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# Extraction and Purification of Glucoamylase and Protease Produced by *Aspergillus awamori* in a Single-Stage Fermentation

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**Summary**

Simultaneous extraction and purification of glucoamylase and protease produced concomitantly by *Aspergillus awamori* Nakazawa MTCC 6652 in a single fermentor using solid-state fermentation (SSF) has been studied. Soaking for 2 h at room temperature (around 30 °C) in 10 % glycerol was found to be most suitable for optimum simultaneous extraction of glucoamylase and protease with the yield of 8645.8 U/g of glucoamylase and 798.6 U/g of protease in dry substrate. Crude extract to acetone ratio of 1:2 yielded optimum simultaneous precipitation of glucoamylase (35.3 %) and protease (61.9 %) with 4.06- and 7.17-fold purification, respectively. Ion exchange chromatography showed specific activities of purified fractions of 253.2 U/mg of glucoamylase and 59.7 U/mg of protease, with 22.1 and 40.8 % recovery, respectively. After gel filtration chromatography specific activity, recovery and purification of glucoamylase were found to be 306.8 U/mg, 4.6 % and 6.25-fold, respectively, whereas those of protease were 85.6 U/mg, 12.9 % and 17.0-fold, respectively. SDS-PAGE and zymogram studies of the purified enzymes indicated the presence of three starch-hydrolyzing isoforms of glucoamylase with molecular mass of approx. 109.6, 87.1, and 59.4 kDa and two types of acid protease with molecular mass of approx. 47.9 and 35.5 kDa. These findings can be very useful for enzyme industry, where glucoamylases and proteases are used concurrently.

*Key words:* extraction, purification, amylase, protease, *Aspergillus awamori*

## Introduction

The necessity of cost-effective production and simultaneous applications of more than one enzyme in enzymatic processes have given thrust to the production of multiple enzymes from a single fermentation. Amylases and proteases are among the most widely used industrial enzymes having various applications in industries like leather, brewing, textile, paper, distilling, food processing, pharmaceuticals, waste decomposition, detergent, *etc*. (*1*). Amylases and proteases are concurrently used in many industries such as soy sauce, bakery, detergent, vegetable oil, soy milk, cheese production, *etc*. Glucoamylases and proteases produced concomitantly in a single-stage fermentation can be very useful for various applications such as detergent, bio-ethanol, animal feed production, *etc*. In view of the wide range of applicability of glucoamylases and proteases, they are considered as the objects of the present investigation.

Efficient extraction and purification of enzymes is vital for their subsequent use and integration in indus-

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trial processes. There are very few reports on the extraction and purification of glucoamylase and protease produced concomitantly in single-stage fermentation. In one such investigation of concurrent extraction and purification of multienzyme complex, Krishna and Chandrasekaran (*2*) reported the extraction of 32 800 U of α-amylase, 380 U of protease and 90 U of cellulase from *B. subtilis* (*2*). Yang and Wang (*3*) reported the production of protease and amylase from *Streptomyces rimosus* TM-55, both in submerged as well as in solid-state fermentation (SSF). Production of multienzymes consisting of alkaline amylase and cellulase by mixed alkalophilic culture and their potential use in the saccharification of sweet potato have been studied by Zhang *et al.* (*4*). Optimization of the production of amylase and protease using evolutionary operation (EVOP) has been reported by Negi and Banerjee (*5*). Amylase and protease from *Streptomyces* species were purified by a combination of ion exchange chromatography and gel filtration, and characterized by Etok and Eka (*6*).

In the present study, glucoamylase and protease are produced concomitantly in solid-state fermentation. Various factors affect simultaneous extraction and purification of both glucoamylase and protease. Therefore, the aim of this study is to optimize various factors that affect the extraction and purification of glucoamylase and protease produced concomitantly in SSF by *A. awamori*, in order to obtain the best possible yield and purification of both enzymes simultaneously.

## Materials and Methods

### *Microorganism and strain maintenance*

A filamentous fungus *Aspergillus awamori* Nakazawa MTCC 6652, isolated from the soil at the Indian Institute of Technology, Kharagpur, India, was explored for the production of extracellular multienzymes protease and glucoamylase. *Aspergillus awamori* Nakazawa was maintained on 2 % malt extract agar slants. The microorganism was allowed to grow in these slants at 37 °C for six days for complete sporulation. It was stored at 4 °C for preservation.

