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Production and characterization of partially purified thermostable α-galactosidases from *Streptomyces griseoloalbus* for food industrial applications

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ABSTRACT

Solid-state fermentation was carried out for the production of extracellular α-galactosidase by *Streptomyces griseoloalbus*. Soybean flour was the best solid substrate for α-galactosidase production. In flask-level optimization, the highest enzyme yield of 111 ± 0.2 U/gds was obtained under optimal conditions. The partially purified α-galactosidase preparation showed highest activity at pH 5.0 and 65 °C. The enzyme was completely stable at pH 5.0 to 7.0 and at 50 and 55 °C for 5 h. The $t_{1/2}$ of the enzyme at 65 °C was 3.5 h. The information obtained from the present investigation is advantageous for food industrial applications of *S. griseoloalbus* α-galactosidases.

1. Introduction

Legumes are excellent sources of proteins, dietary fibres and a variety of micronutrients and phytochemicals. Hence they play a significant role in the traditional diets of many regions throughout the world. Despite their nutritional value, the utilization of legumes for human nutrition is constrained by the presence of the raffinose family oligosaccharides (RFO) which have a tendency to induce flatulence. The production of flatulence is regarded as being due to the lack of ability of the human intestinal tract to synthesize the enzyme α-galactosidase, which is needed to hydrolyze oligosaccharides containing α-galactosidic linkages. Hence, these oligosaccharides tend to remain unhydrolyzed in the upper intestine of human beings. Thus, when the saccharides are fermented by bacteria in the large intestine, they produce gas. It is the production of this gas which leads to the characteristic features of flatulence, namely nausea, cramps, diarrhea, abdominal rumbling, and the social discomfort associated with the ejection of rectal gas (Cristofaro, Mattu, & Wuhrmann, 1974). The problem area in the manufacture of protein foods is, therefore, the breakdown of RFO which are present in the leguminous seeds. Of the various treatments proposed for the treatment of legume foods, enzymatic treatment by α-galactosidase has proved most effective (Mansour & Khalil, 1998; Viana et al., 2005). Usually, legume food processing requires a high operating temperature which necessitates the involvement of thermostable α-galactosidases.

α-Galactosidase or melibiase (α-D-galactoside galactohydrolase, EC 3.2.1.22) is an exo-glycosidase that catalyzes the hydrolysis of terminal non-reducing α-1-6-linked galactosyl residues from a wide range of galactooligosaccharides and polysaccharides (Naumoff, 2004). The suitability of an enzyme for any particular industrial application is determined by its characteristics. We have earlier identified the filamentous actinobacterium, *Streptomyces griseoloalbus,* as a novel source of α-galactosidase (Anisha & Prema, 2006) and the potential of this enzyme in the hydrolysis of flatulence causing oligosaccharides present in soymilk (Anisha & Prema, 2007) and legume seed flours (Anisha & Prema, 2008) was also demonstrated. It is worthwhile to identify the process conditions conducive for enhanced production of this food industrial enzyme by *S. griseoloalbus* under solid-state fermentation (SSF). This study also reports the characteristics of a partially purified enzyme preparation suitable for food industrial applications and the presence of multiple α-galactosidases.

2. Materials and methods

2.1. Microorganism and inoculum preparation

S. griseoloalbus MTCC 7447, isolated in our laboratory, was used for the present study. The organism was maintained at 4 °C on starch casein agar (SCA) slants and was sub-cultured fortnightly.

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Inoculum was prepared by transferring a loopful of culture from fresh SCA slants into sterile medium (100 ml in 250 ml Erlenmeyer flask) composed of (g/l): locust bean gum, 10; yeast extract, 3; $(NH_4)_2HPO_4$, 3.03; KH_2PO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.49; and 1 ml of trace elements solution. The trace elements solution was composed of (g/l): $FeSO_4 \cdot 7H_2O$, 0.1; $MnCl_2 \cdot 4H_2O$, 0.1 and $ZnSO_4 \cdot 7H_2O$, 0.1. The flasks were incubated at 30 °C on a rotary shaker at 175 rpm. A 48 h old culture containing 3×10^6 CFU/ml was used as the inoculum.

