



## PURIFICATION OF A $\alpha$ -L-RHAMNOSIDASE FOR THE TRANSFORMATION OF RUTIN TO ISOQUERCITRIN

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### ABSTRACT

A  $\alpha$ -L-rhamnosidase from the culture filtrate of *F. crookwellence* MTCC-2084 has been purified to homogeneity. The procedure involved ultrafiltration by P10 membrane using Amicon concentration cell, dialysis and cation exchange chromatography on carboxymethyl [CM] cellulose. The molecular mass of the enzyme determined by SDS-PAGE analysis is 142 kDa. The  $K_m$  and  $k_{cat}$  values of the enzyme determined by using naringin as substrate are 0.29 mM and 35.36 s<sup>-1</sup>, respectively. The pH and temperature optima of the enzyme are 8.0 and 65°C, respectively. The enzyme transforms rutin to isoquercitrin which is a pharmaceutically important rare compound of medicinal and food values.

**KEYWORDS:** Rutin, SDS-PAGE, carboxymethyl cellulose, isoquercitrin,  $\alpha$ -L-rhamnosidase.

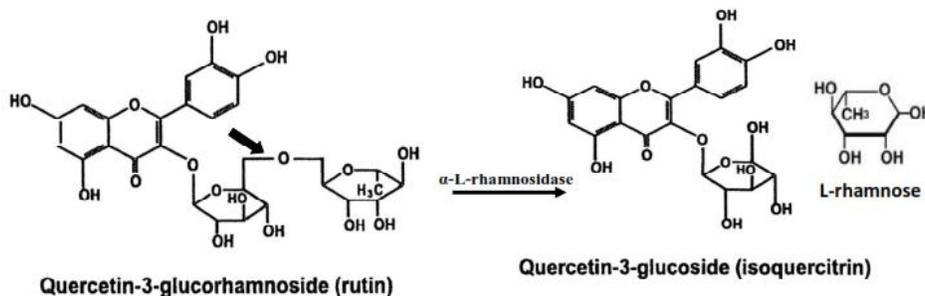
### INTRODUCTION

Rutin is a flavonol glycoside consisting of quercetin and rutinose (Tranchimand *et al.*, 2010, Guo *et al.*, 2007). It is antioxidant vasoprotective, anticancer, hypercholesterol, antidiabetic, anti-hypertensive, exerts renal protective effects and reduce the risk of atherosclerosis (Sharma *et al.*, 2013, Alice *et al.*, 2018) However, the bio-availability of rutin is low due to low solubility (Manludin *et al.*, 2009, Kamel and Mostafa, 2015). Its glysylation by rutinose limits its absorption in the small intestine (Mueller *et al.*, 2018). Derhamnosylation of rutin gives isoquercitrin shown in scheme 1 (Yadav *et al.*, 2010). Through there is structural similarity in rutin, is isoquercitrin and quercetin, they differ in their physical, chemical and biological properties (De Araujo *et al.*, 2013). In comparison with rutin and quercitrin, isoquercitrin is better absorbed in small intestine (Arts *et al.*, 2004). Moreover, isoquercitrin has a more anti-proliferative effect than quercetin or rutin (You *et al.*, 2016). isoquercitrin is a precursor for the enzymatic biosynthesis of enzymatically modified isoquercitrin [EMIQ] which has been approved as a multiple food additive (Makino *et al.*, 2009). Isoquercitrin rarely occurs in nature, its extraction yield is low and therefore it is expensive (Yadav *et al.*, 2018) Thus there is scientific need to develop processes for the preparation of

isoquercitrin from rutin. In an earlier communication (Yadav *et al.*, 2018) we have reported a  $\alpha$ -L-rhamnosidase selective for rutin to isoquercitrin transformation from *Panicillium glyseoroseum* MTCC-92 24 which was active in near neutral pH range. In this communication we report an other  $\alpha$ -L-rhamnosidase selective for rutin to isoquercitrin conversion from *Fusarium crookwellen* MTCC-2084 which is active in alkaline pH range 8.0. The fungal strain has been isolated by D. Ananthpadmanaban and deposited at the Microbial Type Culture Collection center and gene bank, Institute of Microbial Technology, Chandigarh (Catalogue of strains, 2000).

