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α-L-RHAMNOSIDASE FROM *ASPERGILLUS FLAVIPUS* USING CITRUS SOLID WASTE AS INDUCER FOR APPLICATION IN JUICE INDUSTRY

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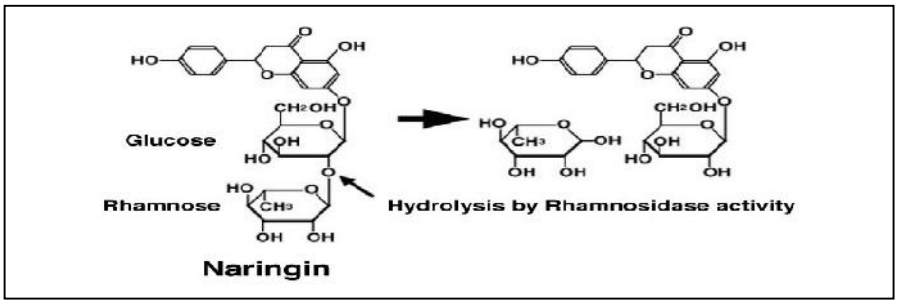
ABSTRACT
α-L- rhamnosidase is an important biotechnologically enzymes that de-rhamnosylated terminal α-L rhamnose from a variety of natural products. The de-rhamnosylated product is rare compound of pharmaceutical importance. In these studies, the effect of different inducers on the production of α-L- rhamnosidase from *Aspergillus flavipus* MTCC-4644 were investigated. Additions of solid citrus waste have been found to increases the production of α-L- rhamnosidase. Addition of 20% (w/v) citrus solid waste and 0.5 % naringin enhance the activity of α-L- rhamnosidase from *A. flavipus.* The pH optima and temperature optima of enzyme were 5.0 and 50°C, respectively. The K_m values using p- nitrophenyl α-L- rhamnopyranoside as the variable substrates in 0.2M Sodium phosphate buffer pH 5.0 at 50 ^{0}C was $0.48x10^3$µM.

KEY WORDS: *Aspergillus flavipus*, naringin, rhamnose, citrus waste, α-L-rhamnosidase.

INTRODUCTION

The presence of bitterness has been a major limitation in the commercial acceptance of citrus juices (Puri and Kalra, 2005). All the processed citrus fruit juices contain naringin (4, 5, 7-tryhydrxyflavonone-7-rhmnoglucoside) which attributes bitterness to the juices (Puri *et al*., 2005). Naringin concentration in immature fruit is high but it decreases as fruit ripens (Yusof *et al*., 1990, Puri and Banergee, 2000). Since naringin is the main bitter component of citrus juices, thus, its hydrolysis with a concomitant decrease in bitterness is of industrial importance. Naringin can be hydrolysed by the α-L-rhamnosidase in to rhamnose and prunin (4, 5, 7-tryhydroxyflavonone-7-glucopyranoside), one third lesser bitterness than that of naringin (Norouzian *et al*., 1999) scheme 1.



The α-L-rhamnosidase is widely distributed in nature and has been reported from animal tissues (Kurosawa *et al*., 1973), Yeast (Qian *et al*., 2005), fungi (Manzanares *et al*., 2000) and bacteria (Jang & Kim 1996). However, for reason of availability, only processes based on microbial α-L-rhamnosidase are practicable (Puri and Banergee, 2000). Keeping in view, the biotechnological application of this enzyme, the objectives of this research were to select a fungus *Aspergillus flavipus* MTCC-4644 with good ability to produce α-L-rhamnosidase and good catalysis at low pH but high temperature, and to optimize the media composition for α-L-rhamnosidase production.

MATERIALS & METHODS

P-Nitrophenyl-α-L-rhamnopyranoside, naringin and L-rhamnose were purchased from Sigma chemical company, St. Louis, (USA). Manganous sulphate, sodium chloride, sodium acetate were from Merck Ltd., Mumbai, (India) and acetic acid, tartaric acids, citric acids succinic acids and other chemicals were from s.d. fine chem. Ltd., Mumbai, (India) and were used without further purifications. Citus fruit peels; lemon peels, baggase, corncob *etc* were procured from local market. The fungal strain *Aspergillus flavipus* MTCC-4644 was procured from MTCC Center and Gene Bank, Institute of Microbial Technology, Chandigarh and were maintained in the laboratory on the agar slants 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: water (MilliQ) 1000 mL, $CaCl_2$ 1 g, $MgSO_4.7H_2O$ 3 g, KH_2PO_4 20 g, $N(CH_2COONa)_3$ 1.5 g, $MnSO_4$ 1 g, $ZnSO_4.7H_2O$ 0.1 g, $CuSO_4.5H_2O$ 0.1 g,.$7H_2O$ 0.1 g, H_3BO_3 10.0 mg, sucrose 40.0 g, ammonium tartrate 8.0 g. One milliliter of the spore suspension (spore density