### *Inoculum preparation*

For inoculum preparation, 25 mL of sterile distilled water were added to the slant grown on malt extract plate for six days at 37 °C and scraped with inoculating loop aseptically. This suspension having spore concentration of approx. $1.3 \cdot 10^6$ cells/mL was used as inoculum for subsequent fermentation.

### *Fermentation process*

Fermentation was carried out in a 500-mL conical flask containing 10 g of wheat bran soaked with 10 mL of salt solution. The medium was mixed properly, autoclaved, and inoculated with optimum spore suspension aseptically. Conical flasks were kept in a humidity chamber where temperature and humidity were maintained during fermentation.

### *Extraction process*

After the fermentation was completed, the fermented substrate was soaked with water and other solutions for extraction of the enzymes. Filtration was done through a cotton cloth and maximum amount of extract was collected. The extract was centrifuged at 9780×*g* to remove the spores and other insoluble particles. The supernatant was kept at 4 °C for the assay of the enzymes.

### *Solid-state fermentation*

A mass of 10 g of wheat bran soaked in 60 mL of Czapek-Dox (containing in g/L: $NaNO_3$ 2.5, $KH_2PO_4$ 1.0, $MgSO_4 \cdot 7H_2O$ 0.5, KCl 0.5) was mixed, autoclaved, then inoculated with optimum number of spores and incubated for four days at 37 °C and 85 % humidity. Fermentation was conducted under various experimental conditions (*5*).

### *Glucoamylase assay*

Glucoamylase activity was measured following the Bernfeld method (*7*). One unit of enzyme activity was defined as the amount of enzyme that released one μmol of glucose as the reducing sugar in one minute under the assay conditions.

### *Protease assay*

Protease activity was measured by caseinolytic method (*8*). One unit of enzyme was defined as the amount of enzyme that liberated peptide fragments equivalent to 1 mg of bovine serum albumin (BSA) under the assay conditions.

### *Extraction*

Optimal extraction conditions were achieved by optimizing different parameters such as solvent selection, volume fraction of the solvent, soaking time, temperature, *etc.* by varying one parameter at a time while keeping the others constant. Fermented substrate was soaked with one of the solvents, and then filtered using cheese cloth to collect the extract. The extract was then centrifuged at 10 000×*g* to remove the insolubles, and the supernatant was stored at 4 °C.

### *Solvent selection*

Various solvents such as water, 0.1 M phosphate buffer (pH=6.5), 10 % aqueous mixture of glycerol, ethanol, salt solution, and acetone were used to determine their effect on the extraction of glucoamylase and protease. During experiments, glycerol was found as the most suitable solvent for optimum simultaneous extraction of glucoamylase and protease, hence, different volume fractions of glycerol were tested further to determine the best fraction for optimum extraction efficiency.

### *Optimization of soaking time and temperature*

Optimum soaking time was determined by incubating the fermented biomass for the time period ranging from 30 min to 3 h, keeping other parameters at optimum level. Similarly, the best temperature for optimum extraction of both enzymes simultaneously was ascertained by conducting the experiments at 15, 30, 40 and 50 °C while keeping the other parameters at optimum level.

### *Purification*

Crude extract of glucoamylase and protease, obtained under optimal conditions, was further concentrated and purified by following the conventional methods of downstream processing such as acetone precipitation, ion exchange chromatography and gel filtration chromatography (GFC).

### *Acetone precipitation*

Precipitation of glucoamylase and protease was carried out at 4 °C using acetone. Crude extract to acetone ratio was varied from 1:0.5 to 1:4 (by volume) to determine the amount of acetone required for optimum precipitation of glucoamylase and protease simultaneously.

Complete precipitation of both enzymes took about one hour, after which the precipitate was centrifuged at 10 000×*g* at 4 °C for 30 min. The precipitated enzymes were then collected, concentrated and suspended in Tris--HCl buffer (0.05 M, pH=6.5). Purification of the concentrated enzymes was carried out by ion-exchange chromatography and followed by gel filtration chromatography.

### *CM Sephadex ion exchange chromatography*

A mass of 5 g of the treated CM Sephadex powder was loaded in a column (20×2 cm) and equilibrated with Tris-HCl buffer (10 mM, pH=6.5). Then, 2 mL of the concentrated enzyme extract were loaded in the column. Subsequently, buffer (50 mM) at pH=7.0 was passed to wash off the unbound protein. After washing the column properly, 1 M sodium chloride salt gradient was added continuously and the active fractions of glucoamylase and protease were collected, pooled for specific activity and further purified by gel filtration chromatography.

