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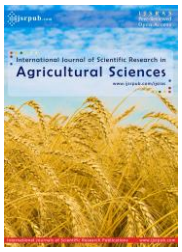

Full Length Research Paper

Kinetic Properties of Extracellular Thermophilic Naringinase Produced by *Rhizophus Stolonifer* Isolated from Palmyrah Fruit Pulp

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Abstract. Bitterness of the fruit juices is a serious challenge in the processing of fruit juices in the food industries. Debittering of the juice using the enzyme naringinase produced by diverse microbes naturally is a promising approach, as it causes minimal damage to the nutritional quality and enhances organoleptic properties. Since Naringinase is an expensive enzyme widely used in the food industry, there is scope for fermentation process development using new isolates, which would result in commercially viable processes. *Rhizophus stolonifer* was isolated from Palmyrah fruit pulp and the extracellular naringinase enzyme was characterized. The crude naringinase enzyme was highly active at 65ºC and it was very stable at 60ºC for at least one hour. Highest naringinase activity was obtained at pH 4.0 and the enzyme was stable at pH 4.5 for at least one hour. The enzyme showed zero order kinetics for 10 minutes. Vmax of the crude naringinase enzyme was 3.125 µmol/mL and Michaelis constant by Lineweaver-Burk Plot for naringin was 3.076 mg/mL under the conditions. Metal ions Mn^{2+}, Cu^{2+} and Ba^{2+} increased the naringinase enzyme activity but Mg^{2+}, Zn^{2+}, Hg^{2+}, Ca^{2+} and Na^{+} reduced the enzyme activity. Naringinase was more stable with Cu^{2+} than Mn^{2+} and Ba^{2+}. The optimum conditions for naringinase activity can be achieved at 65ºC and at pH 4.0 and the enzyme is stable for at least one hour. Therefore the naringinase enzyme from *Rhizophus stolonifer* isolated from palmyrah fruit pulp could be an ideal candidate for the debittering of acidic food items that are produced using moderately high temperatures in the food industries.

Keywords: Debittering, Kinetic properties, Michaelis constant, Naringinase, Palmyrah pulp, *Rhizophus stolonifer*.

1. INTRODUCTION

Commercial fruit juice production is facing serious challenges to minimize the bitterness of the fruit juices. This bitterness is due to the presence of a flavonone called naringin. Naringin concentration is linked with the maturity of the fruit. As the fruit ripens, concentration of the naringin tends to decrease (Yalim et al., 2004). Reduction of naringin content was done by using chemical applications that had several drawbacks resulting in the poor quality of fruit juice. Debittering of the juice using the enzyme naringinase is a promising approach, as it causes minimal damage to the nutritional quality and enhances organoleptic properties (Mukund et al., 2014).

The filamentous fungi are industrially important for their potential to produce large amount of enzymes with limited space, facilities with less cost for production (Guimarães et al., 2006). Among the filamentous fungus, the *Rhizophus stolonifer* is one of the most widely used in industrial application due to its growth characteristics and ability to dissolve growth substrate by its extracellular enzymes (Kapilan, 2015). The species is pathologically significant since it causes soft rot disease on diverse fruits and vegetables (Hernández-Lauzardo et al., 2006). The fungul species *Rhizophus stolonifer* can be easily isolated from decaying fruits and vegetables with low cost, limited facilities. The characterization of the naringinase enzyme produced by *Rhizophus stolonifer* isolated from Palmyrah pulp is important to decide its application in food processing and to utilize the enzyme to its maximum.

Different studies show that different strains of *Rhizophus stolonifer* produce variety of extracellular enzymes such as Ribonuclease (Chacko et al., 1996), protease (Ranganathan, 2015), Polygalacturonase (Lee and West, 1981), alpha-rhamnosidase (Shanmugam and Yadav, 1995) etc. Naringinase is a complex glycolytic enzyme that has both α-L rhamnosidase and β- D-glucosidase activity. Naringinase is an enzyme complex, containing a -L-rhamnosidase and b-glucosidase activity. During naringin hydrolysis there

are two steps involved, where a -L-rhamnosidase first hydrolyzes the naringin to L-rhamnose and prunin and subsequently b-glucosidase hydrolyzes prunin into naringenin and D-glucose. The prunin can be hydrolyzed by the β-D glucosidase into aglyconenaringin and β-D-glucose (Puri, 2012). Naringinase is used in the production of the glycopeptide antibiotic chloropolysporin from *Faenia interjecta*, other than debittering process (Sankyo, 1988). Naringinase can function as a chiral intermediate and widely used as a pharmaceutical and plant protective agent (Daniels et al., 1990). Hence naringinase enzyme makes potential usefulness in pharmaceuticals and food industries (Ribeiro, 2011). With a wide range of applications, naringinase has become one of the biotechnologically important enzymes. Naringinase has been reported in plants, yeasts, fungi, and bacteria (Ribeiro, 2011). Microbial enzymes are getting attractions due to cost-effective production with an economically viable process. Chemical methods of naringin reduction are preferred to replace the microbial naringinases in industries. Though the production of naringinase has been very well studied in fungal sources, very limited reports are available on bacterial naringinase (Mukund et al., 2014, Ni et al., 2011). Since Naringinase is an expensive enzyme there is scope for fermentation process development using new isolates, which would result in commercially viable processes. Therefore the objective of the study was to determine the kinetic properties and stability of the crude naringinase enzyme naturally produced by *Rhizophus stolonifer* isolated from the Palmyrah fruit pulp.