### MATERIALS AND METHODS

Naringin, rutin, hesperidin L-rhamnose, p-nitrophenyl-  $\alpha$ -L-rhamnoside and CM cellulose were purchased from Sigma Chemical Company, St. Louis (USA). The chemical for gel electrophoresis including the medium protein molecular wt. markers used in SDS-PAGE experiment were procured from Bangalore GENEI Pvt. Ltd. Bangalore, (India). All other chemical were either from Merck Ltd Mumbai, (India) or from s.d-fire CHEM Ltd. Mumbai, (India) and were used without further purification. Bagasse and corn cob were procured from local market.



**SCHEME:** Enzymatic hydrolysis of rutin to isoquercitrin by  $\alpha$ -L-rhamnosidase

### The Fungal strain

The fungal strain *F. crookwellence* MTCC-2084, *F. crookwellence* MTCC-2089, *F. moniliforme* MTCC-2092, *F. moniliforme* MTCC-2015, and *F. oxysporum* MTCC-1755 were procured from the Microbial Type Culture Collection Centre and Gene Bank, Institute of Microbial Technology, Chandigarh (India) and were maintained in the laboratory on Agar slants recommended for them (Catalogue of strains, 2000). All the five fungal strains were tested for the secretion of  $\alpha$ -L-rhamnosidase in liquid culture growth medium using the method reported in the literature (Yadav *et al.*, 2011). Out of the above five fungal strain *F. crookwellens* MTCC-2084 was found most promising and therefore, further studies on the  $\alpha$ -L-rhamnosidase of this fungal strain was carried out.

### $\alpha$ -L-rhamnosidase secretion

The  $\alpha$ -L-rhamnosidase secretion by the fungal strain in the liquid culture growth medium was studied using the method reported in the literature (Yadav *et al.*, 2011). The liquid culture growth medium consisted of CaCl<sub>2</sub> 1g, MgSO<sub>4</sub>.7H<sub>2</sub>O 0.1g, 3.0g, KH<sub>2</sub>PO<sub>4</sub> 20.0g, N(CH<sub>2</sub>COONa)<sub>3</sub> 1.5g, MnSO<sub>4</sub> 1.0g, ZnSO<sub>4</sub>.7H<sub>2</sub>O 0.1g, CuSO<sub>4</sub>.5H<sub>2</sub>O 0.1g, FeSO<sub>4</sub>.7H<sub>2</sub>O 0.1g, H<sub>3</sub>BO<sub>3</sub> 10.0mg, sucrose 40.0g, ammonium tartrate 8.0g in 1L of MilliQ water. 1mL of spore suspension (spore density 5x10<sup>6</sup> spore/mL) from the agar slant of the fungal strain was inoculated aseptically in to the sterilized liquid culture growth medium (20mL) kept in a 100mL culture flask. The flasks were incubated in a B.O.D. incubator at 25°C under stationary culture condition. Aliquots of 1mL of the fungal growing cultures were withdrawn at regular intervals of 24 hr, filtered through millex syring filters (0.22 $\mu$ ) and analyzed for the presence of  $\alpha$ -L-rhamnosidase activity using naringin as substrate and monitoring the decrease of naringin by the methods reported by Davis, 1947. The experiment were done in triplicate and the average of enzyme unit/mL present in the growth medium was plotted against the fungus growth time in days. In order to optimize the secretion of  $\alpha$ -L-rhamnosidase in the growth medium, two set of the experiments were performed. In the first set, the above liquid culture medium was amended by adding 4% of each of L-rhamnose or glucose or fructose or sucrose using the liquid culture growth medium without sugars as control. 4% sucrose was found the best inducer of  $\alpha$ -L-rhamnosidase secretion. In the second set of experiment, the liquid culture growth containing 4% sucrose was amended with 0.5 g/20mL of corncob or bagasse particles. Bagasse particles were found the best inducer for the secretion of  $\alpha$ -L-rhamnosidase.

### $\alpha$ -L-rhamnosidase assay

The activity of  $\alpha$ -L-rhamnosidase was assayed by using naringin as the substrate and monitoring the decrease in concentration of the naringin with time by Davis method (Davis, 1947). 2.5mL of 0.86 mM naringin dissolved in 0.1M sodium phosphate buffer pH 8.0 was maintained at 60°C. 0.25mL of the enzyme extract (culture filtrate) was added to the above solution and 0.1mL of aliquot was immediately withdrawn which was added to 2.5mL of 90% diethylene glycol followed by addition of 0.1mL 4N NaOH. Aliquots of 0.1mL of the reaction mixture were withdrawn at regular intervals of 5minutes and the above process was repeated the sample were maintained at room

temperature for 10 minutes and absorption of solutions were measured at 420 nm. The absorbance values were converted to naringin concentration with the help of the calibration curve. The concentrations of naringin were plotted against time and decrease in naringin concentration in  $\mu$ Mole/min was calculated. UV/Visible spectrophotometer Hitachi (Japan) Model U-2900 was used for spectrophotometric measurements. The least count of the equipment was 0.001 absorbance unit. The enzyme unit was defined as the amount of enzyme which decreased the concentration of naringin at the rate of 1 $\mu$ Mol/min under the assay condition defined above.

