GLOBAL JOURNAL OF BIO-SCIENCE AND BIOTECHNOLOGY

www.scienceandnature.org

An α-L- rhamnosiadase from *Aspergillus oryzee* NAIMCC-F-02469 active in neutral pH range

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ABSTRACT
To purify, characterize and assess the biotechnological application of α-L rhamnosiadase of *Aspergillus oryzee* NAIMCC-F-02469. The fungal strain *Aspergillus oryzee* NAIMCC-F-02469 has been isolated from damage rice grain and grown in the liquid culture growth medium using rice grain as an inducer for screening of α-L rhamnosidase. The enzyme has been purified from the culture filtrate of the fungal strain by ammonium sulphate precipitation and cation exchange chromatography on carboxymethyl cellulose. The purified enzyme gave single protein band in both sodium dodecyl sulphate and native polyacrylamide gel electrophoresis indicating that the enzyme was pure. The K_m and k_{cat} values for the enzyme using p- nitro phenyl- α-L rhamnopyranoside were 0.63 m mol l^{-1} and 21.1 s^{-1}, respectively, at optimum pH 7.0 and optimum temperature 60°C. The purified enzyme cleaved L- rhamnose from extract of fresh orange peel. This communication reports a simple procedure for the purification and application of an α-L-rhamnosidase from the culture filtrate of a fungal strain isolated from decayed rice grain. The purified enzyme can be used for specifically cleaving terminal α-L-rhamnose from the natural products for the preparation of pharmaceutically important compounds like prunin, α-L- rhamnose from citrus fruit waste.

KEYWORDS: glycosidase, hesperidin, L- rhamnose, naringin, *Aspergillus oryzee* NAIMCC-F-02469, orange peel.

INTRODUCTION

α-L-rhamnosidase [EC.3.2.1.40] cleaves terminal α-L-rhamnose specifically from a large number of natural glycosides (Chandler et al. 1975; Puri et al. 2000 ; Yadav et al. 2010) such as naringin ,rutin, hesperidin and quarcitrin. The enzyme is widely distributed in nature and has been reported from animal tissues (Kurosawa et al. 1973 ; Qian et al. 2005) Plants (Suzuki 1962 ; Bourbouze et al. 1975) yeasts (Yanai et al. 2000)] fungi (Manzanares et al. 2000 ; Scaroni et al. 2002 ; Puri and Kalra 2005 ; Manzanares et al. 2001; Yu et al. 2002) and bacteria (Jang et al. 1996 ; Victor et al. 1987 ; Park et.al. 2005; Zverlov et al. 2000 ; Orrillo et al. 2007 ; Hashimoto et al. 1998 ; Hashimoto et al. 1999). The enzyme has several biotechnological applications such as debittering of citrus fruit juices caused by naringin (Gray et al. 1981 ; Tsen et al. 1989; Manjon et al. 1985; Yadav et al. 2000, Prakash et al. 2002 ; Busto et al. 2007) hydrolysis of hesperidin to release hesperetin glucoside (Chase 1974) which is an important precursor in sweetener production, preparation of many drugs and drug precursors by derhamnosylating the terminal L- rhamnose containing substrates (Elujoba et al. 1987; Monti et al. 2004; Feng et al. 2005) and preparation of prunin (Roitner et al.1984) and L- rhamnose. The enzyme has also been used in enhancing the aroma of wine (Caldiny et al. 1994) and derived beverages in grape juice (Gunata et al. 1988; Williums et al. 1982). Keeping in view the biotechnological applications of this enzyme, we initiated studies on α-L-rhamnosidases suitable for different applications. Recently, we have reported an α-L-rhamnosidase from *Aspergillus flavus* MTCC-9606, which has pH optimum in alkaline medium (Yadav et al. 2011). In this communication, we report an α-L-rhamnosidase from *Penicillium citrinum* MTCC-8897 which has optimum activity in neutral pH. Attempts have also been made to test the suitability of the enzyme for the removal of L- rhamnose from commercial naringin, and fresh orange fruit juice (scheme 1).

MATERIAL AND METHODS

Chemicals

p-nitrophenyl-α-L-rhamnopyranoside, naringin, L-rhamnose, rutin, CM cellulose were purchased from Sigma Chemical Company, St. Louis (USA). All the chemicals including the protein molecular weight markers used in the polyacrylamide gel electrophoresis were procured from Bangalore GENEI Pvt. Limited Bangalore (India). All other chemicals were either from Merck Limited Mumbai (India) or from s. d. – fine CHEM limited Mumbai (India) and were used without further purifications. The wine sample was from Macleodls distillers Ltd. United Kingdom UK.

Fungal strains

The fungal strain *Aspergillus oryzee* NAIMCC-F-02469 was isolated from damage rice grain and identified from Microbial Type Culture Collection Center and Gene Bank, Institute of Microbial Technology, Chandigarh (India) and maintained in the laboratory on the agar slant as mentioned in the MTCC catalogue-2000.