2. MATERIALS AND METHODS

2.1. Chemical

Naringin was obtained from Sigma, St. Louis, USA. All the other reagents were in analytical grade.

2.2. Microorganism for enzyme production

Crude naringinase enzyme produced by *Rhizophus stolonifer* previously isolated from Palmyrah fruit pulp was used in this kinetic studies. From the 6 day old culture, the spore suspension was made with sterilized 0.85% NaCl solution and the spore size was adjusted up to 10^8spores/mL. It was used as inoculum for activation medium.

2.3. Activation of *Rhizophus stolonifer* and fermentation process

The activation medium was prepared as liquid broth of 100mL in 500mL conical flask with (w/v) 0.2% naringin, 0.5% glucose, 0.7% peptone, 0.1% yeast extract, 0.05% KH_2PO_4, 0.01% $MgSO_4.7H_2O$ and 0.07% $ZnSO_4.7H_2O$, 0.07% $CuSO_4.5H_2O$, 0.07% $FeSO_4.7H_2O$ (Navaratnam et al., 2003, Karuppaija et al., 2016 and Keerthini et al., 2016). The fermentation medium containing (w/v) 0.2% naringin, 0.5% glucose, 0.7% peptone, 0.1%yeast extract, 0.05% KH_2PO_4, 0.01% $MgSO_4.7H_2O$ and 0.07% $ZnSO_4.7H_2O$, 0.07% $CuSO_4.5H_2O$, 0.07% $FeSO_4.7H_2O$ was used. The pH was adjusted to 6.0 (Navaratnam et al. 2003). Twenty five mL of the sterilized liquid medium was taken into 100mL conical flask and 6day old cultures of each organism from potato dextrose agar (PDA) medium was inoculated in the liquid fermentation medium and allowed for fermentation at 200rpm in room temperature until 9^{th} day. The spores were taken out using sterile 0.85% NaCl solution and spore size was adjusted as 10^8 spores/mL.1mL of spore solution was inoculated on duplicate fermentation medium of each fungal strain under aseptic condition. The fermented sample was withdrawn for the enzyme assay at 7th, 8th and 9th day. One unit of naringinase activity is defined as the amount of enzyme that produces 1μmol of reducing sugar in 1 minute at pH 5.0 and 60^{o}C with 10 gL^{-1} naringin (Keerthini et al., 2016 and Karuppaija et al., 2016)

2.4. Extraction of crude naringinase enzyme

The crude naringinase enzyme was extracted from solid fermentation medium (Keerthini et al., 2016 and Karuppaija et al., 2016). The solid medium contained paddy husk and liquid broth at the ratio of 2:10 (Arasaratnam et al., 2001) was used for fermentation. Liquid broth was prepared with (w/v) 1% naringin, 0.5% glucose, 0.7% peptone, 0.1% yeast extract, 0.05% KH_2PO_4, 0.01% $MgSO_4.7H_2O$, 0.07% $ZnSO_4.7H_2O$, 0.07% $CuSO_4.5H_2O$ and 0.07% $FeSO_4.7H_2O$. One hundred mL was taken into 500mL conical flask as duplicate sample and it was sterilized at 121^{o}C 15 psi for 20 minutes. 20 gram of cleaned paddy husk was also sterilized in the autoclave. Sterilized paddy husk was transferred to liquid broth under sterilized condition. 1mL of activation sample has 2×10^7 spores. Therefore 5mL of activated sample was inoculated in each duplicate flask under aseptic condition. Duplicates were allowed to ferment up to 7 days. After 7 days, the fermented samples were withdrawn using muslin cloth. The fermented liquid was centrifuged at 10000rpm for 20 minutes and supernatant was used as the crude enzyme to determine the kinetic properties.

2.4. Enzyme assay

Enzyme assay was done based on the Miller (1959) method that was modified by Roy and Uddin (2004). The end product of glucose by crude naringinase enzyme was determined. The glucose is a reducing sugar that has aldehyde group which reduces 3,5-dinitrosalicylic acid (DNS) to orange coloured 3-amino,5 nitrosalicylic acid in alkaline medium on boiling and oxidizes itself to carboxylic group. Sodium potassium tartarate helps to fix the colour of 3-amino 5-nitrosalicylic acid and intensity of orange colour developed is propotional to the concentration of reducing sugar present in the solution. Based on these principles the naringinase activity was determined by glucose standard curve. The tests and controls were prepared for each experiment separately. For the test, the reaction mixture was prepared with 0.25mL of crude enzyme and 0.25mL of substrate in citrate buffer. The mixture was allowed to incubation. After incubation, the DNS solution of 0.5mL was added to stop the reaction and it was boiled upto 5minutes. Then it was allowed to cool. For control, 0.25mL substrate was mixed with 0.5mL of DNS solution and then 0.25mL of crude enzyme was added. It was allowed to boil up to 5minutes then allowed to cool. Then 5mL of sterilized distilled water was added to each controls and tests and stirred well. The absorbance was read at 550nm. The crude naringinase enzyme activity was calculated as amount of naringin can be hydrolyzed by 1mL of enzyme (U/mL). Then it was converted based on the dry solid substrate (U/gram of dry substrate).