### Purification of $\alpha$ -L-rhamnosidase

For purification of  $\alpha$ -L-rhamnosidase from the fungal culture, it was grown in the liquid culture growth medium containing 4% sucrose and 2.5% bagasse particles in 20x100 flasks each containing 20mL medium of the composition mentioned in section 2.3. Maximum activity appeared on 9<sup>th</sup> day of inoculation of the fungal spores. The culture of all the flasks were pooled, filtered through 4 layers of cheese cloth and centrifuged at 10,000g to remove the fine particles. The centrifuged culture filtrate 280mL was concentrated by ultrafiltration using Amicon Stirred Concentration Cell model 8200 and P10 membrane. The concentrated filtrate (25mL) was dialyzed against 0.1mM NaH<sub>2</sub>PO<sub>4</sub>/NaOH buffer pH 7.0. The dialyzed sample was loaded on a CM Cellulose column of size 1.6 x 15 cm pre-equilibrated with the above buffer. The column was washed with 150 mL of the buffer and eluted with applying 0-1M sodium chloride in the same buffer (75mL buffer +75mL buffer with 1M NaOH). Four mL fractions were collected and were analyzed for the activity and protein concentration by using Davis method (Davis, 1947) and Lowry method (Lowry *et al.*, 1957), respectively. Active fractions were combined and concentrated as mentioned above. The concentrated enzyme was stored in fridge which does not lose activity for three months.

### SDS-Polyacrylamide Gel Electrophoresis [SDS-PAGE]

The purity of the enzyme preparation was checked by SDS-PAGE analysis and the molecular mass of the purified enzyme was determined by the reported method (Weber and Osborn, 1969). The separating gel was 10% acrylamide in 0.375M Tris-HCl buffer pH 8.8 and stacking gel was 5% acrylamide in 0.5 M Tris-HCl buffer pH 6.8. The electrophoresis buffer was 0.025M Tris-glycine of pH 8.6. The molecular weight markers used were phosphorylase B (97.4 kDa), bovine serum albumin (66.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (29.0 kDa) and soyabean trypsin inhibitor (21.1 kDa). The gel was run at constant current of 20 mA using electrogel 50 equipment of Technosource, Mumbai (India).

### Steady-state kinetics of the enzyme and pH, temperature optima

The value of K<sub>m</sub> and V<sub>max</sub> of the purified enzyme were determined using naringin as substrate following the method reported by Davis (Davis, 1947) as described in section 2.4. The steady state velocity of the enzyme catalyzed reaction was determined at different concentrations of naringin in the range of 0.2 to 1mM. K<sub>m</sub> and V<sub>max</sub> value were determined by the linear regression of the data points of double reciprocal plot. The pH

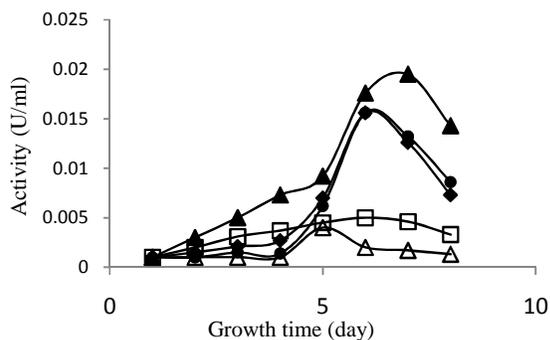
optimum of the enzyme was determined by measuring the steady state velocity of the enzyme catalyzed reaction in solution of varying pH in the range of 6-10 pH unit. The buffer used was 0.1 M sodium phosphate /phosphoric acid 25°C, the steady state velocity was plotted against the pH of the reaction solution and pH optimum was calculated from the graph. The temperature optimum of the enzyme was determined by measuring the steady state velocity of the enzyme catalyzed reaction in solution of varying temperature in the range of 40°C to 80°C at fixed pH of 8.0. A graph was plotted for the steady state velocity versus temperature of the reaction solution and temperature optimum was calculated from the graph.