$6.5x10^6$ spores / mL) from the agar slant were inoculated aseptically into the sterilized liquid culture medium (25 mL) kept in 100-mL culture flasks. The culture flasks were incubated in a BOD incubator at 25°C under stationary culture conditions. Aliquots of one mL of the liquid culture growth medium were withdrawn at the regular intervals of 24 h, filtered through Millex syringe filters (0.22 µM) and were analyzed for the presence of α-L- rhamnosidase activity (Romero et al. 1985). Enzyme unit/mL was plotted against the culture growth time in days. In order to maximize the secretion of α-L-rhamnosidase in the liquid culture growth medium, three sets of experiments were performed. In the first set of experiments, the effects of the presence of 4% (w/v) carbohydrates viz. glucose, rhamnose, fructose and sucrose in the liquid growth medium on the secretion of α-L-rhamnosidase were studied using the liquid growth medium as control. In the second set of experiments, the effects of the presence of hesperedin, rutin, and naringin in the liquid growth medium on the secretion of α-L-rhamnosidase were studied using the liquid culture growth medium containing 4% (w/v) sucrose as the control. Naringin was found to be the best inducer for the secretion of α-L-rhamnosidase. In the third set of experiments, the effect of the presence of some natural inducers like corncob, bagasse, fresh orange peel and rice grain in the liquid culture growth medium containing naringin were studied keeping the liquid culture growth medium containing naringin as the control. In this set of experiments, orange peel was found to be the best inducer for the secretion of α-L-rhamnosidase. For the purification of the enzyme, the fungus was grown in the liquid culture growth medium containing 0.5 % (w/v) naringin and 2.0% (w/v) orange peel.

The activity of α-L-rhamnosidase was assayed using p-nitrophenyl-α-L-rhamnopyranoside as the substrate following the reported method (Romero et al., 1985). The method consisted of taking 0.4 mL of 3.5 mM p-nitrophenyl-α-L-rhamnopyranoside in sodium acetate/ acetic acid buffer of pH 5.0 at 50°C and adding 0.5 mL of the same buffer. A suitable aliquot of the enzyme sample was added, and 100 µL of aliquots were withdrawn at the regular intervals of 2 min and diluted with 3 mL of 0.5 M NaOH, and the absorbance was measured at 400 nm using UV-Visible Spectrophotometer Hitachi (Japan) model U-2000. The molar extinction co-efficient value 21.44 $mM^{-1}cm^{-1}$ of p-nitro phenol was used for the calculation of the enzyme unit. The least count of the absorbance measurement was 0.001.

The MIchaelis-Menten behavior of the purified enzyme for the substrate p-nitrophenyl-α-L-rhamnopyranoside were determined by measuring the steady-state velocity of the enzyme catalyzed reaction at different concentrations of the substrates (0.05–2.0 mM) using the reported method (Romero et al., 1985). The K_m values were calculated by linear regression analysis of the data points (average of triplicate measurements) of the double reciprocal plots. 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 3.0–7.0 of 0.2M sod acetate/acetic acid buffer. The steady-state velocity was plotted against pH of the reaction solution, 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 (30–70°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.

RESULTS & DISCUSSION

To maximize the secretion of α-l-rhamnosidase by *A. flavipus* MTCC-4644 for the purification of the enzyme, the effects of addition of glucose, fructose, rhamnose and sucrose in the liquid culture growth medium on the secretion of α-l-rhamnosidase were studied using the medium having no carbohydrate in the medium as control. The results are shown in fig. 1(a).

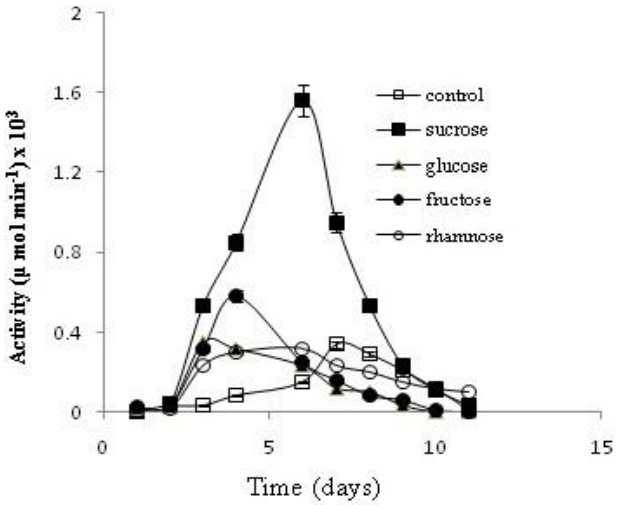


FIGURE 1 a. Secretion of α-L-rhamnosidase in the culture medium of *Aspergillus flavipus* MTCC-4644.