2.5. Kinetic properties and stability of crude naringinase enzyme

2.5.1. Effect of temperature

The substrate for determination of enzyme activity was prepared with 1% naringin in citrate buffer (pH 5.0). The optimum temperature for naringinase activity was determined by incubating supernatant and substrate mixture (pH 5.0) at temperature ranging from 35ºC - 75ºC, with 10ºC interval for 10 minutes, where substrate concentration and pH were maintained as 0.8% and 5.0 respectively. Optimum temperature was determined by calculating the resulted reducing sugar (glucose) from enzyme and substrate reaction described above.

2.5.2. Effect of pH

The effect of pH on naringinase activity was determined by incubating supernatant with substrate at different pH values ranging from 4.0 – 7.0, with the interval of 1.0 for 10 minutes, where the substrate concentration and temperature were 0.8% and 65^{o}C respectively. The different pH of the substrate was obtained by mixing 0.1 M citric acid buffer and 0.2 M Na_2HPO_4 at different volume combination.

2.5.3. Effect of substrate concentration

The optimum substrate concentration was determined by incubating the crude enzyme with substrate at different concentration that ranging from 2g/L - 12g/L with the interval of 2g/L for optimum incubation time, where pH and temperature were maintained at optimum (65^{o}C & pH 4.0 for 10minutes). The optimum substrate concentration was determined based on the naringenase assay results.

2.5.4. Effect of time

Optimum time for naringinase enzyme activity was measured by incubating the crude enzyme with substrate at the optimum pH and temperature (65^{o}C and pH 4) for different time, ranging from 5-25 minutes, with an interval of 5 minutes. Optimum time was determined based on the naringinase assay results.

2.5.5. Effect of ions

The effect of various minerals on the enzyme activity were determined by using Cu^{2+}, Ca^{2+}, Mn^{2+}, Mg^{2+}, Hg^{2+}, Zn^{2+}, Na^{+} and Ba^{2+} with the concentration of 2mM. Each ions were added to the incubation mixture at pH 4.0 and 10g/L of substrate concentration. Each test was incubated at optimum temperature (65^{o}C) for optimum time (10minutes). The enzyme activity was determined for every ions based on the naringenase assay described above.

2.5.6. Thermal Stability

Thermal stability was determined at various temperatures near the optimum temperature of enzyme activity which was identified as 65^{o}C. The crude enzyme was allowed to pre incubate at 60, 65 and 70^{o}C for 1 hour and this pre incubated enzyme was used to determine the enzyme activity after 60 minutes. The incubation mixture was allowed to react up to 10 minutes. The substrate concentration used was 10g/L at pH 4.0 with citrate buffer.

2.5.7. Stability for pH

When the optimum temperature (65^{o}C) and substrate concentration (10g/L) were kept as constant, the pH

stability was determined at pH 3.5, 4.0 and 4.5 for 1 hour of pre incubation at 65^{o}C.

2.5.8. Stability for Metal ions

Based on the study on the effect of metal ions on naringinase enzyme activity, the three metal ions were selected to proceed and to determine the enzyme stability. The 2mM of metal ions such as Mn^{2+}, Cu^{2+} and Ba^{2+} were used with the crude naringinase enzyme and 10g/L of substrate adjusted to pH 4.0 using citrate buffer were used at 65^{o}C with 1 hour incubation for the stability determination.

3. RESULTS

3.1. Effect of temperature

The crude enzyme showed highest activity at 65ºC, where different temperatures were assayed ranging from 35ºC- 75ºC (Figure 1). Enzyme activity was significantly higher at 65ºC than the other temperatures tested. After 65ºC, the activity of crude naringinase declined with the increasing temperature.

3.2. Effect of pH

The optimum pH of crude naringinase activity was observed at pH 4.0 (Figure 2) in citrate buffer when 1% naringin as substrate and optimum temperature at 65^{o}C for 10 minutes of incubation. Citrate-phosphate (pH 3.0, 4.0, 5.0 and 6.0), sodium phosphate (pH 7.0), Tris (pH 8.0), Glycine NaOH buffer (pH 9.0) buffers were used. The pH values of the reaction mixtures were measured. When the pH was varied from 3.0-7.0, the activity of naringinase was highest at pH 4.0 and further increase of pH, decreased the enzyme activity (Figure 2). However naringinase activity was higher in acidic pH, ranging between pH 4.0 and 6.0. Since the highest activity was obtained at pH 4.0, this value was selected for further studies.

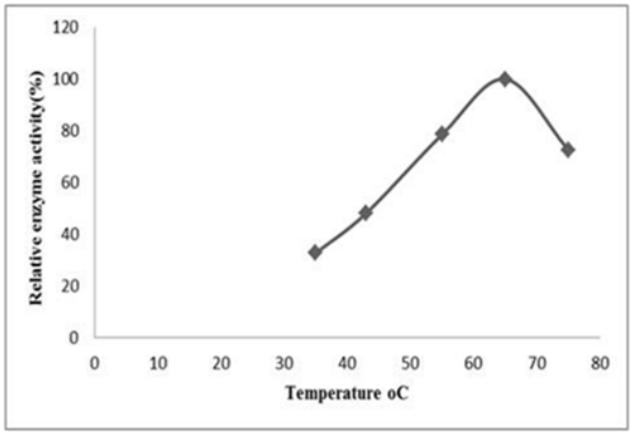

Fig. 1: Effect of temperature on the activity of crude naringinase produced by *Rhizophus stolonifer* isolated from Palmyrah fruit pulp