#### Transformation of rutin to isoquercitrin

200  $\mu$ L of the enzyme stock 0.43U/mL was added to one mL of 1mM solution of rutin in to 0.1mM sodium phosphate buffer pH 8 and the reaction was incubated at 60°C for 1hour. The transformation of rutin was tested by thin layer chromatography on silica gel on glass plate. The identity of isoquercitrin was confirmed by purifying isoquercitrin by preparative TLC and analyzing it by HPLC-Mass spectrometry. The mobile phase used in TLC was butanol: acetic acid: water in volume ratio 30:15:55. Detection was done by putting the silica gel glass plate in iodine chamber. Isoquercitrin was extracted from the silica gel by ethanol HPLC-Mass spectrometry was done at Sophisticated Analytical Instrumentation Facilities (SAIF) Central Drug Research Institute, Lucknow.

## RESULTS AND DISCUSSION

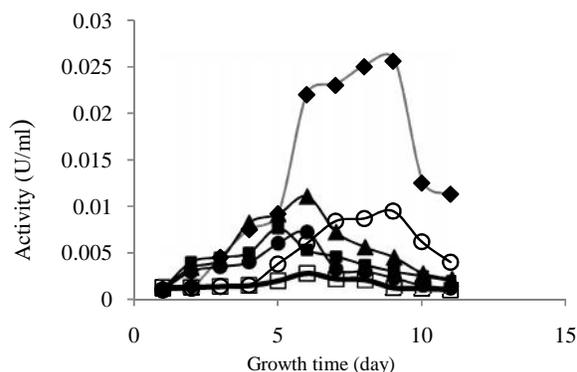
The results of the studies on the secretion of  $\alpha$ -L-rhamnosidase in the liquid culture medium of *F. crookwellence* MTCC--2084 is shown in Fig 1(a) where the enzyme unit secreted per mL is plotted against the



**FIGURE 1.(a)** Effect of carbohydrates on the secretion of  $\alpha$ -L-rhamnosidase. from upside Control, Rhamnose, Glucose, Fructose, Sucrose

The Michaelis-Menten constant of the purified enzyme using naringin as the substrate is calculated by double reciprocal plot (graph not shown). The calculated  $K_m$  and  $k_{cat}$  values are 0.29 mM and  $35.36s^{-1}$  giving  $k_{cat}/K_m$  value of  $12.19 \times 10^4 M^{-1}s^{-1}$  which is far below the perfectly evolved enzymes, the limiting values for which are in the range  $10^8$ - $10^9 M^{-1}s^{-1}$  (Ferst,1999). Thus there is scope for improving the catalytic efficiency of this enzyme by directed evolution techniques (Arnol and Geogion, 2003, Arnol and Geogion, 2003) of molecular biology.  $K_m$  values of  $\alpha$ -L-rhamnosidases using naringin as the

number of days of the fungal growth. It is clear from the figure that among all the carbohydrates tested, sucrose is the best inducer. In order to enhance the secretion of  $\alpha$ -L-rhamnosidase further by the fungal strain secretion of the enzyme was tested by amending liquid culture growth medium containing 4% sucrose with 2.5% corn cab particles or bagasse particles or rutin the results are drawn in Fig 1(b). Among all these, bagasse particles were found the best inducer of secretion of the enzyme. For the purification of the enzyme, the fungus was grown in the reported culture medium amended with 4% sucrose and 2.5% bagasse particles. The results of purification of the enzyme from the culture filtrates of the fungal strain are summarized in table 1. It involved concentration of the culture filtrate by ultra filtration, dialysis, and chromatography on C.M. cellulose. The enzyme binds to CM cellulose in 10 mM sodium phosphate buffer pH 7.0 and is eluted by the linear gradient of NaCl in the same buffer in the range 0-1M Six fold purification with 15% yield is achieved. The results of SDS-PAGE analysis of the purified enzyme is shown in Fig-2 in which lane 1 contains the protein band of medium range protein molecular weight markers and lane 2 contains bands of the purified enzyme. The presence single protein band in lane 2 indicates that the purified enzyme is pure. The molecular weight of the purified enzyme calculated from the positions of protein molecular weight markers is 142 kDa which compares with molecular weight reported for the  $\alpha$ -L-rhamnosidase of thermophilic bacteria PRI-1686 (Birgisson *et al.*, 2004) reported in the literature. The molecular weights of  $\alpha$ -L-rhamnosidases reported in the literature are in the range of 41 kDa to 240 kDa (Yadav *et al.*, 2010).

