewable energy sources. Amylase conversion of raw and soluble starch into energy and other related products will be a superior alternative to the conventionally used acid hydrolysis-based methods in terms of energy utilisation and process simplicity[3,4,5].

α-amylases are universally distributed in all organisms. The enzymes from fungal, bacterial and yeast sources have dominated applications in industrial sector. Extracellular raw-starch digesting amylases from *Aspergillus* spp. have been found important in application of bioconversion of starch and starch-based substrates[3,5]. However, to date, only few microorganisms have been reported to possess ability to produce raw starch degrading α-amylases[5,6,7].

This paper reports the results of a study on identification and isolation of fungal species, having extra-cellular α-amylase activity on raw cassava starch and also the purification and characterisation of α-amylases produced from such species with the long term objective of commercial utilisation of this enzyme.

METHODS AND MATERIALS

Isolation of α -amylase producing fungi: Sliced and macerated cassava and potato, kept separately in Petri dishes were exposed to air and soil solutions at room temperature for the initial isolation of the fungi. Soil solutions used for inoculation were prepared by mixing approximately 5 g of top soil samples collected from different locations of the Makandura premises of the

*Corresponding author (dpstga@yahoo.com)

Wayamba University of Sri Lanka with 100 mL of distilled water. After the sedimentation of the large particles, 5 drops of the soil suspension were applied to the sliced material. For the macerated material, 1 mL of the soil suspension was applied. The isolation of amylolytic fungi grown on raw cassava and potato were done by selectively transferring the fungal colonies to the Potato Dextrose Agar (PDA) medium until pure cultures of the fungi were obtained. Two day old fungal colonies with different morphological characters were identified by examining their sporulating structures under the microscope by slide culture technique[7]. Purified cultures were identified to the species level at the Department of Botany, University of Kelaniya and the National Plant Quarantine Centre, Department of Agriculture. Pure cultures were maintained by sub-culturing monthly, on PDA slants.

α -amylase assay: α-amylase activity on raw starch and soluble starch was assayed by the method described by Henry *et al* [8]. Raw starch flour was prepared from cassava in the laboratory following the method described by Jansz *et al* [9]. Soluble potato starch and raw cassava starch in 0.1M phosphate buffer (pH 7) were used as substrates for enzyme assay. One unit of amylase was defined as the amount of enzyme, which liberates 1 mg of reducing sugar per min under the assay conditions.

Preparation of glucose standard curve: Standard glucose curve was plotted using working glucose concentrations of 1 mg/mL, 2 mg/mL, 3 mg/mL, 4 mg/mL and 5 mg/mL[8]. Amount of reducing sugar liberated by the enzyme was determined with respect to the concentration of standard glucose.

Assay of protein: Protein content was determined by measuring the absorbance at 280 nm as compared to a standard curve prepared using Bovine Serum Albumin (BSA) protein dilution series.

Optimizing culture conditions for α -amylase secretion: Eight cultures from each of the isolated fungi species were grown in Erlenmeyer flasks containing 50 mL sterilized fermentation medium (1.5% soluble starch, 0.2% yeast extract, 0.01% $MgSO_4$, 0.01% $CaCl_2$, 0.26% KH_2PO_4, pH 4.0) at 32°C in a rotary shaker at 100 rpm for 96 h. The α-amylase activity, pH and the protein content of the culture supernatant of each of the fungal species were measured by sampling a single culture from each of the fungal species at 12 h intervals. Uninoculated cultures incubated at the same conditions were used as the reagent blank.

Extraction and purification of α -amylase: Fungal species giving the highest enzyme activity on raw starch were selected and grown in 250 mL fermentation medium for 72 h under the same conditions given above. The supernatant was collected and the protein content, total α-amylase activity on both raw and soluble starch were assayed. The proteins in the supernatant were concentrated by 50% $(NH_4)_2SO_4$ saturation and the redissolved precipitate after dialysis was assayed for the enzyme activity and protein content.