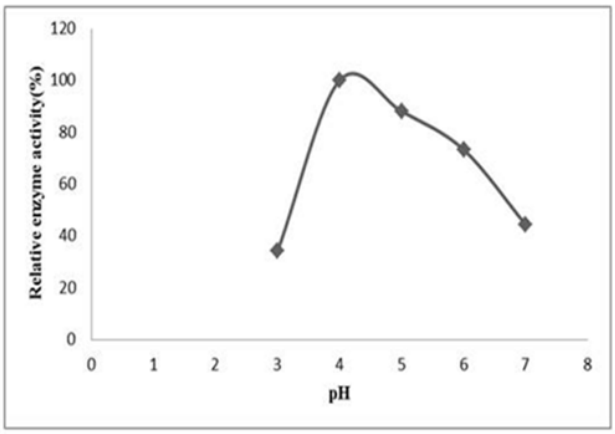

Fig. 2: Effect of pH on the activity of the crude naringinase produced by *Rhizophus stolonifer* at 65^{o}C. The organism was isolated from Palmyrah fruit pulp

3.3. Effect of substrate concentration

When the substrate concentration was increased from 2(g/L) to 10(g/L), the crude enzyme activity was also increased. Beyond 8 g/L of the substrate concentration, naringinase activity remained more or less same. The activity started to show a constant value when the temperature was at 65^{o}C and pH 4.0 for 10 minutes of incubation (Figure 3). Lineweaver-Burk plot was used to determine the Km and Vmax values of the crude naringinase enzyme (Figure 4). The Michaelis constant by Lineweaver-Burk Plot for naringin for the crude naringinase was 3.076 mg/mL and Vmax was 3.125µmole/mL at pH 4.0 and at 65^{o}C.

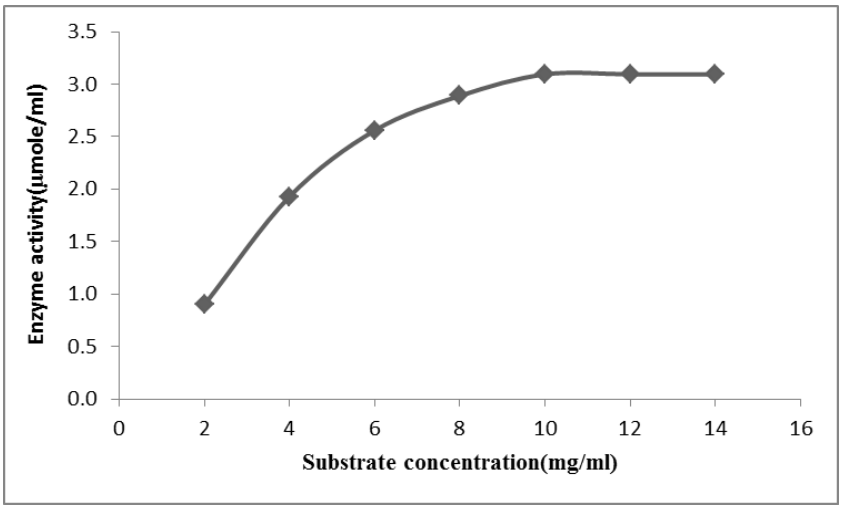

Fig. 3: Effect of substrate concentration on the activity of the crude naringinase produced by *Rhizophus stolonifer* at pH 4.0 and at 65^{o}C. The organism was isolated from Palmyrah fruit pulp.

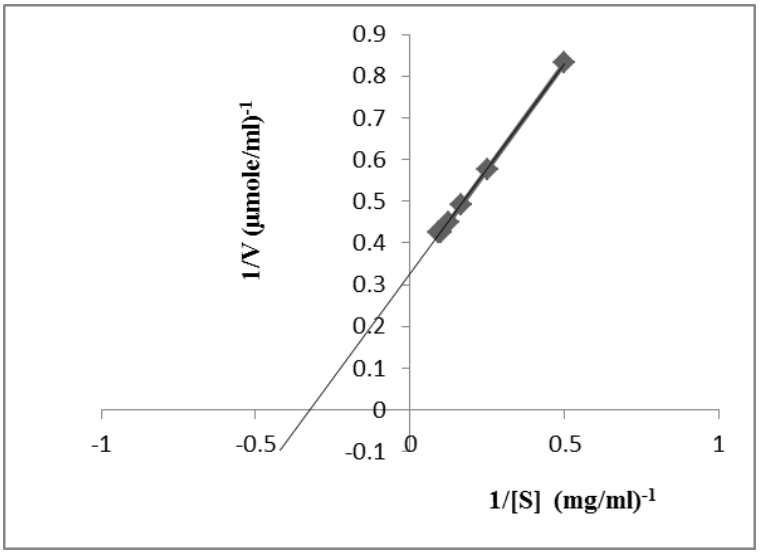

Fig. 4: Lineweaver-Burk plot for the activity of the crude naringinase produced by *Rhizophus stolonifer* at pH 4.0 and at temperature 65^{o}C. The organism was isolated from Palmyrah fruit pulp. Vmax of the crude naringinase enzyme was 3.125µmol/mL and the Michaelis constant by Lineweaver-Burk Plot for naringin was 3.076mg/mL under the above mentioned conditions

3.4. Effect of incubation time

The effect of incubation time was determined at 65^{o}C and pH 4.0 with 10g/L of naringin was used as substrate.Incubation time was influenced on the crude enzyme activity (Figure 5). The enzyme preparation showed a linear relationship between the time and product formation up to 10 minutes. Hence, it was decided to fix the reaction time for 10min. Even though the enzyme activity was increased from 5 minutes to 10 minutes at optimum pH, temperature and substrate concentration, it showed decreased activity after 10 minutes.