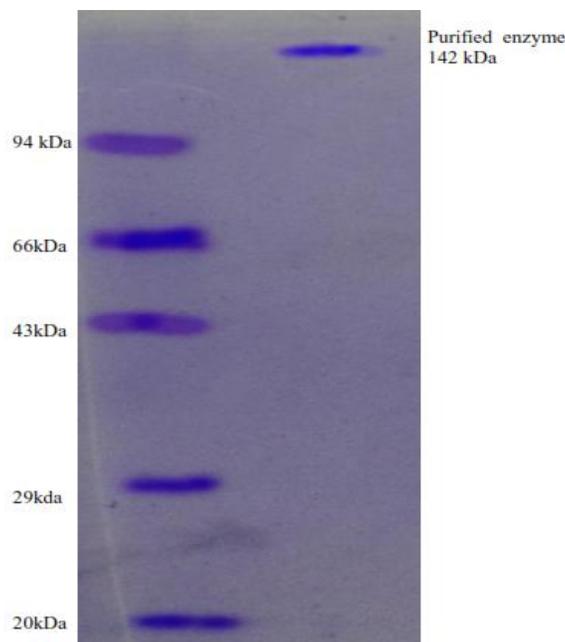


**FIGURE 1.(b)** Effects of substrates on the secretion of  $\alpha$ -L-rhamnosidases Corn—Cab, Rutin, Bagasse

substrate are 0.89mM for Bacteriodes jY-6 enzyme (Jangand Kim, 1966) 0.17mM for *Pseudomonas paucimobilis* FP2001 enzyme (Mike *et al.*, 2000) and 1.9mM for *Aspergillus niger* enzyme (Puri and Kalva, 2005). Thus  $K_m$  value of the purified enzyme using naringin as the substrate 0.29mM is lower than some of the reported  $K_m$  values (Jang and Kim,1966, Puri and Kalva, 2005) and greater than  $K_m$  value reported for the  $\alpha$ -L-rhamnosidases of *Pseudo Monas paucimobilis* FP2001 (Mike *et al.*, 2000). Thus the affinity of the purified enzyme for naringin is in the middle of other

reported  $\alpha$ -L-rhamnosidases. Though the  $k_{cat}$  values using naringin as the substrate for the reported  $\alpha$ -L-rhamnosidases are not available in the literature (Yadav *et al.*, 2010) but those could be calculated from the reported specific activities and molecular weights of the enzymes. For the  $\alpha$ -L-rhamnosidases of *A. niger* (Puri and Kalra,

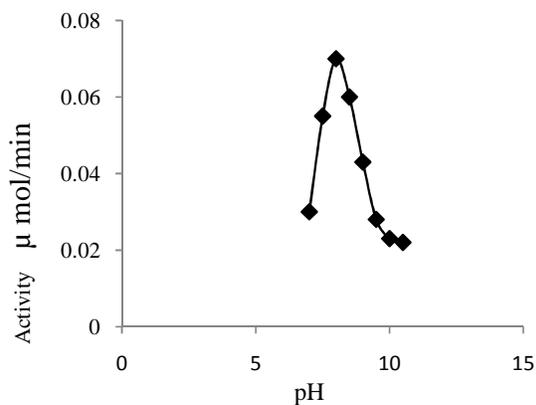
2005), it comes out  $58.82s^{-1}$  which is in the same order as the value of  $k_{cat}$   $35.36 s^{-1}$  as calculated for the purified enzyme. However, for the bacterial  $\alpha$ -L-rhamnosidases  $k_{cat}$  values are  $0.34s^{-1}$  and  $0.96s^{-1}$  for *pseudomonad panicimobilis* FP2001 (Mike *et al.*, 2000) & *Fusobacterium K-6* (Park *et al.*, 2005) enzymes, respectively.



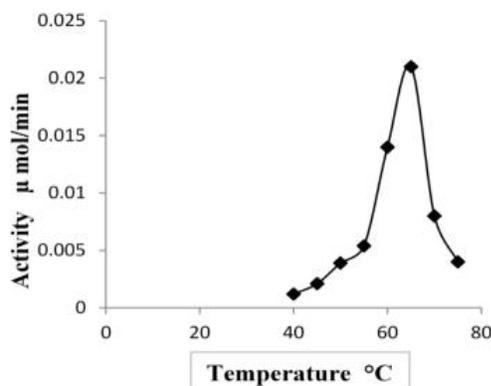
**FIGURE 2:** Results of SDS-PAGE analysis  
Lane 1: Purified enzyme 10 $\mu$ g  
Lane 2: Protein molecular wt markers.