### *Gel filtration chromatography*

The glucoamylase fraction isolated using the ion exchange chromatography was further concentrated to 1 mL by dialyzing against 4 M sucrose solution and loaded on the gel filtration column equilibrated with acetate buffer, pH=4.0. The elution was done in 50 mM acetate buffer and the active fractions were collected. Similarly, partially purified, concentrated and dialyzed protease fractions were loaded to gel filtration column eluted with 50 mM Tris-HCl at pH=6.5.

### *Determination of molecular mass of protease by GFC*

The relative molecular mass of glucoamylase and protease was determined by GFC using a calibration curve plotted on semi-log graph paper between the log of molecular mass and relative mobility of standard proteins: phosphorylase b (185 kDa), bovine serum albumin (67 kDa), lysozyme (14 kDa), cytochrome c (12.6 kDa), determined with respect to blue dextran ($M_r$=2000 kDa).

### *Molecular mass determination through SDS-PAGE*

The active fractions of amylase and protease collected after gel filtration were pooled, dialyzed and concentrated against 4 M sucrose solution and subjected to SDS-PAGE to get the accurate molecular mass using the standard markers: phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14 kDa). The electrophoresis was carried out by using 10 % (by mass per volume) running gel and 3 % (by mass per volume) stacking gel. A plot of the log of molecular mass and relative mobility of the standard markers in SDS-PAGE was used to estimate the molecular mass of the enzymes.

Activities of glucoamylase and protease isoforms were further studied through zymogram study by incubating the gel obtained after SDS-PAGE in the starch and casein solution overnight.

## Results and Discussion

A number of factors such as the type of solvent, solvent fraction, soaking time, temperature, *etc*. affect the extraction of enzymes from the fermented mass (*9*,*10*). Hence, the effects of different parameters on the extraction efficiency have been studied to optimize it. Effects of different solvents on extraction efficiency indicate that both glucoamylase and protease were leached out at their maximum level in glycerol solution (Fig. 1). Therefore,

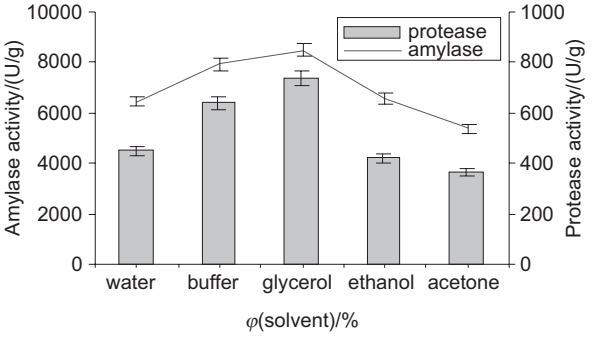


**Fig. 1.** Effect of different solvents on the extraction of glucoamylase and protease

glycerol solution was considered as the most suitable solvent for optimum extraction of both enzymes simultaneously and it was used for further investigation. Lower dielectric constant of glycerol probably helped in improving the interaction between the enzyme and glycerol, leading to better extraction. Organic solvents have often been reported as the most suitable solvent for the extraction of enzymes (*11*). Furthermore, 10 % glycerol solution yielded maximum amylase as well as protease extraction (Fig. 2). At lower fractions, hydroxyl group of the glycerol

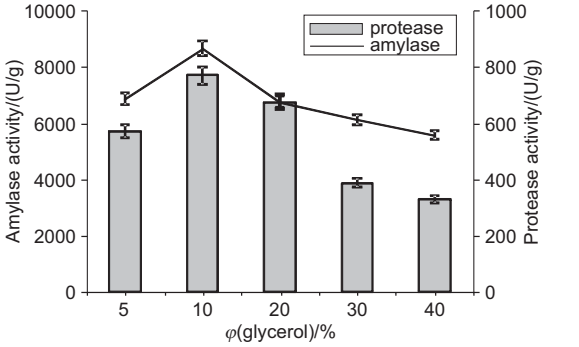


**Fig. 2.** Effect of glycerol fraction on the extraction of glucoamylase and protease

was more likely to form hydrogen bond with the protein molecules, giving better stability to the enzyme molecules (*10*).