The secretion of α-L-rhamnosidase by the fungal strain was studied in the liquid culture medium having composition: $CaCl_2$ 1.0 g, $MgSO_4.7H_2O$ 3.0 g, KH_2PO_4 20.0 g, $N(CH_2COONa)_3$ 1.5 g, $MnSO_4$ 1.0 g, $ZnSO_4.7H_2O$

0.1 g , $CuSO_4.5H_2O$ 0.1g, $FeSO_4.7H_2O$ 0.1 g, H_3BO_3 10.0 mg, sucrose 40.0 g, ammonium tartarate 8.0 g. water (MilliQ)1000 ml. One ml of the spore suspension (spore density 6 x 10^5 spores/ml) from the agar slant was inoculated aseptically into the liquid culture medium (25ml) kept in 100 ml culture flask which also contained 0.5 % of naringin or rutin or 5 g/ 25ml of corn cob or bagasse or rice grains as inducers. The experiments were performed in triplicates. The culture flasks were incubated in a Biological Oxygen Demand (BOD) incubator at 27°C and the fungal strain was allowed to grow under stationary condition. One ml of the growth medium were withdrawn at the regular intervals of 24 h, were filtered through Millex syringe filters (0.22µm) and were analyzed for the presence of α- L-rhamnosidase activity as given below. Enzyme units /ml were plotted against growth time in days. The enzyme unit/ml plotted in the graph indicated the relative amounts of the enzyme secreted in the medium.

Enzyme assay

The activity of α-L-rhamnosidase was assayed using p-nitrophenyl-α- L- rhamnopyranoside as the substrate and monitoring the liberation of p- nitrophenol spectrphotometrically at λ= 400nm using molar extinction coefficient value of 21.44 $nm^{-1}cm^{-1}$ by using reported method (Romero and Manjon et al. 1985). The assay solution 1.0 ml contained 0.4m mol l^{-1} p-nitrophenyl-α-L- rhamnopyranoside, 100 µl of the culture filtrate in 0.5M sodium phosphate buffer pH 7.2 at 60°C.All Spectrophotometric measurements were made with UV/Vis spectrophotometer Hitachi (Japan) model U-2000 which was connected to electronic temperature control unit. The least count of the absorbance measurements was 0.001 absorbance unit. One enzyme unit is the amount of the enzyme which librates one µmole of p- nitrophenolate ion/min at 60°C under the assay conditions specified above. The steady state velocity measurements were reproducible within 5% standard deviation.

Purification of α-L-rhamnosidase

For the purification of α-L-rhamnosidase, the fungal strain was grown in fifteen sterilized 100 ml culture flasks each containing 25 ml liquid culture medium amended with 0.5% naringin. The maximum activity of α-L-rhamnosidase appeared on eighth day of the inoculation of the fungal spores. The fungal cultures in all the culture flasks were pooled and the mycelia were removed by filtering the culture medium through four layers of cheese cloth. The culture filtrate was centrifuged using Sigma refrigerated centrifuge model 3K30 at 8,000 × g for 20 min at 4 °C to remove the particles. The desired enzyme was precipitated from the clear culture filtrate (350 ml) by slowly adding fine powdered ammonium sulphate up to 85% saturation at 4°C.The precipitate was collected by centrifugation as mentioned above. The precipitate was dissolve in 0.1 mol l^{-1} sodium acetate/ acetic acid buffer pH 5.0 and was dialyzed against 0.01 mol l^{-1} sodium acetate/ acetic acid buffer of pH 5.0 using 1: 1000 excess of the buffer with three changes in the 24 hours. The appropriate condition for the binding of α-L-rhamnosidase on CM cellulose was determined experimentally by the reported method (Pharmecia 1983). The 5.0 ml of dialyzed enzyme was loaded on CM-cellulose column of size 2.5 X 15 cm equilibrated with 0.01 mol l^{-1}sodium acetate/ acetic acid buffer of pH 5. The column was washed with the same buffer and α-L-rhamnosidase activity was eluted using the linear NaCl gradient of 0-1.0 mol l^{-1} in the same buffer (50 ml of the buffer + 50 ml buffer containing 1.0 mol l^{-1} NaCl). Fractions of 4.0 ml size were collected and were analyzed for the activity of α-L-rhamnosidase using the reported method (Romero and Manjon, et al. 1985) and for protein concentration using Lowry's method (Lowry et al. 1951). The α-L-rhamnosidase active fractions were combined and concentrated by putting in dialysis bag which was kept in solid powdered sucrose. The purified, concentrated enzyme sample 3.0 ml was stored in the refrigerator and was used whenever required.

SDS- PAGE and Native PAGE analysis of the purified enzyme

The homogeneity of the enzyme preparation was checked by SDS-PAGE analysis using the reported method (Weber and Osborn 1969). The resolving gel was 12% acrylamide in 1.5 mol l^{-1} Tris-HCl buffer of pH 8.8 and stacking gel was 5% acrylamide in 0. 5 mol l^{-1} Tris-HCl buffer of pH 6.8. The electrophoresis buffer was 0.025 mol l^{-1} Tris - glycine buffer of pH 8.5. The gel was run at constant current of 20mA. The enzyme was visualized by silver staining. The molecular weight markers used were phosphorylase B (97.4 kDa), bovine serum albumin (66.0 k Da), ovalbumin (43.0 kDa) , carbonic anhydrase (29.0) and soyabean trypsin inhibitor (20.1 kDa). The molecular mass of the purified enzyme was determined by plotting the log of the molecular masses of the protein molecular mass markers verses relative distances travelled by the protein molecular mass markers on the SDS polyacrylamide gel. The log of the molecular mass of the purified enzyme was read corresponding to the relative mobility of the purified enzyme and the molecular mass was calculated from the corresponding value of the log of molecular mass of the enzyme.

The native polyacrylamide gel electrophoresis was done using the reagent kit supplied by Bangalore GENEI Pvt. Limited Bangalore (India). The resolving gel was 10% acrylamide in1.5 mol l^{-1} Tris – HCl buffer of pH 8.8 and the stacking gel was 5% acrylamide in 0.5 mol l^{-1} Tris – HCl buffer of pH 6.8. The molecular weight markers used were phosphorylase B (97.4 kDa) and bovin serum albumin (67.0kDa). The proteins were located by silver staining.