The results of pH and temperature optima determination are shown in Fig. 3(a) and 3(b) in which enzyme activity has been plotted against pH and temperature of the reaction solutions, respectively. The determined pH and temperature optima are 8.0 and 65 $^{\circ}$ C, respectively. The reported pH optima for  $\alpha$ -L-rhamnosidases in the literature (Yadav *et al.*, 2010) are in the range 2.8 to 7.9 though one

$\alpha$ -L-rhamnosidases with reported pH optimum 10.0 has recently been reported (Yadav *et al.*, 2018). Most of the  $\alpha$ -L-rhamnosidases have temperature optima in the range 40 $^{\circ}$ C-80 $^{\circ}$ C (Yadav *et al.*, 2010). Thus the temperature optimum of the purified enzyme 65 $^{\circ}$ C lies in the range temperature for the reported for the most of the  $\alpha$ -L-rhamnosidases (Yadav *et al.*, 2010).



**FIGURE 3a**



**FIGURE 3b**

a) Reaction solutions containing 1mM naringin of varying pH (7 to 10) at 60 $^{\circ}$ C were used and activity was determined as mentioned in section 2.4.

b) Reaction solutions containing 1mM naringin at pH 8.0 at different temperature (40-75 $^{\circ}$ C) were used and activity was determined at mentioned in section 2.4.

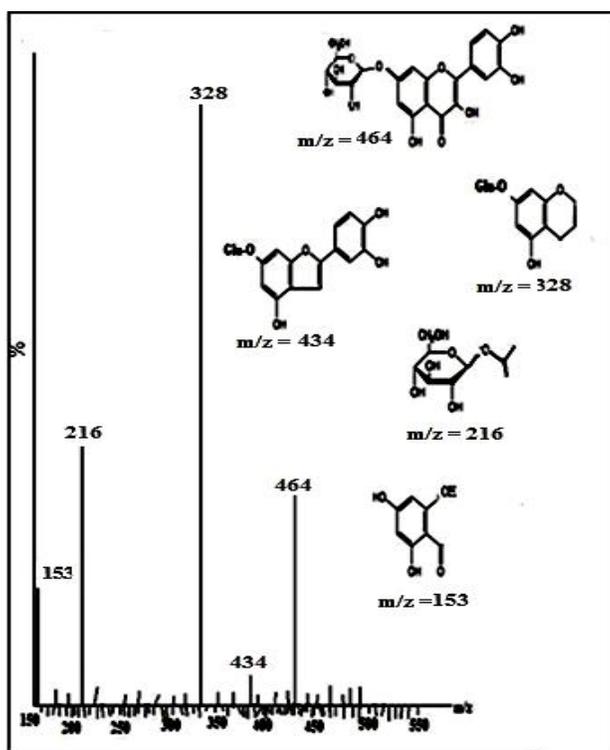


FIGURE 4. Determination of pH and temperature optima

#### Transformation of Rutin to isoquercitrin

The importance of isoquercitrin as a pharmaceutically important compound has already been mentioned in the introduction section of this communication. Keeping that point in view the transformation of rutin to isoquercitrin using the purified  $\alpha$ -L-rhamnosidase was tested by the procedure described in section 2.8. The results of HPLC-Mass spectrometry analysis of the product are shown in Fig. 4 which confirms the identity the product as isoquercitrin. In 1mM rutin enzymatic conversion was complete and spot for the reactant was not observed in TLC analysis. The reaction time was only 11 hr. at 60°C indicating that the purified enzyme has a potential for rutin to isoquercitrin transformation.

Recently, a number of enzymatic processes for the transformation of rutin to isoquercitrin have been reported (Wang, J. *et al.*, 2013, and Wang, J. *et al.*, 2015) and some of them (Wang, J. *et al.*, 2013 and Wang, J. *et al.*, 2015) use additional techniques like ionic liquid as co-solvent and biphasic system to accelerate isoquercitrin production. Such additional techniques combined with this reported  $\alpha$ -L-rhamnosidase may prove a better system for isoquercitrin production from rutin

#### CONCLUSION

This communication reports a new  $\alpha$ -L-rhamnosidase for the transformation of rutin to isoquercitrin which is a pharmaceutically important rare compound of medicinal and food values.

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