Ion-exchange chromatography: The enzyme was further purified by cation exchange chromatography using Dowex basic cation exchange matrix. The $(NH_4)_2SO_4$ fractionated samples were applied to the column at the rate of 1 mL/min. The column was eluted with 20 mL of phosphate buffer (pH 7) with increasing ionic strength (0.05M, 0.06M, 0.08M, 0.1M, 0.12M, 0.14M, 0.16M, 0.18M, 0.2M and 0.2M + 15% NaCl). Two milliliter fractions were collected manually. For each fraction the absorbance at 280 nm and α-amylase activity on both raw and soluble starch were determined. The fractions giving higher enzyme activity peaks were pooled and the protein content and total α-amylase activity on both raw and soluble starch were measured.

End-product analysis: Fifty milliliters of the culture medium (1.5% soluble starch, 0.2% yeast extract, 0.01% $MgSO_4$, 0.01% $CaCl_2$, 0.26% KH_2PO_4, pH 4.0) was inoculated with *G. candidum* and incubated at 32 º C in a rotary shaker at 100 rpm for 72 h. Iodine test was done to confirm the presence or absence of starch by taking 1 ml samples at 12 h intervals.

RESULTS

Identification of fungal species

Three fungal species with different morphological characters were isolated from the culture media exposed to air and soil suspension. They were designated as PSA06 (Potato Sliced Air exposed, Figure 1a), PMSS06 (Potato Macerated Soil Solution exposed, Figure 1b) and CMSS06 (Cassava Macerated Soil Solution exposed, Figure 1c).

Under light microscope, PSA06 and PMSS06 had filamentous, branched and septate hyphae and long delicate sporangiophores. Based on the sporulating structure and colour of the spores of these two species, PSA06 was identified as *Mucor* spp. and PMSS06 as *Aspergillus* spp. (Figures 2a & 2b). Species CMSS06 under light microscope clearly showed conidia (arthrospores) with variable width, length and cell wall structure. Slightly convex arthrospores round off to form chain of globes to sub-globes could be observed.

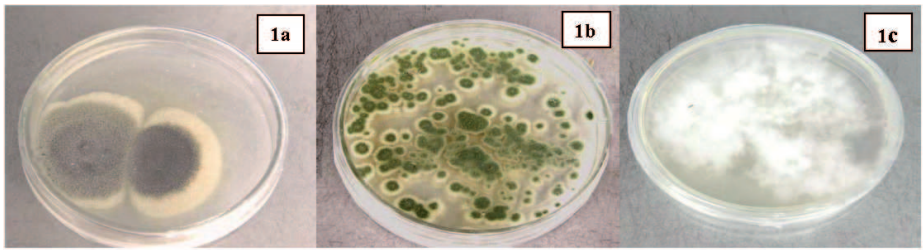

Figure 1: Morphological characters of isolated fungal colonies PSA06 (1a), PMSS06 (1b) and CMSS 06 (1c)

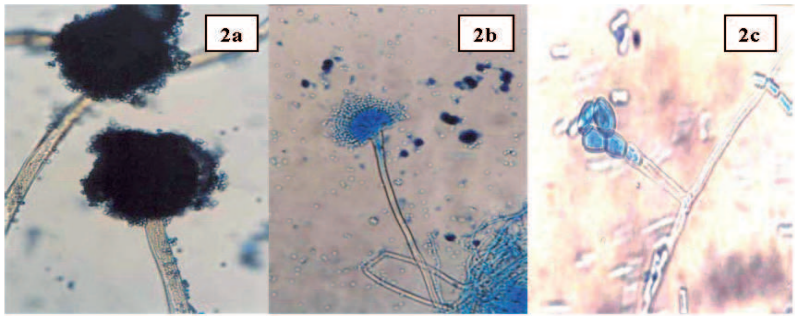

Figure 2: Structural features of isolated fungal species PSA06 (2a), PMSS06 (2b) and CMSS06 (2c) under light microscope (magnification 10 x 40)

Colonies were white and yeast-like but later produced white cottony aerial mycelium. Based on the structure and colour of the spores this fungal species CMSS06 was identified as *G. candidum* (Figure 2c).