Enzymatic characteristics

The Michaelis- Menten behavior of the purified enzyme for the substrate p-nitrophenyl-α-L-rhamnopyranoside was determined by measuring the steady state velocity of the enzyme catalyzed reaction at different concentrations of p-nitrophenyl-α-L-rhamnopyranoside (0.05 m mol l^{-1} to 1.5 m mol l^{-1}) using the reported method. The Km and Vmax value were determined by linear regression analysis of the data points of the double reciprocal plot.

The pH optimum of the purified enzyme was determined by using p-nitrophenyl-α-L-rhamnopyranoside as the substrate and measuring the steady state velocity of the enzyme catalyzed reaction in solutions of varying pH in the range 5 to 13. The buffers used were 0.5 mol l^{-1} NaH_2PO_4/NaOH. The steady state velocity was plotted

against pH of the reaction solutions and pH optimum was calculated from the graph.

The temperature optimum was determined by measuring the steady state velocity of the enzyme catalyzed reaction in solutions of varying temperatures (40-70°C) using p-nitrophenyl- α-L-rhamnopyranoside as the substrate. The steady state velocity of the enzyme catalyzed reaction was plotted against the temperature of the reaction solution and temperature optimum was calculated from the graph.

The pH stability of the enzyme was determined by incubating the enzyme in the buffer of different pHs for 24 h at 25°C. The residual activity were assayed and plotted vs. pHs to which the enzyme was exposed for 24 h.

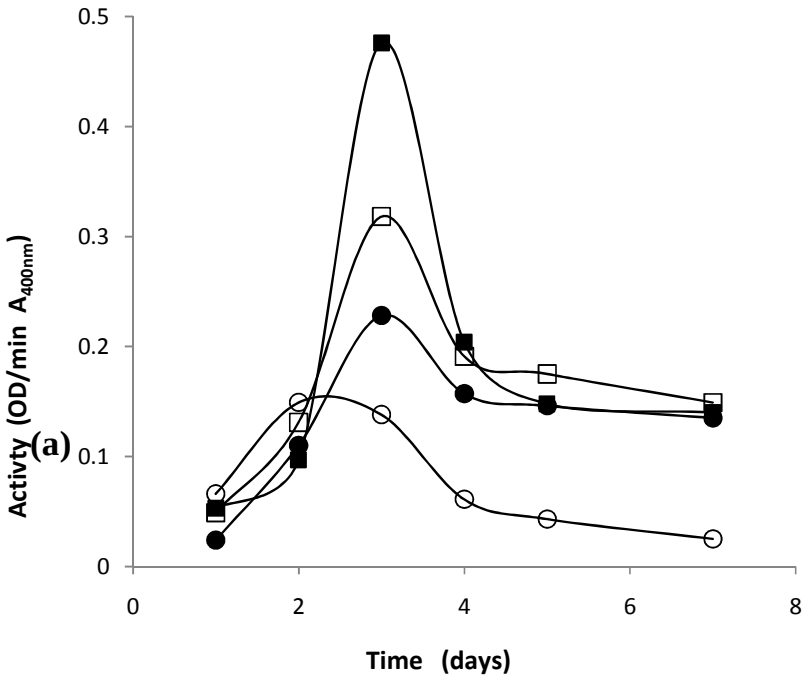
The thermal stability was determine by incubating the aliquots of the enzyme at different temperatures (viz. 20°,30°,40°,50°,60°and 70°C)and assaying the residual activity at the interval of 20 min for two h. The residual activity was plotted against the time for which the enzyme was exposed at that temperature.

Studies on the release of L- rhamnose from the orange peel by the purified enzyme

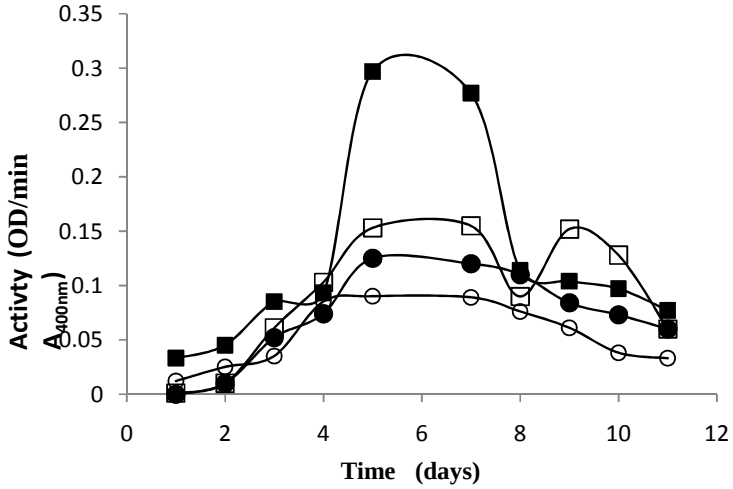
In 1.0 ml solution of 1.0 m mol l^{-1} naringin in 0.5 mol l^{-1} sodium phosphate buffer pH 7.0 at 30°C, 20µl of the purified enzyme stock 0.54 IU/ml was added and UV/Vis spectrum was recorded in the range 200-800 nm at regular intervals of 15 min . The reaction solution was left over night and the release of L- rhamnose was detected by thin layer chromatography using silica gel on glass plates. The mobile phase used was chloroform-methanol mixture 70:30 (v/v). The detection was made in iodine chamber. Another experiment was performed using fresh orange juice. The pH of solution of orange juice was maintained at pH 4.5 by sodium acetate acetic acid buffer and treated by α-L-rhamnosidase (0.82 IU/mL) at 50ºC. The reaction solution was left overnight and the release of L-rhamnose was detected by thin-layer chromatography using silica gel on glass plates

RESULTS

The secretion of α-L-rhamnosidase in the liquid culture medium amended by the addition of the probable inducers is shown in two steps fig.1 (a and b). It is obvious from the figure 1(a) of first step experiment that the peak value of enzyme secreted in the liquid culture mediums amended with naringin, rutin, and hesperidin in which growth medium containing 0.5% naringin is maximum on 3rd day after incubation of the fungal spores. The second steps of experiment shown in figure 1(b), the peak value of secretion of enzyme at 5th day in liquid culture growth medium amended with 4% rice grain (w/v).