Two hours of soaking was found sufficient for optimum extraction of both enzymes simultaneously (Fig. 3).

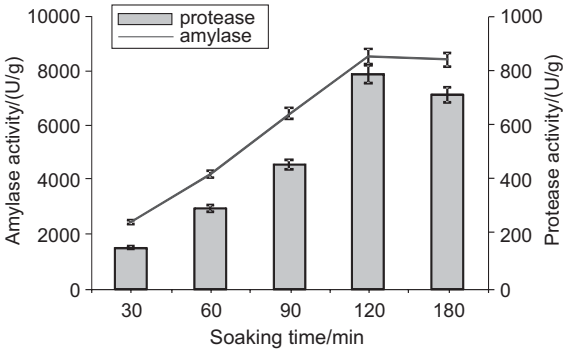


**Fig. 3.** Effect of soaking time on the extraction of glucoamylase and protease

Extraction of protease at 30 °C was found to be maximum, whereas optimum amylase extraction was obtained between 30 and 40 °C (Fig. 4). At higher temperatures enzymes might become denatured, causing the fall in the enzyme activity (*9*). Room temperature was found to be optimal for simultaneous extraction of glucoamylase and protease. Therefore, extraction for further investigations was carried out at room temperature (around 30 °C). Activities of glucoamylase and protease in crude extract were found to be 760.9 and 82.8 U/mL, respectively (Table 1).

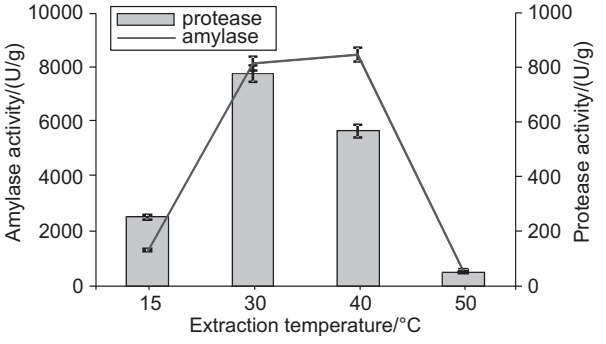


**Fig. 4.** Effect of temperature on the extraction of glucoamylase and protease

Precipitation is one of the fundamental methods of recovery and purification of proteins. Both glucoamylase and protease were precipitated with acetone at 4 °C, which resulted in better enzyme recovery compared to other precipitating agents like ethanol and $(NH_4)_2SO_4$. Crude extract to acetone ratio of 1:2 was found to be most suitable for the optimum precipitation of glucoamylase and protease simultaneously (Table 1). Precipitation of other unwanted proteins at higher ratio of acetone (1:2 and higher) might be the reason for the decrease in specific activities of glucoamylase and protease. After acetone precipitation, activities of glucoamylase and protease were found to be 2670 U/mL with 35.3 % recovery and 513 U/mL with 61.9 % recovery, respectively (Table 2).

Ion exchange chromatography is the most widely used chromatographic technique for purification of biological products. This technique is very powerful and offers high capacity relative to other types of chromatography. Therefore, in the present work a cationic exchanger (CM Sephadex) was employed. Better results were obtained when the elution was carried out by 0.5 M NaCl solution. Chromatograms shown in Figs. 5 and 6 show single peaks representing amylase (fraction numbers 7 to 13) and protease (fraction numbers 4 to 9) activity, respectively. After ion exchange chromatography, 22.1 % of glucoamylase and 40.8 % of protease were recovered with 5.16- and 11.9-fold purification, respectively. Specific activities of purified glucoamylase and protease after this stage were found to be 253.2 and 59.7 U/mg, respectively (Table 2).

Gel filtration chromatography was chosen for further purification of samples from the excess salt and to sort them out according to their molecular size. Wanderley *et al.* (*12*) used this technique for the purification of glucoamylase produced by *Cryptococcus flavus*. Mohamed *et al.* (*13*) purified glucoamylase using gel filtration and SDS-PAGE. Minami and Kilikian (*14*) characterized glucoamylase of *A. awamori* by size-exclusion chromatography. After gel filtration chromatography, 4.6 % glucoamylase with 6.25-fold purification and 12.9 % protease with 17.0-fold purification was recovered (Table 2).