3.5. Effect of metal ions

The influence of various minerals on crude enzyme activity is presented in the Figure 6. The enzyme activity increased when 2mM of Mn^{2+},$Ba^{2+}$and Cu^{2+} were added to the reaction mixture and the activity was decreased when Hg^{2+}, Zn^{2+}, Ca^{2+}, Na^{+} and Mg^{2+} were added. Therefore addition of 2 mM of Mn^{2+}, $Ba^{2+}$and Cu^{2+} increased the naringinase activity.

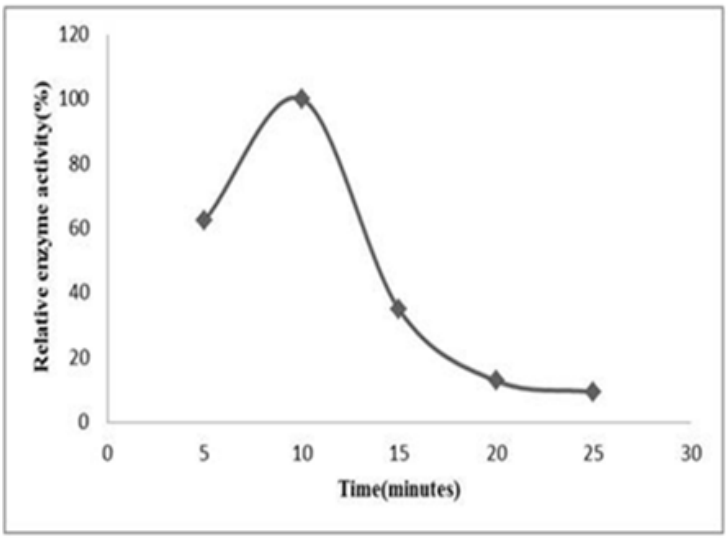

Fig. 5: Effect of time on the activity of the crude naringinase produced by *Rhizophus stolonifer* at pH 4.0 and at 65°C. The organism was isolated from Palmyrah fruit pulp

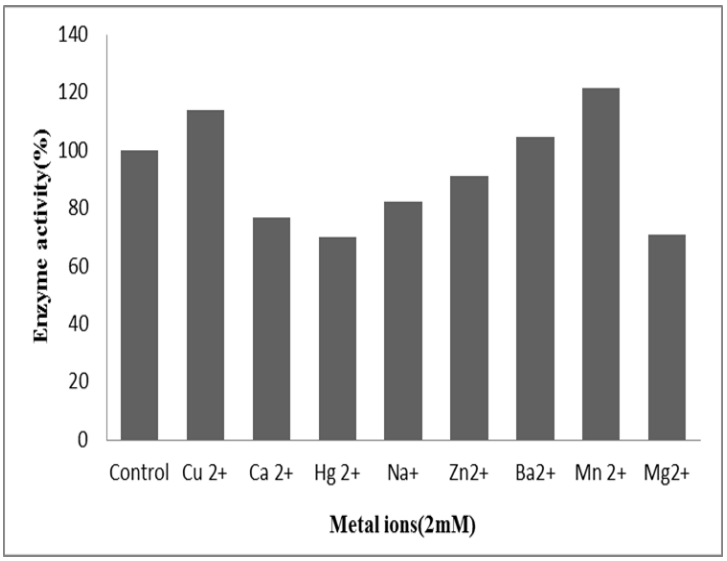

Fig. 6: Effect of different Metal ions on the activity of crude naringinase produced by *Rhizophus stolonifer* at pH 4.0 and at temperature 65°C

3.6. Stability for temperature

Thermal stability was determined at 60, 65 and 70°C for 1 hour of incubation**.** Crude enzyme of naringinase showed highest stability at 60°C, during 1hour incubation and the recorded activity was 22.04% (Figure 7). When the enzyme was pre-incubated at 65°C for 1 hour the residual activity left was 7.66%. Hence the temperature 60°C was chosen as the best temperature in terms of thermal stability of the crude naringinase from *Rhizophus stolonifer*.

3.7. Stability for pH

pH stability was determined at pH3.5, 4.0 and 4.5 in citrate buffer at 10g/l of substrate concentration for 1 hour. Enzyme was more stable at pH 4.5. The crude enzyme showed 17.37% of relative activity at pH 4.5 when incubated for one hour (Figure 8). At pH 3.5 and 4.0, there was significantly low residual activity showed by the enzyme, after 1 hour of pre-incubation.