Optimum culture conditions for extracellular α-amylase production

The culture supernatant of *Mucor* spp. showed maximum activity of 5232.6 mU/mL and 3853.4 mU/mL for raw and soluble starch respectively at 84 h incubation period. However, the protein content at 84 h is comparatively low indicating the recovery of protein will be poor at this stage. pH of the culture supernatant dropped from 4.0 to 3.04 (Figure 3).

The culture supernatant of *Aspergillus* spp. showed maximum activity of 1233.5 mU/mL for raw starch at 96 h and 1116.6 mU/mL for soluble starch after 84 h. pH of the medium dropped from 4 to 3.89 during the incubation period (Figure 4).

G. candidum CMSS06 showed maximum activity of 6540 mU/mL for raw starch at 72 h incubation period. The activity of the culture supernatant for soluble starch at this stage was 5094.7 mU/mL. The protein content was comparatively high at this stage according to the absorbance values observed at 280 nm. pH of the culture supernatant has dropped from 4.0 to 3.46 (Figure 5).

α-amylase activity on culture supernatant

Based on the results, 72 h old culture supernatant of *G. candidum* CMSS06 which gave the highest raw starch hydrolysing activity was selected for purification of α-amylase.

α-amylase activity of culture supernatant at 72 h was 89 mU/mL and 82 mU/mL for raw and soluble starch respectively. Total volume used for extraction was 150 mL. Therefore, the total α-amylase activity in the culture volume was 13350 mU/mL for raw starch and 12300 mU/mL for soluble starch. The total protein content was 351 mg.

Ammonium Sulphate fractionation

Earlier studies conducted on $(NH_4)_2SO_4$ fractionation of α-amylase indicated that 0-50% saturated precipitate contained higher α-amylase activity than 50-100% saturated precipitate[10]. Therefore, 72h old culture supernatant was immediately brought up to 50% saturation. This precipitate was dissolved in a minimum amount of 0.01M phosphate buffer and dialysis was done against the same buffer to remove the salts from the purified protein.

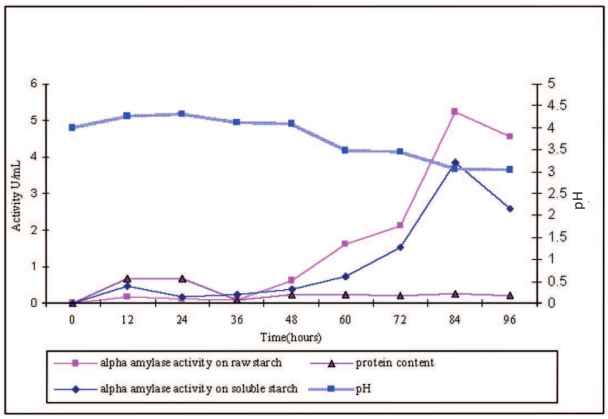

Figure 3: α-amylase activity, pH and protein content of the culture supernatant of *Mucor* spp.

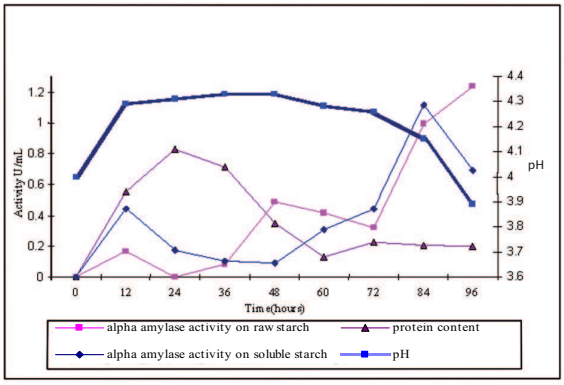

Figure 4: α-amylase activity, pH and protein content of the culture supernatant of *Aspergillus* spp.