2.2. Solid-state fermentation

Ten grams of solid substrate, in a 250 ml Erlenmeyer flask, were moistened with mineral salt solution (g/l: KH_2PO_4, 1; $MgSO_4$, 0.4; pH 7.0), thoroughly mixed and autoclaved at 121 °C for 30 min. The cooled medium was inoculated with 48 h old inoculum (2.25×10^6 CFU/g initial dry substrate) and incubated at 30 °C for 5 days. The moisture content of the medium after inoculation was 50%. Unless otherwise specified, these fermentation conditions were maintained throughout the experiment.

2.3. Evaluation of solid substrates

Different agro-industrial materials, such as soymeal, soybean, chick pea, guar seeds, maize, sorghum, wheat bran, rice bran, groundnut oil cake and coconut oil cake, were obtained from the local market, ground to flour and used as solid substrates to evaluate their suitability in sustaining good production of α-galactosidase by *S. griseoloalbus*. The best solid substrate chosen was used for further optimization experiments.

2.4. Optimization of process parameters

Fermentation parameters, such as particle size, moisture content, initial pH, incubation temperature and inoculum size, were optimized at flask-level. Care was taken to keep the amount of mineral salt solution constant while varying the moisture level. Different carbon supplements (glucose, galactose, melibiose, lactose, raffinose, xylan and locust bean gum; 1%, w/w), organic nitrogen supplements (yeast extract, tryptone, corn steep liquor, urea; 0.3%, w/w) and inorganic nitrogen supplements ($(NH_4)_2SO_4$, NH_4Cl, $(NH_4)_2HPO_4$, KNO_3 and $NaNO_3$; final concentration of nitrogen 0.004%, w/w) were incorporated in the medium to investigate their effects on α-galactosidase production. At each step of optimization, the process conditions resulting in the highest enzyme yield were chosen as the control for the next experiment.

2.5. Enzyme extraction

The fermented matter was thoroughly mixed with distilled water (1:5, w/v) on a rotary shaker at 200 rpm for 1 h. It was then filtered through muslin cloth and the filtrate obtained was centrifuged at 10,000 rpm for 20 min at 4 °C. The resultant supernatant was used as the enzyme preparation.

2.6. Enzyme assay

The activity of α-galactosidase was routinely determined using *p*-nitrophenyl α-D-galactopyranoside (*p*NPG), as reported earlier (Dey & Pridham, 1969; Anisha & Prema, 2007). One unit (U) of enzyme activity was defined as the amount of enzyme that liberated one μmol of *p*-nitrophenol/min under the assay conditions. α-Galactosidase yield under SSF was expressed as U/g of dry fermented substrate.

2.7. Partial purification of α-galactosidase

The proteins in the crude enzyme extract were concentrated by ammonium sulphate precipitation (0–80% saturation). The precipitate obtained by centrifugation (10,000 rpm, 20 min) was dissolved in the minimal amount of buffer and dialyzed against the same buffer overnight at 4 °C. The α-galactosidase-active fractions were pooled and used as partially purified enzyme preparation for characterization.

2.8. Effect of pH on activity and stability of partially purified enzyme preparation

The optimum pH for α-galactosidase activity was determined in the assay mixture over a pH range of 4.0–9.0. For pH stability determination, enzyme preparations were incubated at pH values in the range of 4.0–9.0 for 5 h at optimum temperature and residual activity was determined at regular time intervals of 30 min.

2.9. Effect of temperature on activity and stability of partially purified enzyme preparation

The optimum temperature for enzyme activity was determined by incubating the assay mixture at different temperatures ranging from 40 to 75 °C. Enzyme preparations were incubated at temperatures in the range 50–75 °C, for 5 h at optimum pH, and residual activity was determined as described previously.

2.10. Native PAGE and zymogram analysis

Native PAGE (10%, w/v) was done under non-denaturing conditions in a vertical slab gel apparatus. α-Galactosidase activity was visualized by incubating gel at 65 °C for 10 min in 0.15 M citrate phosphate buffer (pH 5.0) containing the fluorescent substrate, 4-methyl-umbeliferyl-α-D-galactopyranoside (MU-α-gal, 1 mM). The activity band was visualized as a fluorescent band under a UV transilluminator. Corresponding protein bands in the other half of the gel were visualized by staining with Coomassie Brilliant Blue R-250.

2.11. Biomass estimation

The biomass was estimated by determining the *N*-acetyl glucosamine released from the cell wall of the actinomycete by acid hydrolysis (Sakurai, Lee, & Shiota, 1977) and was expressed as mg of glucosamine/g of dry fermented substrate.