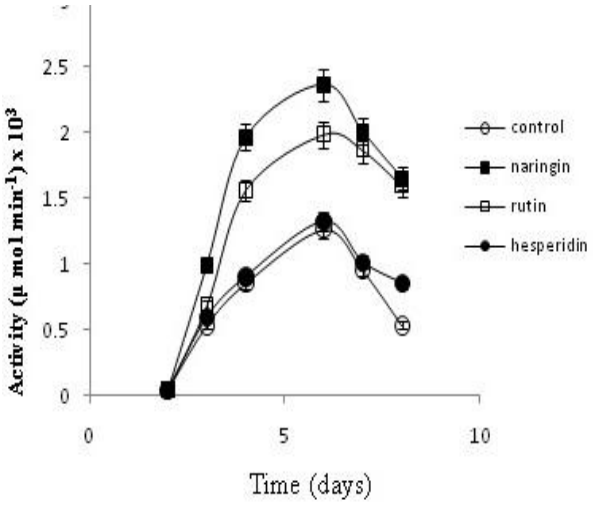


FIGURE 1b. Secretion of α-L-rhamnosidase in the culture medium of *Aspergillus flavipus* MTCC-4644.

The order of the peak values of the enzyme secreted in the growth media was medium containing sucrose > fructose > rhamnose > glucose > control. The effects of the presence of naringin, rutin and hesperidin in the liquid culture growth medium on the secretion of α-L-rhamnosidase by *A. flavipus* MTCC-4446 are shown in Fig. 1(b). The maximum activity of the enzyme appeared in case of the medium containing 0.5% (w/v) naringin and it was on the 6th day of the growth of the fungal strain. Fig. 1(c) shows the results of the effects of the presence of corncob, bagasse, rice grain, and orange peel in the liquid culture growth medium containing 0.5% naringin on the secretion of α-L-rhamnosidase by the fungal strain. The maximum activity of the enzyme appeared in the medium containing 0.5% (w/v) naringin and 20% (w/v) orange peel. The maximum activity was approximately 10 times greater than the activity of the enzyme observed in case of the medium containing only 0.5% naringin. Since not much is known about the structure and regulation of α-L-rhamnosidase genes (Tomayo-Romos and Orejas 2014), the reason for the enhancement of α-L-rhamnosidase secretion in the presence of orange peel cannot be discussed. For the purification of α-L-rhamnosidase, the fungal strain was grown in the liquid culture growth medium containing 0.5% (w/v) rutin and 20% (w/v) orange peel.

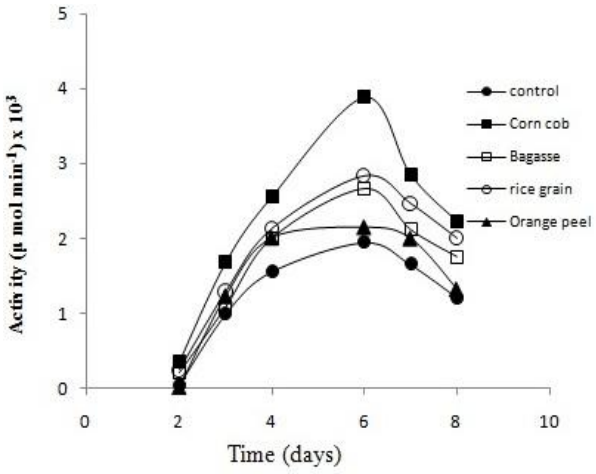


FIGURE 1c. Secretion of α-L-rhamnosidase in the culture medium of *Aspergillus flavipus* MTCC-4644.

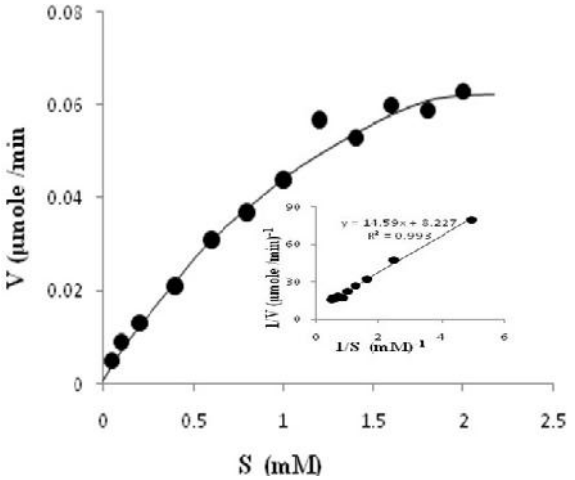


FIGURE 2. Michelis –Menten behavior of α-L-rhamnosidase using p- nitrophenyl-α-L-rhamnopyranoside as substrate.