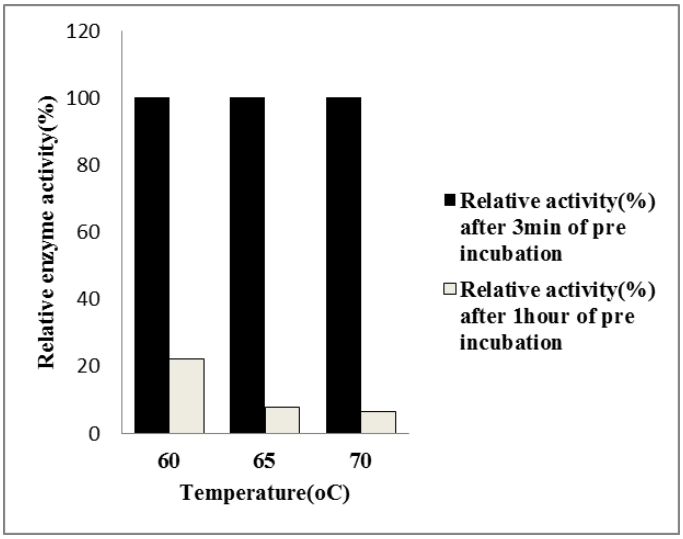

Fig. 7: Relative activity of naringinase enzyme from *Rhizophus stolonifer* after 3 minutes and 1 hour pre-incubation period at different temperature under optimized conditions

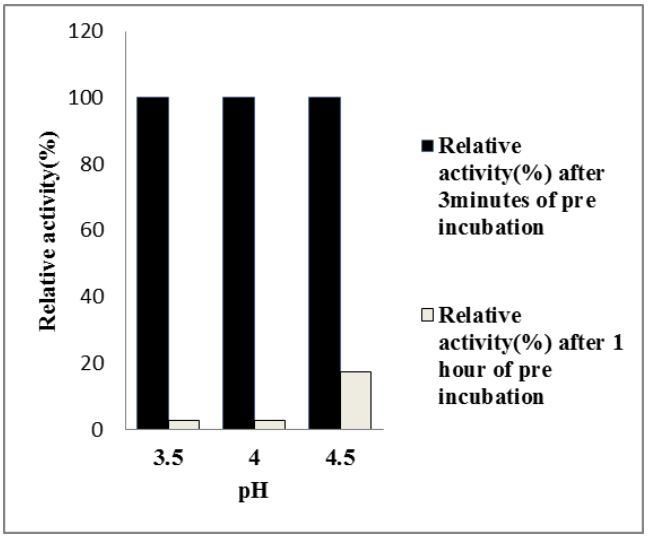

Fig. 8: Relative activity of naringinase enzyme from *Rhizophus stolonifer* after 3 minutes and 1 hour pre-incubation period at different PH values under optimized conditions

3.8. Stability for metal ions

Stability of the metal ions was determined after 1 hour of pre-incubation and the results were showed in figure 9. After 3minutes of pre-incubation, the metal ion Mn^{2+} was showed highest enzyme activity but no any significance difference among other metal ionsand after 1hour of pre-incubation, the metal ion Cu^{2+} was given highest enzyme activity. Hence Cu^{2+} was best stability.

4. DISCUSSION

The present study was conducted to characterize the crude naringinase produced by *Rhizophus stolonifer*. When *Rhizophus stolonifer* strain was grown in the solid state fermentation system using paddy husk as media, naringinase production was higher (269.84 U/gram of dry substrate) in seven days (Karuppaija et al., 2016b). The activity of the crude naringinase obtained from this strain was maximum at 65^{o}C. Activity of naringinase from *Aspergillus flavus* and *Aspergillus niger* were optimum at 50^{o}C (Radhakrishnan and Sampath, 2013 and Puri and Kalra, 2005). Another study indicates that the activity of crude naringinase from *Aspergillus niger* and *Penicillium decumbens* were optimum at 55^{o}C (Norouzian et al., 2000 and Thammawat et al., 2008). The optimum temperature for the activity of naringinase enzyme from *Rhizophus stolonifer* was higher than what was obtained in the previous studies. Differences in the optimum temperature may be due to the nature of the crude naringinase enzyme which produced by different fungal strains (Karuppaija et al., 2016a). Hence the naringinase enzyme can be worked at wide range of temperatures. The crude naringinase enzyme was stable at 60^{o}C for 1hour. The stability of crude naringinase produced by *Aspergillus niger* 1344

at 37°C for 72 hours which showed its optimum activity at 50°C and at pH 4.0 (Puri and Kalra, 2005). The stability was determined at low temperature (37°C) but in the present study, stability was determined at 60°C.

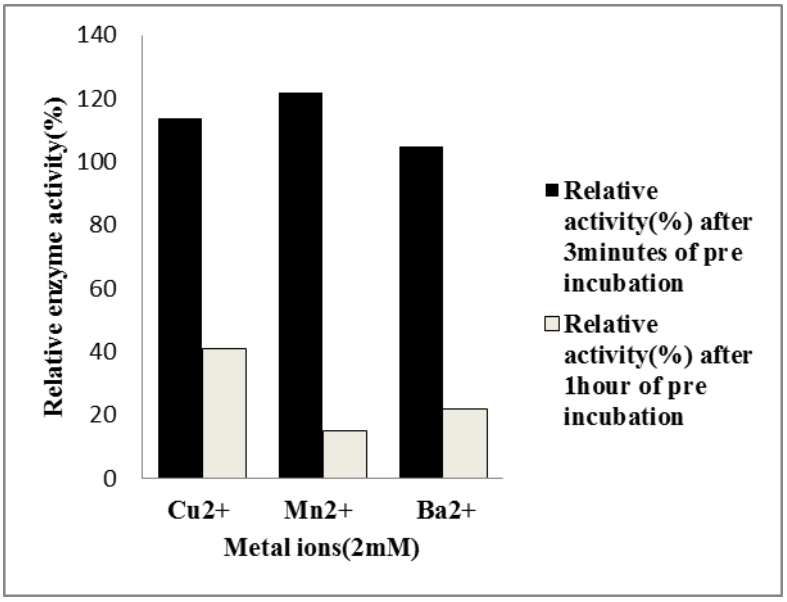

Fig. 9: Relative activity of naringinase enzyme produced by *Rhizophus stolonifer* after 3 minutes and 1 hour pre-incubation period with 2mM of Cu^{2+}, Mn^{2+}& Ba^{2+} under optimized conditions.