2.12. Statistical analysis

All experiments were carried out in triplicate to check the reproducibility of results. The data presented here are the average of triplicate determinations ± SD.

3. Results and discussion

3.1. Selection of substrates for α-galactosidase production

Of the various solid substrates screened, soybean flour proved to be most suitable for the colonization of *S. griseoloalbus*, as indicated by the maximum visible growth on the surface of substrate and the highest enzyme yield (80.5 ± 0.08 U/gds). The increased α-galactosidase yield could be attributed to the presence of α-galactosides in soybean flour. Unlike the reports of Cruz and Park (1982), using ground soy products directly as the solid substrate is

much more economical than extracting, fermenting and recovering the oligosaccharides and then adding them to the SSF system. Developing an α-galactosidase production process based upon soybean flour as the solid substrate is very attractive, since it is a readily available source of carbon. All the other tested legume-based substrates, such as soymeal (73.6 ± 0.06 U/gds), guar flour (72.6 ± 0.03 U/gds), and chickpea flour (59.8 ± 0.04 U/gds), and oil cakes, such as groundnut oil cake (66.4 ± 0.17 U/gds) and coconut oil cake (57.9 ± 0.1 U/gds) supported good α-galactosidase yield. Wheat bran (37.9 ± 0.2 U/gds), rice bran (8.1 ± 0.25 U/gds), maize flour (23.7 ± 0.11 U/gds) and sorghum flour (21.6 ± 0.09 U/gds) gave only poor results.

3.2. Optimization of process parameters

Soybean flour in the particle size range of 0.5–1 mm (Fig. 1A) and an initial moisture level of 40% (Fig. 1B) supported the highest α-galactosidase yield by *S. griseoloalbus*. Though filamentous microorganisms are generally reported to grow best when the substrate moisture content is between 50 and 75% (Pandey, Soccol, Rodriguez-Leon, & Nigam, 2001), Annunziato, Mahoney, and Mudgett (1986) reported 35% initial moisture to be the optimum for highest α-galactosidase yield from *Aspergillus oryzae*. The decrease in moisture level is advantageous since the chance of contamination in the fermentation medium is reduced. However, decrease in moisture level below the optimum results in minimized heat exchange, oxygen transfer, reduced solubility and low availability of nutrients to the culture. Higher initial moisture in SSF decreases porosity, changes the particle structure and promotes development of stickiness due to agglomeration of the substrate. This reduces the mass transfer process and gas exchange, subsequently restricting the supply of oxygen for the growth of microorganism and leading to suboptimal product formation (Narahara et al., 1982; Pandey et al., 2001). The moisture content of the substrate in SSF greatly depends on water activity of the substrate which, in turn, is highly dependent on the water binding properties of the substrate (Krishna, 2005). For a substrate like soybean flour, the water activity is very high and hence even a slight increase in water content by addition of mineral salt solution can lead to agglomeration of the substrate. The importance of moisture