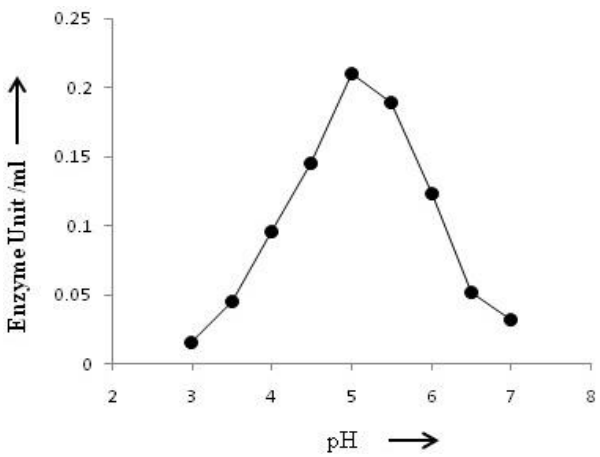


FIGURE 3. Effects of pH on the activity of the enzyme. The assay solution 1.0 mL contained 0.4 mM substrate, 1.50 g of the enzyme in 0.2 M Sod. /acetate acetic acid buffer at varying pH range 3-7.

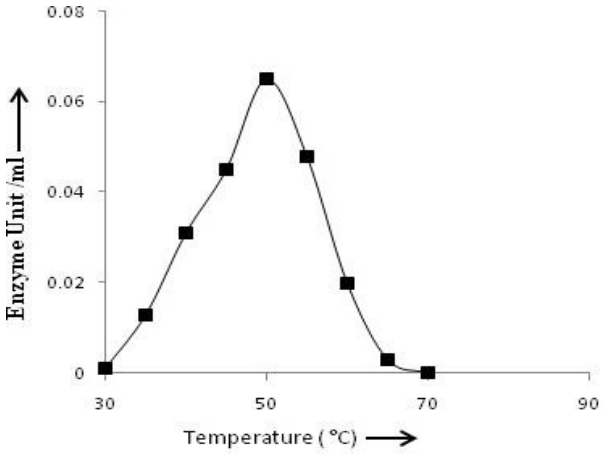


FIGURE 4. Effects of temperature on the activity of the enzyme. The assay solution 1.0 mL contained 0.4 mM substrate, 1.50 g of enzyme in 0.2 m Sod. /acetate acetic acid buffer pH 5.0 at varying temperatures (30–70 °C).

The Michaelis–Menten behaviour of the purified enzyme using p-nitrophenyl-α-L-rhamnosidase as the substrate was determined (shown in fig 2). The calculated K_m value for this enzyme using p-nitrophenyl- α-L-rhamnopyranoside as the substrate was $0.48x10^3$µM at 50°C in 0.2 M Sodium acetate /acetic acid buffer pH 5.0. The K_m values for α-L-rhamnosidases purified from *Pseudomonas paucimobilis* FP 2001 (Maik *et al*., 2000) from *Fusobacterium* K-60 (Park *et al*., 2005), from *Aspergillus aculeatus* RhaA and RhaB (Manzanares *et al*., 2001), from *Aspergillus flavus* MTCC-9606 (Yadav *et al*., 2011) and from *Penicillium citrinum* MTCC-8897 (Yadav *et al*., 2011) have been reported to be 1.18, 0.06, 0.30 and 2.80, 1.89 and 0.36 mM, respectively. Thus, the purified α-L-rhamnosidase has intermediate affinity for p-nitrophenyl-α-L-rhamnopyranoside as compared to reported α-L-rhamnosidases (Yadav *et al*., 2010).

The results of the studies on the variation of activity of the purified enzyme with the variation in the pH of the reaction solution are shown in Fig. 3. The pH optimum of the purified enzyme was 5.0 using p-nitrophenyl- α-L-rhamnopyranoside as the substrate. Most of the α-L-rhamnosidases reported so far have pH optima in the acidic pH range (Yadav *et al*., 2010). Only α-L-rhamnosidases of pig liver (Qian *et al*., 2005) and *P. citrinum* MTCC-8897 (Yadav *et al*., 2012) have pH optima in the neutral pH range. The results of the variation in the activity of the purified enzyme with temperature of the reaction solution are shown in the Fig. 4. The temperature optimum of the purified enzyme was 50°C, which lies in the range of temperature optima reported for the other fungal α-L-rhamnosidases (Yadav *et al*., 2010).
Though the secretion of α-L-rhamnosidase in the liquid culture medium containing *A. flavipus* MTCC-4644 was not very high, with the development of in the area of molecular biology it would be possible to isolate the gene of the above vector so that the amount of enzyme needed for commercial applications could be produced. The reported studies in this communication will be useful in achieving the above objective.

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