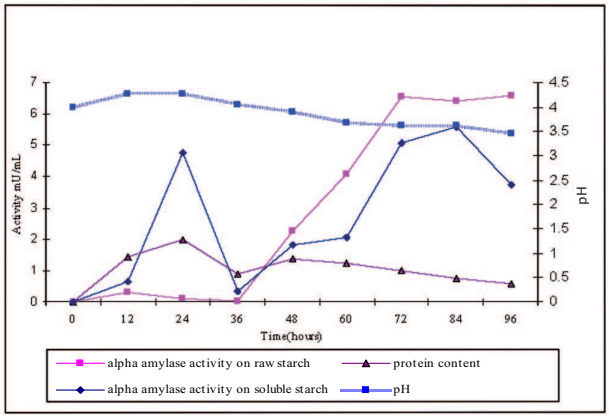

Figure 5: α-amylase activity, pH and protein content of the culture supernatant of *Geotrichum* spp.

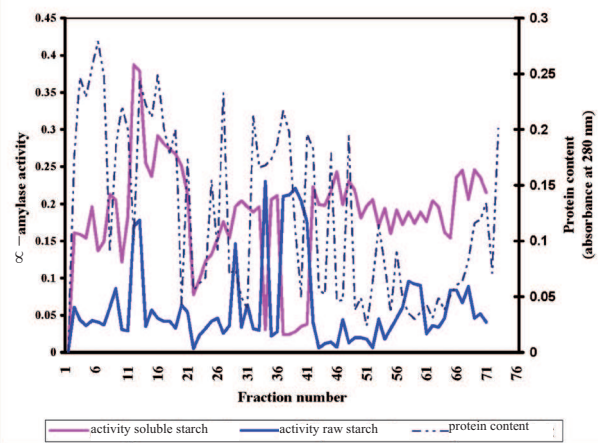

Figure 6: α-amylase activity on raw and soluble starch and protein content of the collected fractions

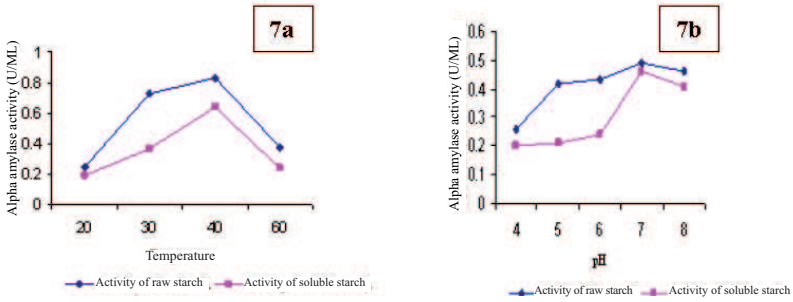

Figure 7: Effect of temperature (7a) and pH (7b) on α-amylase activity from *G. candidum* CMSS06

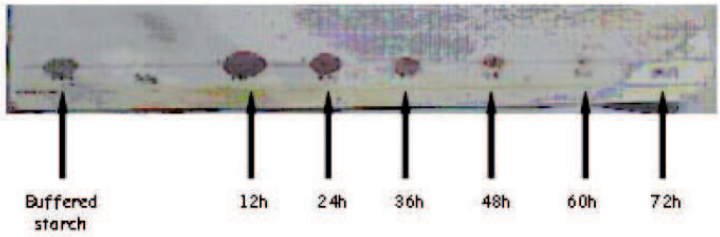

Figure 8: End product analysis done at 12 h intervals with iodine test

Table 1: Details of the purification steps of raw starch hydrolysing α-amylase from *G. candidum* CMSS06

Purification step	Volume (ml)	Protein content of raw starch	Total activity(mU/ml)		Raw starch		Soluble starch		Purification fold	
			Raw starch	Soluble starch	Specific activity (mU/ml)	Recovery %	Specific activity (mU/ml)	Recovery %	Raw starch	Soluble starch
Culture medium	150	327	13350	12300	40.8	100	35.0	100	1	1
50%$(NH_4)_2SO_4$ Fractionation	10	6.2	1200	1000	193.54	9	128.2	8.1	4.74	3.6
Ion Exchange Chromatography										
SS1	4.5	3.42	-	1350	-	-	394.7	10.9	-	11.2
RSH1	4.5	1.98	270	-	136.3	2	-	-	3.34	-

Final volume obtained for $(NH_4)_2SO_4$ fractionated, dialysed sample was 10 mL. α-amylase activity of the sample was 120 mU/mL and 100 mU/mL for raw starch and soluble starch with the total activity of 1200 mU and 1000 mU respectively. Total protein content of this fraction was 7.8 mg.