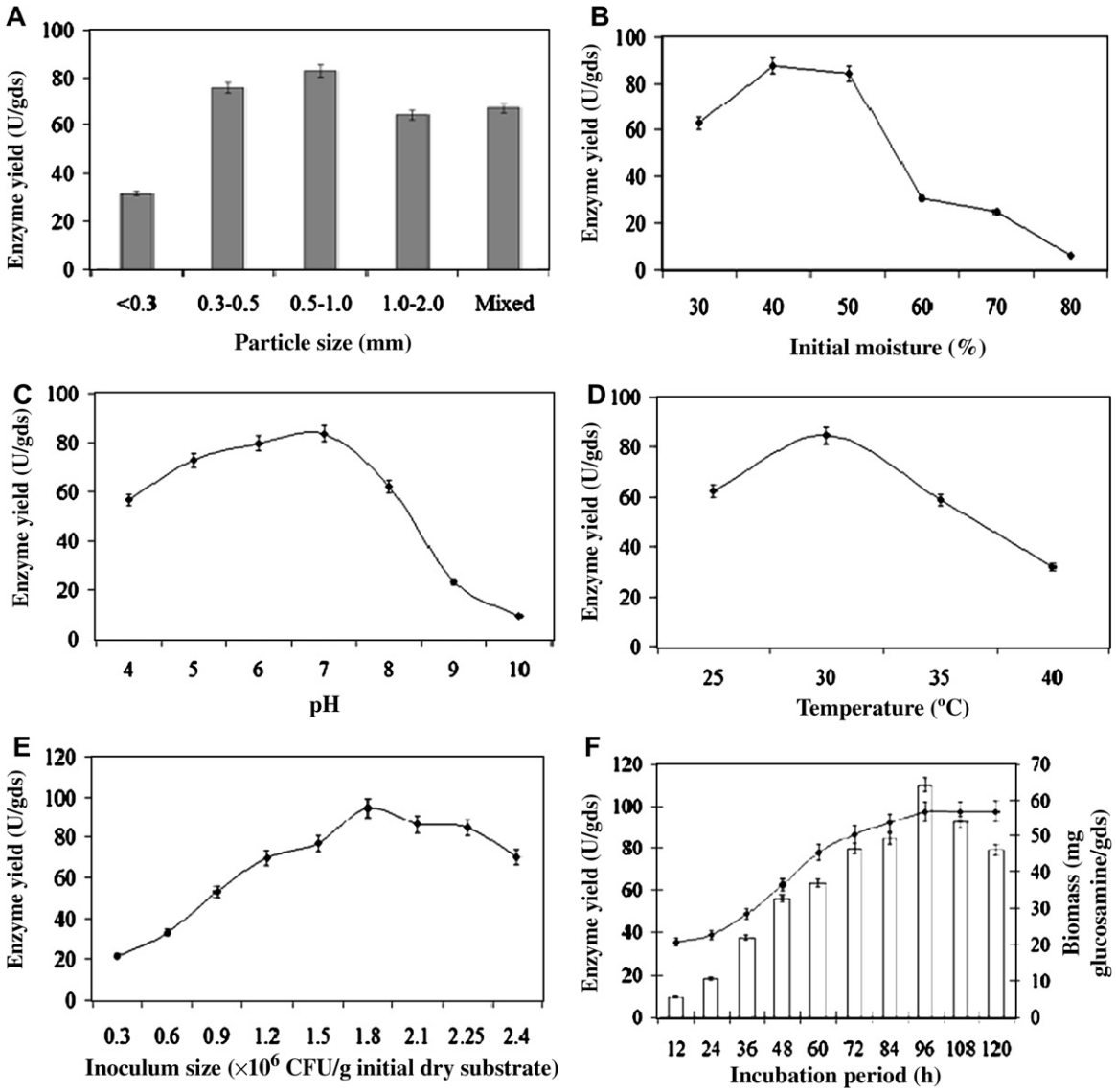

Fig. 1. Effects of (A) particle size, (B) initial moisture (care was taken to keep the amount of mineral salt solution constant while varying the moisture level), (C) initial pH of medium, (D) incubation temperature, (E) inoculum size (3×10^6 CFU/ml) on α-galactosidase production and (F) fermentation profile of *Streptomyces griseoloalbus* showing α-galactosidase yield (□) and growth kinetics (♦) at different hours of incubation.

and water activity implies that it is necessary to consider the exact quantities to be added to the substrate, while preparing a substrate (Gowthaman, Krishna, & Moo-Young, 2001).

The optimum pH of medium (Fig. 1C) and incubation temperature (Fig. 1D) for α-galactosidase production were found to be 7.0 and 30 °C, respectively. The α-galactosidase yield was comparatively higher in the acidic range than in the alkaline range. At pH 10.0, the enzyme yield was drastically reduced. α-Galactosidase yield increased with increase in incubation temperature from 25 to 30 °C and higher temperatures were detrimental to the growth and α-galactosidase production by *S. griseoloalbus*, which may be attributed to the mesophilic nature of the microbe.

The optimum inoculum size for α-galactosidase production by *S. griseoloalbus* was 1.8×10^6 CFU/g initial dry substrate (Fig. 1E). Adequate inoculum can initiate fast mycelium growth and product formation, thereby reducing the growth of contaminants. A decrease in enzyme production was observed when the inoculum size was increased beyond the optimum level. Enzyme production attains its peak when sufficient nutrients are available to the biomass. Conditions with a misbalance between nutrients and proliferating biomass result in decreased enzyme synthesis (Ramachandran, Roopesh, Nampoothiri, Szakacs, & Pandey, 2005).