(a). Control (○); growth medium+ naringin (■); growth medium+ rutin (□); growth medium+ Hesperidin (●);



(b). Control (○); growth medium+ rice grain (■); growth medium+ bagasse (□); growth medium+ corn cob (●);

FIGURE 1: Secretion of α-L-Rhamnosidase in the culture medium amended with inducers.

The purification procedure of the α-L-rhamnosidase from the culture filtrates of *Aspergillus oryzee* NAIMCC-F-02469 is summarized in table-1 and elution profile of the enzyme from the CM-Cellulose column is shown about 18 fold purification of the enzyme with 25% recovery of the activity was achieved Figure not shown. The result of SDS-PAGE analysis is shown a single protein band showed that the enzyme was pure. The relative molecular mass calculated from the SDS-PAGE analysis was 56.0 kDa.

TABLE 1: Summary of purification procedures

Steps	Vol.(ml)	Activity (IU/ml)	Protein (mg/ml)	Total activity (IU)	Total protein (mg)	Specific activity	Purification fold	% Yield
Crude	150	0.22	0.10	33	14.25	2.32	1	100
Ammonium sulphate conc.	5	1.14	0.43	5.69	2.15	2.65	1.14	17.24
Dialysis	9	0.67	0.16	6.03	1.44	4.19	1.96	18.27
CM cellulose	15	0.54	0.02	8.12	0.1	42.74	18.42	24.60

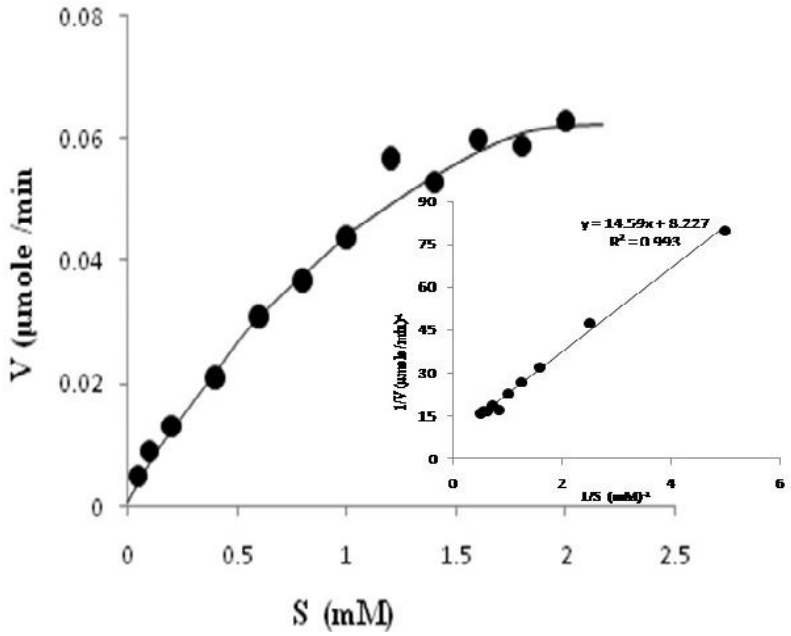


FIGURE 2: (a) Michalis- Menten and (b) Double reciprocal plots of the purified enzyme using p-nitrophenyl α- L- rhamnopyranoside as the substrate. The assay solution 1.0 ml contained (0.05 m mol l^{-1} to 1.5 m mol l^{-1}) substrate, 0.56 µg of pure enzyme in 0.5 mol l^{-1} sodium phosphate buffer pH 7.2 at 50°C.