Ion exchange chromatography

According to the values of α-amylase assay, the highest α-amylase activity for raw starch was given by the fractions from 37 to 40. These fractions were eluted by 0.08M phosphate buffer (pH 7.0). They were pooled and labelled as RSH I. The highest activity on soluble starch given by the fractions from 11 to 19 were pooled and labelled as SS I which was eluted from 0.06M phosphate buffer (pH 7.0). Most of the other proteins were eluted by 0.05M ionic strength according to the absorbance value observed at 280 nm (Figure 6).

Total volumes of the pooled fractions were 4.5 mL. α-amylase activity of RSH I and SS I were 60 mU/mL and 300 mU/mL with the total activity at 270 mU and 1350 mU for raw and soluble starch respectively.

Specific activity of the enzyme RSH 1 increased from 40.8 mU/mg to 136.3 mU/mg and purification fold was 3.34 (Table 1). The purified enzyme showed maximum activity at pH 7.0 and 40ºC (Figure 7).

End-product analaysis

Complete disappearance of starch was observed from the medium after 72 h incubation period (Figure 8).

DISCUSSION

Among the three fungal species isolated in this study, *G. candidum* CMSS06 gave the highest extracellular α-amylase activity on both potato soluble starch and raw cassava starch. This species required lesser incubation period for maximum amylase activity when compared to the other two species studied (*Mucor* spp. and *Aspergillus* spp.). The fact that two enzyme activities specific to soluble starch and raw starch were obtained suggests the presence of two different forms of enzyme of the amylase family. This study confined only to the extracellular α-amylase available in the culture supernatant. Low intracellular amylase activity in fungi species has been reported previously[10].

Different α-amylase activities were obtained in *G. candidum* CMSS06 cultures used for enzyme activity optimisation and enzyme extraction. This can be attributed to the variations in culture conditions such as initial inoculum concentrations which were not controlled in the present study.

Raw starch hydrolysing enzyme purified in this study had the specific activity of 136.6 mU/mL, 2% recovery and 3.34 purification fold.

The activity of the enzyme reported in this study was maximum at 40ºC in agreement with earlier observations for other fungal starch digesting amylases[3,11,12].Most raw starch digesting amylases are known to exhibit temperature optima between 40ºC and 60ºC and are remarkably stable against high temperatures[13]. Further studies should be carried out to study the stability of the crude enzyme identified in this study in different buffers, so that the temperature optimum may be further enhanced to make use of this enzyme for industrial uses at higher temperatures.

G. candidum CMSS06 after 72 h incubation with starch has shown complete disappearance of starch from the medium indicating its ability to convert starch into

alpha limit dextrins. A similar study conducted in Nigeria using *Aspergillus niger* AM07 have shown the capacity to hydrolyse raw starch to sugars with higher conversion efficiency[11].

G. candidum has been used as a starter culture for protection of barley in malts[14] and for biotreatment of raw wastewaters, especially in starch processing[15] and distillary wastewater effluent[16]. Its ability to remove nitrogen from the effluent is significant[17], indicating the possibility of utilisation of agroindustrial effluents as a component of a low cost medium for α-amylase production using *G. candidum* .

Appropriate combinations of amylolytic enzymes completely depolymerise starch to glucose. Detection and purification of raw starch hydrolysing enzyme in *G. candidum* CMSS06 species isolated in this study therefore shows the potential to be used in bioconversion of raw starch to low molecular weight sugars for alcoholic fermentation contributing to the global search for production of renewable energy resources.

Acknowledgement

The authors wish to acknowledge the advice given by Dr S.D.P. Kannangara, Department of Botany, University of Kelaniya, Dr D.B.Kelaniyangoda, Department of Horticulture and Landscape Gardening, Wayamba University of Sri Lanka and the staff of the National Plant Quarantine Center, Department of Agriculture for their assistance in identification of the fungi. Assistance provided by Mr. H.P.R.N. Pathirathna, Department of Biotechnology, Wayamba University of Sri Lanka is also acknowledged.