Among the different carbon supplements tested, galactose had the highest inducing effect on α-galactosidase synthesis by *S. griseoloalbus* in SSF (110 ± 0.2 U/gds). SSF systems are resistant to catabolite repression, even at high sugar concentration. This is because thin layers of microbial cells growing on a solid support exhibit changes in cell permeability to sugars, thereby creating a microenvironment where sugar concentration is optimal for growth and enzyme production (Viniegra-González & Favela-Torres, 2006). None of the nitrogen supplements enhanced α-galactosidase production. The soybean flour is a rich source of nitrogen, in the form of protein, which accounts for about 40% of dry soybeans by weight. This was sufficient to meet the nitrogen demand of the culture, thereby avoiding the need for nitrogen supplementation.

The time courses of biomass and α-galactosidase production by *S. griseoloalbus* are shown in Fig. 1F. There was a lag phase up to 24 h, after which the biomass increased rapidly in the exponential growth phase from 24 to 96 h, followed by a stationary phase. The initial α-galactosidase biosynthesis by *S. griseoloalbus* was correlated with the growth profile of the culture and reached the maximum at 96 h when the organism had established itself well in the deeper layers of the solid medium. A decrease in enzyme titre was observed after 96 h of fermentation which could be due to the depletion of nutrients for enzyme synthesis and proteolytic degradation of already synthesized enzymes. At this stationary phase of growth, nutrients might have been utilized mostly for cell multiplication and compensation of cell death. The partially purified enzyme preparation, which was free of cell biomass, was stable for more than six months (data not shown).

3.3. Partial purification and characterization

Partial purification by ammonium sulphate precipitation increased the specific activity 13.7-fold. α-Galactosidase activity was obtained in 50–80% ammonium sulphate precipitated fractions.

Characterization studies of the partially purified enzyme preparation indicated that the optimum pH for highest α-galactosidase activity was 5.0 (Fig. 2). The enzyme was active over a wide range of pH (4.0–9) with more activity toward the acidic range than toward alkaline range. At pH 4.0, the enzyme showed 85% of the activity obtained at pH 5.0. Only a negligible amount of activity was obtained at pH 9.0. The enzyme activity remained stable for 5 h over a wide pH range (5.0–7.0) (Fig. 2). This is an appreciable character for application in the treatment of legume-based food.

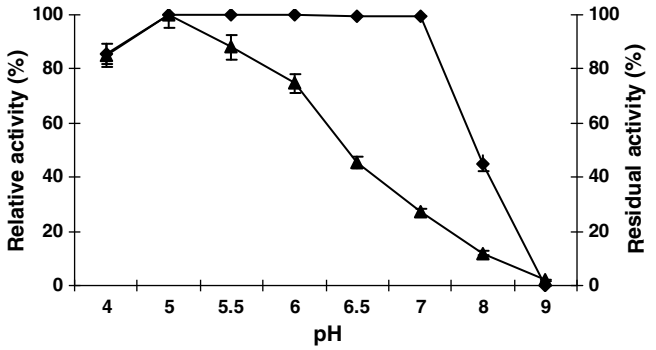

Fig. 2. Effects of pH on activity (▲) and stability (♦) of partially purified α-galactosidase. For the determination of optimum pH, relative α-galactosidase activity was determined in the assay mixture over a pH range of 4.0–9.0. For pH stability determination, enzyme preparations were incubated at pH values in the range 4.0–9.0 for 5 h at optimum temperature and residual activity was determined at regular time intervals of 30 min.

The pH of legume slurries meant for enzymatic treatment varies considerably. An enzyme with activity over a wide range of pH is advantageous, because it will eliminate the necessity for pH adjustment, which may cause precipitation of proteins, leaving a sour taste in such food (Gote, Umalkar, Khan, & Khire, 2004). Mansour and Khalil (1998) have reported α-galactosidase activity in *A. oryzae* and *A. niger* that is stable over the pH range 4.0–7.0 and *Cladosporium cladosporides* α-galactosidase is stable over the pH range 5.0–7.0.