Table 1. Effect of enzyme to acetone ratio on fold purification of amylase and protease

| $\psi$(crude enzyme: acetone) | $\gamma$(protein) / mg/mL | Activity / U/mL | | Specific activity / U/mg | | Increase in activity / % | |
|---|---|---|---|---|---|---|---|
| | | Amylase | Protease | Amylase | Protease | Amylase | Protease |
| 1:0 | 16.50 | 760.9 | 82.8 | 46.1 | 5.02 | 100.0 | 100.0 |
| 1:0.5 | 8.75 | 1010.6 | 164.9 | 115.5 | 18.85 | 132.8 | 199.2 |
| 1:1.0 | 10.20 | 1711.5 | 180.5 | 167.8 | 17.70 | 224.8 | 218.0 |
| 1:1.5 | 13.50 | 2438.0 | 408.0 | 180.6 | 30.22 | 320.4 | 492.7 |
| 1:2.0 | 14.25 | 2670.0 | 513.0 | 187.4 | 36.00 | 350.9 | 619.6 |
| 1:2.5 | 14.60 | 2364.4 | 452.1 | 161.9 | 30.97 | 310.7 | 546.0 |
| 1:3.0 | 14.12 | 1898.0 | 258.0 | 134.4 | 18.28 | 249.4 | 311.7 |
| 1:3.5 | 13.10 | 1508.0 | 178.6 | 115.1 | 13.63 | 198.2 | 215.7 |
| 1:4.0 | 11.92 | 720.0 | 67.5 | 60.4 | 5.66 | 94.6 | 81.5 |

Table 2. Purification profiles of glucoamylase (Amy) and protease (Pro)

| Purification step | $V$/mL | $m$(total protein) | Total activity | | Specific activity | | Recovery | | Purification | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | mg | U | | U/mg | | % | | fold | |
| | | | Amy | Pro | Amy | Pro | Amy | Pro | Amy | Pro |
| Crude filtrate | 20 | 330.0 | 15218 | 1656 | 46.1 | 5.02 | 100.0 | 100.0 | 1.00 | 1.00 |
| Acetone | 2 | 28.5 | 5340 | 1026 | 187.4 | 36.00 | 35.3 | 61.9 | 4.06 | 7.17 |
| Ion exchange chromatography | 20 | 13.3 | 3367 | 675 | 253.2 | 59.70 | 22.1 | 40.8 | 5.16 | 11.90 |
| Gel filtration | 16 | 2.3 | 705 | 214 | 306.8 | 85.60 | 4.6 | 12.9 | 6.25 | 17.00 |