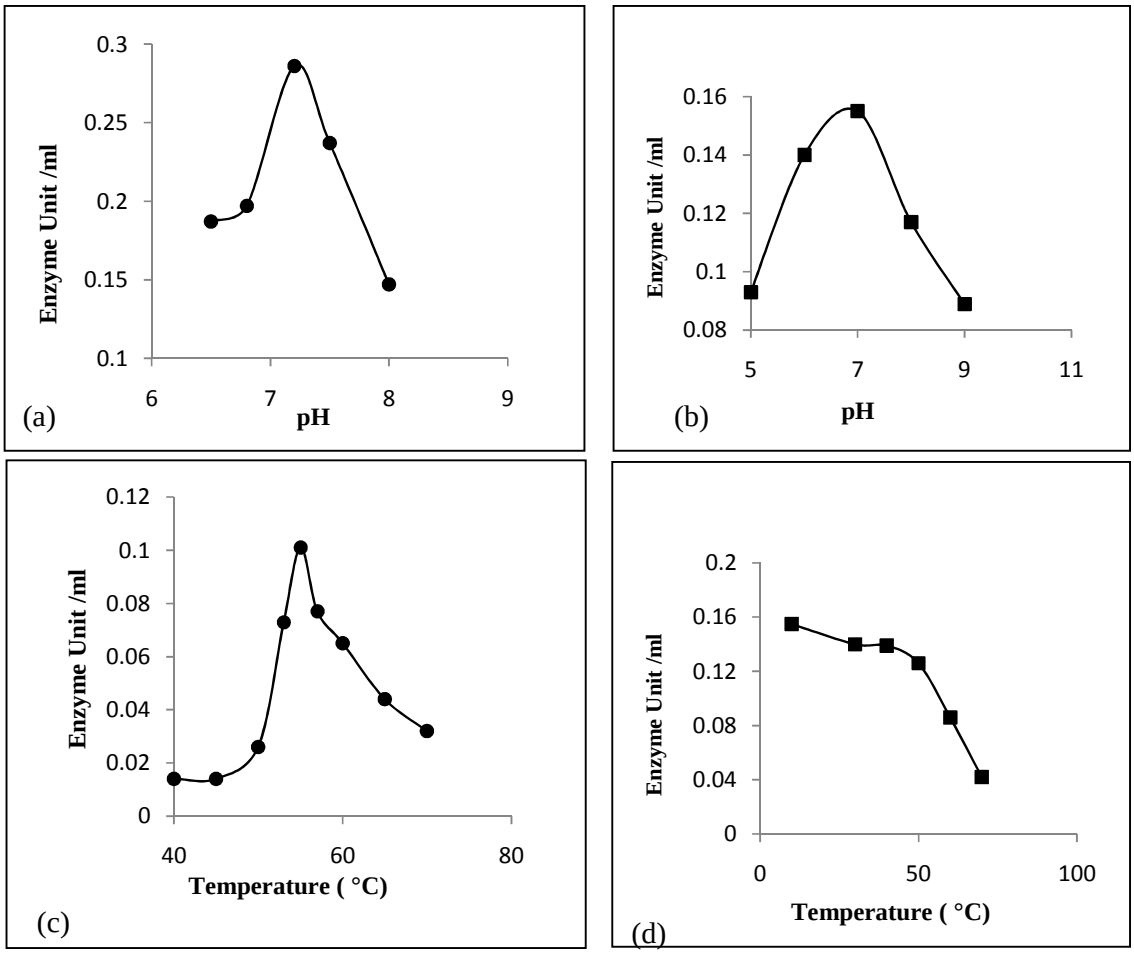


Fig. 3: Effects of (a) reaction pH (b) pH stability , (c) reaction temperature and (d) temperature stability on the activity of the enzyme .
(a) The assay solution 1.0 ml contained 0.4m mol substrate, 0.56 µg of pure enzyme in 0.5 mol l^{-1} sodium phosphate buffer of varying pH in the range 5 to 13 at 50°C.
(b) The assay solution 1.0 ml contained 0.4m mol substrate, 0.56 µg of pure enzyme in 0.5 mol l^{-1} sodium phosphate buffer pH 7.2 at varying temperatures (40-70°C).

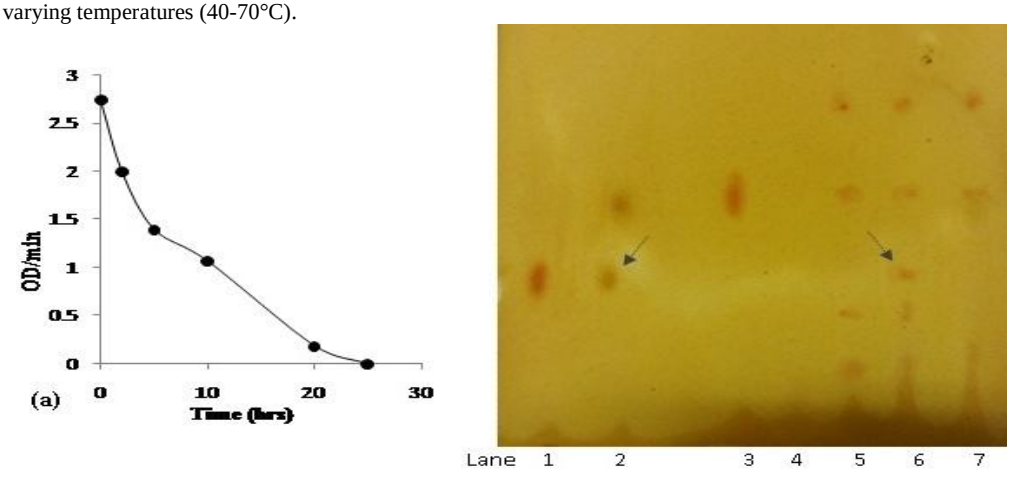
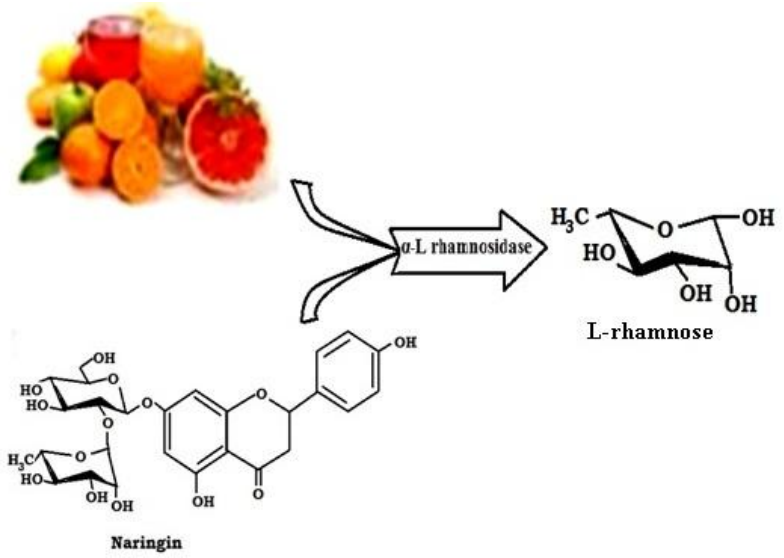


Fig. 4: Studies on the release of L- rhamnose from naringin and fresh orange juice by the purified enzyme.
(a). Enzymatic hydrolysis of naringin. A_{400} of naringin solution decreases with time.
Lane-1; pure L-rhamnose ,Lane-2; release of L- rhamnose from naringin treated with enzyme, lane -3 Stander sample of glucose, lane - 4 stander sample of naringin, lane -5 fresh orange juice, lane- 6 orange juice treated with enzyme lane-7 orange juice heated with water.