The optimum temperature of the partially purified α-galactosidase preparation was determined at pH 5.0. The enzyme preparation was most active at 65 °C and exhibited 74% activity at 70 °C (Fig. 3). Thermostability of α-galactosidase was determined by studying the time-dependent thermal inactivation of enzyme, as shown in Fig. 4. The enzyme was found to be completely stable at 50 and 55 °C for 5 h. The half-life ($t_{1/2}$) of inactivation of α-galactosidase at 65 °C was 3.5 h. Enzyme activity decreased rapidly at 70 and 75 °C. The high thermostability of the enzyme is advantageous for industrial application, especially when removing RFO from soymilk and other soy-based products, as the high temperature (usually 65–70 °C), used during the pasteurization step following the soybean processing, leads to denaturation of thermolabile enzymes. α-Galactosidases of hyperthermophilic bacteria *Thermotoga maritima* ($t_{1/2}$ 6 h at 85 °C) (Miller et al., 2001) and *Thermotoga neapolitana* (75% activity after 4 h at 85 °C) (King, Yernool, Eveleigh, & Chassy, 1998) are reported to have activity and prolonged stability above 75 °C.

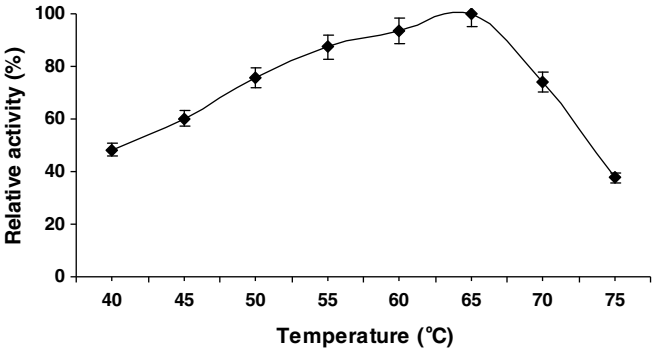

Fig. 3. Effect of temperature on activity (♦) of partially purified α-galactosidase. The optimum temperature for enzyme activity was determined by incubating the assay mixture at different temperatures ranging from 40 to 75 °C.

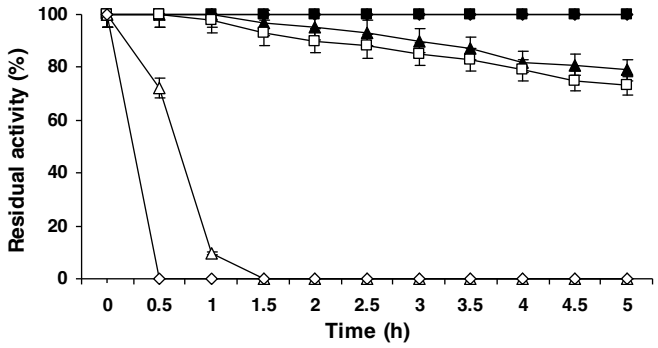

Fig. 4. Time-dependent thermal inactivation of partially purified α-galactosidase at 50 (♦), 55 (■), 60 (▲), 65 (□), 70 (△) and 75 °C (◇). For stability determination, enzyme preparations were incubated at temperatures in the range of 50–75 °C for 5 h at optimum pH and residual activity was determined at regular time intervals of 30 min.

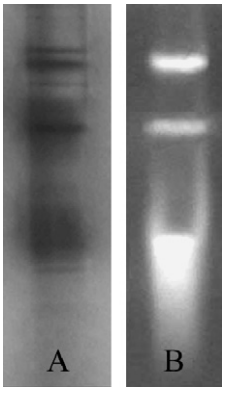

Fig. 5. Native PAGE (A) and zymogram analysis (B) of partially purified α-galactosidase. α-Galactosidase activity was visualized by incubating gel at 65 °C for 10 min in 0.15 M citrate phosphate buffer (pH 5.0) containing fluorescent substrate, 4-methyl-umbeliferyl-α-D-galactopyranoside (1 mM).

3.4. Native PAGE and zymogram analysis

The native PAGE and zymogram analysis, with MU-α-gal, of the partially purified enzyme preparation showed the presence of three enzymes having α-galactosidase activity (Fig. 5). The wide range of pH and temperature stabilities of the partially purified enzyme preparation could be attributed to the presence of multiple α-galactosidases. The presence of multiple α-galactosidases has been previously reported by Ademark, Larsson, Tjerneld, and Stálbrand (2001) in *A. niger*.

4. Conclusions

The results obtained during the course of this study indicate the scope for utilization of thermostable α-galactosidases from *S. griseoloalbus* for the processing of legume foods. The relatively wide range of pH and temperature for activity and stability exhibited by the α-galactosidase preparation from *S. griseoloalbus* is interesting and deserves further study. Purification and characterization of the three α-galactosidases is in progress.

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