References

1. Windish W.W. & Mhatre N.S. (1965). Microbial amylases. *Advances in Applied Microbiology* **7**: 273-304.
2. Karkalas C. & John S.P. (1985). An improved enzymatic method for the determination of native and modified starch. *Journal of the Science of Food and Agriculture* **36**(10): 1019.
3. Fogarty W.M. (1983). Microbial amylases. In: *Microbial Enzymes and Biotechnology* (Ed. W.M. Fogarty) pp. 1–92. Applied Science Publishers, London, UK.
4. Achi O.K. & Njoku-Obi A.N.U. (1992). Production of raw starch saccharification amylase by *Bacillus alvei* grown on different agricultural substrates. *World Journal of Microbiology and Biotechnology* **8**(2): 206-207.
5. Okolo B.N., Ezeogu L.I. & Mba C.I. (1995). Production of raw starch digesting amylase by *Aspergillus niger* grown on native starch sources. *Journal of the Science of Food and Agriculture* **69**: 109-115.
6. Abe J., Bergmann F.W., Obeta K. & Hizukuri S. (1988). Production of the raw starch digesting amylase of *Aspergillus* spp. K-27. *Applied Microbiology and Biotechnology* **27**: 447- 450.
7. Radley J.A. (1953). *Starch and its Derivatives*. Vol.1. 3rd Edition. pp. 369-401. John Wiley and Sons, New York, USA.
8. Henry R.J. & Chiamori N. (1960). Study of the saccharogenic method for the determination of serum and urine amylase. *Clinical Chemistry* **5**: 434.
9. Jansz E.R., Pierie N., Jeyaraj E.E. & Abeyratne D.J. (1976). A process for improved manioc chips and flour. In: *Industrial Microbiology Section-Annual Report of the Ceylon Institute of Scientific and Industrial Research* 3rd Edition. pp. 3-5. Industrial Technology Institute, Colombo.
10. De Silva S.N.T. (1997). Purification and characterization of raw starch hydrolysing α-amylase from *Aspergillus* spp. *M.Sc Thesis.* Faculty of Medicine, University of Colombo, Colombo.
11. Omemu A.M., Akpan I., Bankole M.O. & Teniola O.D. (2004). Hydrolysis of raw tuber starches by amylase of *Aspergillus niger* AM07 isolated from the soil. *African Journal of Biotechnology* **4**(10): 19-25.
12. Okolo B.N., Ire F.S., Ezeogu L.I., Anyanwu C.U. & Odibo F.J.C. (2000). Purification and some properties of a noval starch digesting amylase from *Aspergillus carbonarius*. *Journal of the Science of Food and Agriculture* **81**(3): 329-336.
13. Forgarty W.M. & Kelly C.T. (1979). Developments in microbial extracellular enzymes. In: *Topics in Enzyme and Fermentation Biotechnology.* Vol. 3 (Ed A.Wiseman). pp. 45-108. Applied Science Publishers, London, UK.
14. Foszczyńska B., Dziuba E. & Stempniewicz R. (2004). The use of *Geotrichum candidum* starter culture for protection of barley and its influence on biotechnological qualities of malts. *Electronic Journal of Polish Agricultural Universities* **7**(2): 4.
15. Jin B., van Leeuwen J., Yu Q. & Patel B. (1999). Screening and selection of microfungi for microbial biomass protein production and water reclamation from starch processing wastewater. *Journal of Chemical Technology and Biotechnology* **74**(7): 106-110.
16. Kida K., Morimura S., Abe N. & Sonoda Y. (1995). Biological treatment of Shochu distillery wastewater. *Process Biochemistry* **30**(2): 125-132.
17. Lacina C., Germain G. & Spiros A.N. (2003). Utilisation of fungi for biological treatment of water. *African Journal of Biotechnology* **2**(12): 620-630.