SCHEME 1: Enzymatic hydrolysis of naringin.

The Michealis-Menten behavior of the purified enzyme using p-nitrophenyl-α-L-rhamnosidase as the substrate is shown in fig.2. The calculated K_m value for this enzyme using p-nitrophenyl- α-L-rhamnopyranoside as the substrate was 0.36m mol l^{-1} and V_{max} value was 22.54 µmole/min/mg at 50°C in 0.5mol l^{1} sodium phosphate-NaOH buffer pH 7.0.

The results of studies on the variation of activity of the purified enzyme with the variation of the pH of the reaction solution are shown in figure -3(a). The pH optima of the purified enzyme was 7.0 using p-nitrophenyl- α-L-rhamnopyranoside as the substrate. The variation of the activity of the purified enzyme with temperature of the reaction solution is shown in the fig-3(c). The temperature optimum of the enzyme was 50ºC.

In order to find out the suitability conditions for the preservation of the purified enzyme, pH and temperature stability of the enzyme were studied but results are shown in figure 3 (b) and (d). The enzyme was most suitable at pH 7.0 and the activation energy of thermal denaturation of the enzyme was 23.56 $kJ^{-1}mol^{-1}°K$.

The α-L-rhamnosidase has been used in the preparation of L-rhamnose from the natural glycosides containing terminal α-L- rhamnose. This was tested by treating the solutions of naringin, rutin and hesperidin with the purified enzyme and analyzing the formation of L-rhamnose in the reaction solution using thin layer chromatography and the standard samples of L-rhamnose. The results are shown in figure 4.0. It is obvious from the figure that the purified enzyme is cleaving terminal α-L-rhamnose from natural glycosides.

DISCUSSION

In order to enhance the secretion of α-L-rhamnosidase by *A. oryzee* in its liquid culture growth medium, the effects of the presence of naringin, rutin, corn cob, bagasse particles and rice grains in the growth medium were studied. The results (fig. 1) clearly indicate that the peak value of the enzyme secreted in the liquid culture medium is higher only in case of naringin in comparison to the control. In all other cases, the peak values of the enzyme secreted in the medium were lower than the peak value of the enzyme secreted in the case of control. On the basis of these results, for the purification of α-L-rhamnosidase, *A. oryzee* was grown in the liquid culture medium containing 0.5% naringin 4% rice grain as solid support.

The purification procedure (table 1) of α-L-rhamnosidase from the culture filtrate of *P. citrinum* is simpler than the procedures reported for α-L-rhamnosidase from other sources (Yadav and Yadav 2010). It involved only concentration by ammonium sulfate precipitation and chromatography on carboxy methyl cellulose. The enzyme binds on CM cellulose in 10.0 m mol l^{-1}sodium acetate/acetic acid buffer pH 5.0 and is eluted by the linear gradient of NaCl in the range 0.4 mol l^{-1} to 0.8 mol l^{-1}. Both the SDS and native PAGE gave single protein band confirming that the purity of the enzyme. The molecular weight determined by SDS-PAGE analysis was 56.0 kDa which is the range of molecular wts 41.0-240.0 kDa reported for α-L-rhamnosidases in the litrature (Yadav and Yadav 2010).

The K_m and k_{cat} values calculated for the purified enzyme using p-nitrophenyl -α- L-rhamnopyranoside (fig 4) are 0.36m mol l^{-1} and 17.1 s^{-1},respectively, giving k_{cat} / K_m 4.74×10^4 $mol^{-1}1s^{-1}$. The K_m values for α-L-rhamnosidases purified from *Fagopyrum esculeutu* (Bourbouze etal. (1975), from *Bacteroides* JY-6(Jang and Kim 1996), from *Pseudomonas paucimobilis* FP 2001(Miake etal.2000), from *Fusobacterium* K-60 [Park and Kim et al. 2005), from *Penicillium decumbens* (Romero and Manjon et al. 1985) and from *Aspergillus aculeatus* RhaA and RhaB (Manzanares et al.2001) have been reported to be 0.33, 0.29, 1.18 0.06, 1.52 and 0.30 and 2.80m mol l^{-1}, respectively. Thus the purified α-L-rhamnosidase has intermediate affinities for p-nitrophenyl-α-L-rhamnopyranoside as compared to reported α-L-rhamnosidases.