The enzyme activity is influenced by different factors such as temperature, pH, substrate concentration, enzyme concentration, inhibitors, metal ions and incubation period. Thermal properties (activation energy and thermal stability) of enzyme influence the catalytic activity which is peak at its optimum temperature (Norouzian et al., 2000). The optimum pH of an enzyme activity is important and it determines the usage of the enzyme in acidic or alkalinic environment (Saranya et al., 2009). A small amount of substrate is enough to saturate the enzyme and at saturation point enzyme activity would be its maximum and above this point, increase in substrate concentration will not increase the enzyme activity (Puri and Kalra, 2005). In our study, the activity of naringinase by *Rhizophus stolonifer* was optimum at pH 4.0 and the enzyme was stable for at least one hour at pH 4.5. Variation of initial medium pH and temperature had significant effects on growth and naringinase production by *Bacillus methylotrophicus* (Mukund et al., 2014). Acidic pH values such as 3.0, 3.5, 4.0, and 4.5 did not support the increase in biomass production and naringinase enzyme production, where as pH 5.0 and above supported growth and highest enzyme synthesis by *Bacillus methylotrophicus*. This is similar to the activity of naringinase enzyme produced by *Aspergillus niger* (Puri and Kalra, 2005). The activity of naringinase enzyme produced by *Aspergillus niger*, was optimum at pH 4.5 (Thammawat et al., 2008). Naringinase enzyme produced by *Aspergillus flavus* (Radhakrishnan and Sampath, 2013) *Aspergillus oryzae* (Chen et al., 2010) and *Penicillium decumbens* (Norouzian et al., 2000) had an acidic pH range for optimum enzyme activity. The optimum pH range for naringinase production reported so far was 4.0 -6.0. The naringinase enzyme is generally active in acidic pH ranges (Puri and Kalra, 2005, Chen et al., 2010). The crude naringinase enzymes are active at acidic pH range and this feature could be used for their industrial application of foods that are acidic in nature.

In this study the substrate concentration was at peak at 10g/L and above this level enzyme activity remained constant. The Vmax of substrate was 3.125µmole/mL and Km was low 3.076mg/mL which indicated the crude naringinase enzyme produced by *Rhizophus stolonifer* has high affinity for substrate (Berg et al., 2002).

The metal ions were showed inductive effect on the enzyme activity. The crude naringinase enzyme produced by *Rhizophus stolonifer* had higher enzyme activity when treated with 2mM of Mn^{2+}, Ba^{2+} and Cu^{2+} but the enzyme activity was inhibited by Na^{+}, Ca^{2+}, Zn^{2+}, Mg^{2+} and Hg^{2+}. And it due to the metal ions Mn^{2+}, Ba^{2+} and Cu^{2+} were increase the catalytic action. Hence these ions could be used in the culture medium to increase the enzyme activity. Mn^{2+} reduced the enzyme activity at the concentration of 10mM but Ca^{2+} and Mg^{2+} increased the enzyme activity of *Aspergillus flavus* (Radhakrishnan and Sampath, 2013). The present study was conducted with 2mM of metal ion concentration; deviation of the results from

literature may be due to the fugal strains or ion concentrations used.

Amylase enzyme activity from *Rhizophus stolonifer* was optimum at pH 7.0 and at 40^{o}C but the temperature could be raised even to 90^{o}C (Sivaramakrishnan et al., 2006). The protease enzyme from *Rhizophus stolonifer* showed its optimum activity at pH 8.0 and temperature at 30^{o}C (Ranganathan, 2015). Hence the *Rhizophus stolonifer* could be used to produce wide range of enzymes with wide range of applications due to their ability to be active in diverse environmental conditions.

5. CONCLUSION

The *Rhizophus stolonifer* isolated from decayed Palmyrah fruit pulp showed optimum naringinase activity at 65^{o}C, pH 4.0, 10g/l of substrate concentration. Enzyme showed zero order kinetics for 10 minutes of incubation. At these optimum conditions, enzyme was stable for at least one hour. Metal ion Mn^{2+} at 2mM concentration promoted enzyme activity whereas Hg^{2+} inhibited the enzyme activity and the stability. Naringinase was more stable with Cu^{2+} than Mn^{2+} and Ba^{2+}. Therefore Naringinase enzyme produced by *Rhizophus stolonifer* could be a potential candidate for the debittering of acidic food items that are produced using moderately high temperatures in the food industries.

Authors' declaration

Authors declare that they have no competing interests.

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References

Arasaratnam V, Mylvaganam K, & Balasubramaniam K (2001). Glucoamylase production by *Aspergillus niger* in solid state fermentation with paddy husk as support. Journal of food science and technology, 38(4): 334-338.

Berg JM, Tymoczko JL, Stryer L (2002). Biochemistry. 5thdition. Edited by Freeman WH. New York.

Chacko R, Deshpande M, Shankar V (1996). Extracellular ribonuclease production by *Rhizopus stolonifer*: influence of metal ions. Current microbiology, 32(5): 246-251.