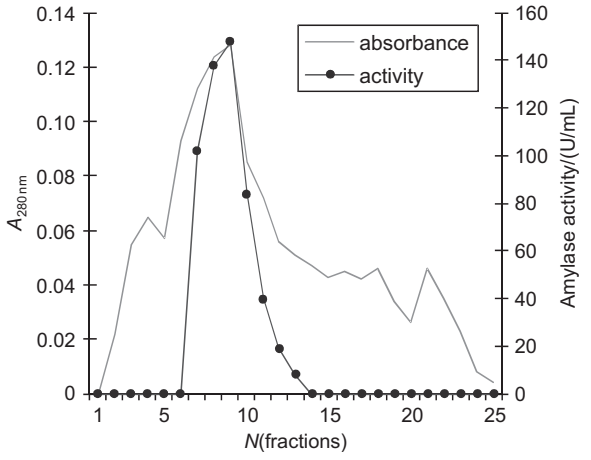


**Fig. 5.** CM-Sephadex ion exchange chromatogram of amylase

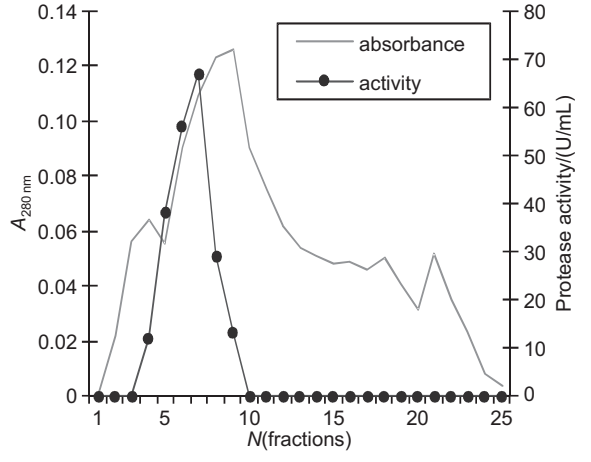


**Fig. 6.** CM-Sephadex ion exchange chromatogram of protease

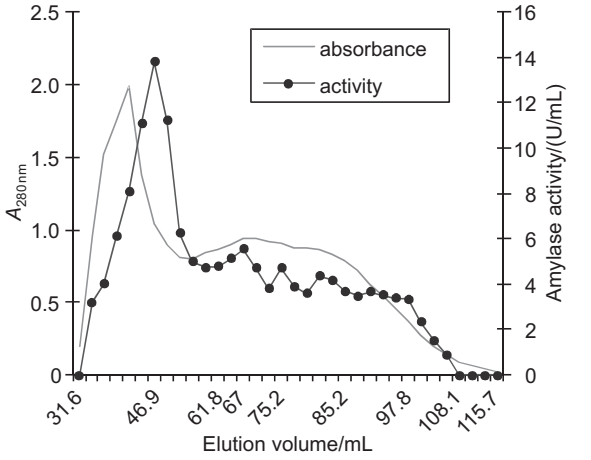


**Fig. 7.** Sephadex G-100 gel filtration chromatogram of amylase

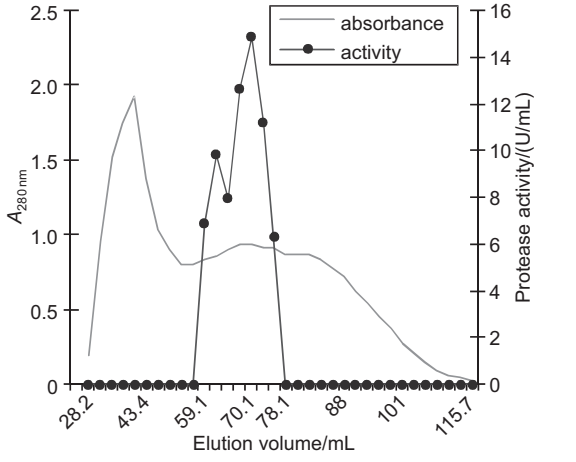


**Fig. 8.** Sephadex G-100 gel filtration chromatogram of protease

Fig. 7 shows three peaks of amylase indicating three isoforms of glucoamylase synthesized by *Aspergillus awamori* Nakazawa, whereas Fig. 8 shows two types of protease. Glucoamylase isoforms present in the elution volume of 46.9 to 61.8 mL had a significant activity, whereas other two peaks had a very low activity. There are many other reports mentioning that glucoamylase from *Aspergillus* sp. exists in two to four isoforms, which vary from species to species (*15*). The chromatogram of purified protease indicated two types of proteases, confirmed by two peaks in GFC in close vicinity. SDS-PAGE of the samples collected after ion exchange chromatography also showed two bands and confirmed caseinolytic activity and homogeneity of purified protease.

Molecular mass ($M_r$) determination by GFC indicated three glucoamylase isoforms with $M_r$ of 100, 84 and 61 kDa, and two proteases with $M_r$ of 41 and 28 kDa, approximately. Using SDS-PAGE, molecular mass of the enzymes was estimated to be 109.6, 87.1 and 59.4 kDa for glucoamylases and 47.9 and 35.5 kDa for proteases. Selvakumar *et al*. (*16*) reported the purification of four different forms of glucoamylases having apparent molecular mass of 112, 104, 74 and 61 kDa produced by *Aspergillus niger*, grown on wheat bran in solid cultures. Tunga *et al*. (*17*) reported the purification of an extracellular alkaline serine protease produced by *Aspergillus parasiticus* in solid-state fermentation with the molecular mass of protease of 23 kDa estimated by SDS-PAGE.

## Conclusion

From these findings it can be concluded that optimum simultaneous extraction of glucoamylase and protease produced by *Aspergillus awamori* Nakazawa in a single-stage fermentation can be achieved in 10 % glycerol solution at room temperature with 2 h of soaking. Furthermore, concurrently produced glucoamylase and protease can be purified considerably better using acetone precipitation, ion exchange chromatography and GFC in laboratory conditions. Results of this study revealed that both these enzymes can be produced efficiently by a single microorganism in a single-stage fermentation and both can be extracted and purified efficiently under the similar fermentation conditions using the same techniques. Therefore, findings of this study can be very useful for enzymatic processes involving simultaneous use of multienzymes, particularly glucoamylases and proteases.

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