Most of the α-L-rhamnosidases reported so far have pH optima either in the acidic (Kurosawa et al. 1973 ; Manzanares et al. 2001) or in basic (Zverlov et al. 2000; Hashimoto et al. 1998; Miake et al. 2000) pH range. Only α-L-rhamnosidases of *Bacteroides* JY-6 (Jang et al 1996),

pig liver (Qian et al. (2005), *Bacillus* sp. GL1 (Hashimoto et al. 1999) have pH optima in the neutral pH range. The purified α-L-rhamnosidase of *A. oryzee* is another enzyme having pH optimum in neutral pH range. The temperature optimum of this enzyme is 50ºC which is in the range 40-80ºC reported for other α-L-rhamnosidases (Yanai and Sato 2000; Scaroni et al. 2002; Miake et al. 2000). The results of pH stability of the purified α-L-rhamnosidase show that the enzyme retained most of its activity in buffers of the pHs 6.0-7.0 if left for 24 hrs at room temperature. The studies on the thermal stability of the purified enzyme has given the value of energy of activation for thermal denaturation of the enzyme as indicating that the enzyme could be stored refrigerated at pH 7.0.

One of the important applications of the α-L-rhamnosidase is in preparation of L-rhamnose and bioactive compounds by specific removal of terminal α -L-rhamnose from natural products. The purified enzyme cleaved L-rhamnose from naringin and fresh orange juice (fig 4) indicating that it has potential application in the preparation of L-rhamnose from natural products containing terminal α-L-rhamnose and also converting such natural products into pharmaceutically import compounds.

In conclusion, this communication reports the purification of an α-L-rhamnosidase from the culture filtrate of a novel fungal strain *A. oryzee* using a simpler procedures compared to the procedures reported in the literature. The enzyme is active in the neutral pH range and can be used for the preparation of L-rhamnose from the natural rhamnosides containing terminal α-L- rhamnose and also converting them to pharmaceutically import compounds.

ACKNOWLEDGEMENTS

Authors acknowledge the financial support of UGC, New Delhi in the form of a **Post Doctoral Fellowship no. PDFWM-2014-15-OB –UTT- 23606 dated 30.01.2015 awarded to Dr. Sarita Yadav**, under which this research work has been done.

REFERENCES

Bourbouze, R., Pratviel-Sosa, F., Percheron, F. (1975). Rhamnodiastase et α-L-Rhamnosidase De *Fagopyrum Esculentum* Phytochemistry,14,1279-1282 .

Busto, M.D., Meza V., Mateos, N.P.(2007). Immobilization of naringinase from *Aspergillus niger* CECT 2088 in poly (vinyl alcohol) cryogels for the debittering of juices. Food Chem, 104, 1177-1182.

Caldiny, C.,Bonomi, F., Pifferi, P.G., Langirini, G., Alante, Y.M.(1994).Kinetic and immobilization studies on the fungal glycosidase for the aroma inhancement of wine. Enzyme Microb. Technol 16, 286-291.

Chandler, B.V., Nicol, K.J.(1975) . Debittering Citrus products with enzyme. CSIRO Food ResQuart, 35, 79–88.

Chase, T.(1974). Flavour enzyme.Adv.Chem.Ser., 136,241-266.

Elujoba, A.A., Hardman, R.(1987). Diosgenin production by acid and enzymatic hydrolysis of fenugreek. Fitoterapia, 58, 299–303.

Feng, B., Ma, B., Kang, L., Xiong C., Wang S.(2005). The microbiological transformation of steroidal saponins by *Curvularia lunata*. Tetrahedron., 61, 11758-63.

Giovanni, S., Riccardo, N. B., Angella, M., Pier, G. P. (2000). A simple method for purifying glycosidases: α-L-Ramno-pyranosidase from Aspergillus niger to increase the aroma of Moscato wine, 27, 522-530.

Gray G.M., Olson A.C.(1981). Hydrolysis of High Levels of Naringin in Grape Juice Using a Hollow Fiber Naringinase Reactor. J Agric Food Chem. 29(6), 1299-1301.

Gunata, Z., Bitteur, S., Brillouet, J.L Bayonove, C. and Cordonnier, R.(1988). Sequential enzymatichydrolysis of potential aromatic glycosides from grape, Carbohydrate Res.,184, 139-149.

Hashimoto, W., Murata, K.(1998). α-L-Rhamnosidase of *Sphingmonas* sp. R1 producing an unusual Exopolysaccharide of Sphingan. Biosc Biotechnol Biochem, 62(6), 1068-1074.

Hashimoto, W., Nankai, H., Sato, N., Kawai, S., Murata, K.(1999). Characterization of α –L- Rhamnosidase of *Bacillus* sp. GL1 Responsible for the Complete Depolymerization of Gellan. Arch Biochem Biophys. 368, 56-60.

Jang, I.S., Kim, D.H.(1996). Purification and characterization of alpha-L-rhamnosidase from *Bacteroides* JY-6, a human intestinal bacterium. Biol Pharm Bull; 19: 1546-1549.

Kurosawa, Y., Ikeda,K., Egami, F.(1973). alpha-L-Rhamnosidases of the liver of *Turbo cornutus* and *Aspergillus niger* J. Biochem, 73, 31-37.

Lowry, O.H., Rosebrough, N.J., Farrand,A.L., Randall, R.j.(1951). Protein measurement with the Folin-phenol reasent,J,of Biologycal Chem, 193,265-275.

Manjon, A., Bastida, J., Romero,C., Jimeno,A., Iborra J.L.(1985). Immobilization of Naringinase on Glycophase-Coated Porous Glass. Biotechnol Lett, 7(7), 477-482.

Manzanares, P., Orejas, M., Ibanez, E., Valles, S., Ramon, D.(2000). Purification and characterization of an alpha-L-rhamnosidase from *Aspergillus nidulans.* Lett Appl Microbiol, 31, 198-202.

Manzanares,P., Broeck, H.C.V., Graaff, L.H.D., Visser J.(2001)Purification and Characterization of Two Different α-L-Rhamnosidases, RhaA and RhaB from *Aspergillus aculeatus.* Appl Environ Microb; 67(5): 2230-34.