Chen D, Niu T, Cai H (2010). Optimizing culture medium for debittering constitutive enzyme naringinase production by *Aspergillus oryzae*. African Journal of Biotechnology, 9(31): 4970–4978.

Daniels L, Linhardt RJ, Bryan BA, Mayerl F (1990). Pickenhagen, M. Methods for Producing Rhamnose. U.S. Patent 4: 933,281.

Guimarães LHS, Peixoto-Nogueira SC, Michelin M, Rizzatti ACS, Sandrim VC, Zanoelo FF, Polizeli MDL (2006). Screening of filamentous fungi for production of enzymes of biotechnological interest. Brazilian Journal of Microbiology, 37(4): 474-480.

Hernández-Lauzardo AN, Bautista-Baños S, Velázquez-del Valle MG, Trejo-Espino JL (2006). Identification of *Rhizopus stolonifer* (Ehrenb.: Fr.) Vuill, causal agent of *Rhizopus* rot disease of fruits and vegetables. Revista Mexicana de Fitopatología, 24(1): 65-69.

Kapilan R (2015). Enzyme Production by *Rhizophus stolonifer* Isolated from Bread and Kinetic Properties of the Extracellular Amylase. Jacobs Journal of Enzymology and Enzyme Engineering, 1(1):1-5.

Kapilan R, Arasaratnam V (2011). Paddy husk as support for solid state fermentation to produce xylanase from *Bacillus pumilus*. Rice Science, 18(1): 36-45.

Karuppaija S, Kapilan R, Seevaratnam V (2016a). Characterization of best naringinase producing fungus strain isolated from palmyrah (*Borrasus flabellifer*) fruit pulp, International Journal of Biological Research, 4(2): 97-101.

Karuppaija S, Kapilan R, Seevaratnam V (2016b). Optimization of naringinase production by *Rhizophus stolonifer* in solid state fermentation media using paddy husk as support, Scholars Academic Journal of Biosciences, 4(6): 507-514.

Keerthini S, Kapilan R, &Vasantharuba S (2016). Characterization of best naringinase producing fungus isolated from citrus fruits. International Journal of Biological Research, 4(2): 83-87.

Lee SC and West CA (1981). Polygalacturonase from *Rhizopus stolonifer*, an elicitor of casbenesynthetase activity in castor bean (*Ricinus communis* L.) seedlings. Plant Physiology, 67(4): 633-639.

Miller GL (1959). Use of Dinitrosalicylic acid reagent for determination of reducing sugar. Analytical chemistry, 31(3): 426-428.

Mukund P, Belur PD, and Saidutta MB (2014). Production of naringinase from a new soil isolate, *Bacillus methylotrophicus*: Isolation,

optimization and scale-up studies. Prep. Biochem. Biotechnol., 44: 146-163.

Navaratnam P, Ketheeswary N, Senthuran A, Arasaratnam V (2003). Preliminary studies on the isolation of naringinase producing fungus. Proceedings in the annual scientific session of the Jaffna Science Association.

Ni H, Li L, Xiao A, Cao Y, Chen Y, Cai H (2011). Identification and Characterization of a New Naringinase Producing Strain, *Williopsis californica* Jmudeb007. World Journal of Microbiology and Biotechnology, 27(12): 2857–2862.

Norouzian D, Hosseinzadeh A, Inanlou DN, Moazami N (2000). Production and partial purification of naringinase by *Penicillium decumbens* PTCC 5248. World Journal of Microbiology and Biotechnology. 16(5): 471-473.

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 Journal of Microbiology and Biotechnology, 21(5): 753-758.

Puri M (2012). Updates on naringinase: structural and biotechnological aspects. Applied microbiology and biotechnology, 93(1): 49-60.

Radhakrishnan I, Sampath S (2013). Isolation and characterization of enzyme naringinase from *Aspergillus flavus*. International Journal of Advanced Biotechnology and Research. 1(4): 208-212.

Ranganathan Kapilan (2015). Characterization of the Protease Produced by *Rhizophus stolonifer* Purified from Bread. Journal of Progressive Research in Biology, 2(1):43-49.

Ribeiro MH (2011). Naringinases: occurrence, characteristics, and applications. Applied microbiology and biotechnology, 90(6): 1883-1895.

Sankyo (1988). Preparation of Antibiotic Chloropolysporin C. Japanese Patent, 63:146,797.

Saranya D, Shanmugam S, Sathish T, Thayumanavan B, Rajasekaran P (2009). Purification and characterization of naringinase from *Candida tropicalis*. Advanced Biotechnology, 3: 11-13.

Shanmugam V, Yadav KD (1995). Extracellular production of alpha-rhamnosidase by *Rhizopus nigricans*. Indian journal of experimental biology, 33(9): 705-707.

Sivaramakrishnan S, Gangadharan D, Nampoothiri KM, Soccol CR, Pandey A (2006). a-Amylases from microbial sources–an overview on recent developments. Food Technol Biotechnol, 44(2): 173-184.

Thammawat K, Pongtanya P, Juntharasri V, Wongvithoonyaporn P (2008). Isolation, preliminary enzyme characterization and optimization of culture parameters for production of naringinase isolated from *Aspergillus niger* BCC 25166. Kaestsart J. Nat. Sci., 42: 61-72.

Yalim S, Ozdemir Y, Ibrahim Ekiz H (2004). Naringin in Turkish Orange Juices and Its Reduction by Naringinase. Journal of Food and Drug Analysis, 12: 273–276.

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