Miake, F., Satho,T., Takesue, H., Yanagida, F., Kashige, N.,Watanabe, K.(2000).Purification and characterization of intracellular alpha-L-rhamnosidase from *Pseudomonas paucimobilis* FP2001. Arch Microbiol, 173, 65-70.

Microbial Type Culture Collection, Catalogue of Strains-2000, Institute of Microbial Technology, Chandigarh, India.

Monti, D., Pisvejcova, A., Kren, V., Lama, M., Riva, S.(2004). Generation of an α-L-Rhamnosidase Library and its Application for the Selective Derhamnosylation of Natural Products;Biotechnology and Bioengineering, 87 (6), 763-771.

Orrillo, A.G., Ledesma, P., Delgado, O.D., Spagna, G., Breccia, J.D.(2007). Cold-active α-l- rhamnosidase from psychrotolerant bacteria isolated from a sub-Antarctic ecosystem. Enz Microb Technol,40, 236-241.

Park, S., Kim, J., Kim, D.(2005). Purification and characterization of quercitrin-hydrolyzing alpha-L-rhamnosidase from *Fusobacterium* K-60, a human intestinal bacterium. J Microb Biotech. 15, 519-24.

Pharmecia F.C.A.B.,(1983). Ion exchange chromatography;principle and methods,Box,175,S- 75104 Uppsala,Sweden.Prakash S., Singhal R.S., Kulkarni P.R.(2002). Enzymic debittering of Indian grapefruit (*Citrus paradisi*) juice. J Sci Food & Agric, 82, 394-397.

Prakash, S., Singhal, R.S., Kulkarni, P.R.(2002). Enzymic debittering of Indian grapefruit (*Citrus paradisi*) juice. J Sci Food & Agric, 82, 394-397.

Puri, M., Banerjee,U.C. (2000). Production, purification, and characterization of the Debittering enzyme naringinase. Biotechnology Advances, 18, 207–217.

Puri, M., Kalra, S.(2005). Purification and characterization of naringinase from a newly isolated strain of *Aspergillus niger* 1344 for the transformation of flavonoids. World J Microb Biotech 21, 753-58.

Qian, S., Yu, H., Zhang, C., Lu, M., Wang, H., Jin, F. (2005).Purification and characterization of Dioscin-α-l-rhamnosidase from *pig liver*. Chem. Pharm. Bull, 53(8), 911-914.

Roitner, M., Schsalkhammer, T.H. and Pittner, F.(1984). Preparation of pruning with help of immobilized naringinase pretreated with alkaline buffer, J.Applied Biochem.&Biotech.,9, 483-488.

Romero,C., Manjon, A. et.al.(1985).A method for assaying the rhamnosidase activity of naringinase. Ana. Biochem., 149,566-571.

Scaroni, E., Cuevas,C., Carrillo, L., Ellenrieder, G. (2002). Hydrolytic properties of crude alpha-L- rhamnosidases produced by several wild strains of mesophilic fungi. Lett Appl Microb, 34, 461-65.

Suzuki, H.(1962). Hydrolysis of Flavonoid Glycosides by Enzymes (Rhamnodiasase) from *Rhamnus* and Other Sources. Arch Biochem Biophys , 99, 476-483.

Tsen, H.Y., Tsai, S.Y., Yu ,G.K.(1989). Fiber Entrapment of Naringinase from *Penicillium* sp. and Application to Fruit Juice Debittering. J Ferment Bioengineer; 67(3):186-189.

Victor D.B., Cedric H.L.S., Jeanette W.(1987). Hydrolysis of dietary flavonoid glycosides by strains of intestinal Bacteroides from humans. Biochem J. 248, 953-56.

Weber, K.,& Osborn, M.,(1969). The reliability of molecular weight determination by dodecyl sulphat polyacrylamide gel electrophoresis. J. of Biol.Chem.,244, 4406-4412.

Williums, P.,C. Strauss, and B.(1982). Wilson.Studied on the hydrolysis of *Vitis vinifera* monoterpene precursor compounds and model monoterpene –D- glucosidase rationalizing the monoterpene composition of graps.J.Agri. Food Chem.,30, 1219-1223.

Yadav, S., Yadav, K.D.S.(2000). Secretion of a-L-Rhamnosidases by *Aspergillus terreus* and its role debittering of orange juice; J. of Scientific & Industrial Research., 59, 1032-1037.

Yanai, T., Sato, M. (2000). Purification and Characterization of α-L-Rhamnosidases from *Pichia Angusta* X349. Biosci Biotech Biochem, 64(10): 2179-2185.

Yadav,V., Yadav,P.K., Yadav,S .,&Yadav,K.D.S. (2010), α-l-rhamnosidase : A review, Process Biochem, 45, 1226-1235.

Yadav V.,Sarita, Y., Yadav S.,& Yadav K.D.S. (2011).α-L-Ramnosidase from *Aspergillus flavus* MTCC-9606 isolated from lemon fruit peel. I. J. of Food Sci. and Technol.,46 m, 350-357.

Yu, H.,Gong, J., Zhang, C., Jin, F.(2002). Purification and Characterization of Ginsenoside - α – L-hamnosidase.Chem Pharm Bull, 52(2), 175-178.

Zverlov, V.V., Hertel, C., Bronnenmeier, K., Hroch, A., Kellermann, J., Schwarz, W.H.(2000). the thermostable α-L-rhamnosidase RamA of *Clostridium stercorarium*: biochemical characterization and primary structure of a bacterial a-L-rhamnoside hydrolase, a new type of inverting glycoside hydrolase. Mol Microb. 35(1), 173-79.