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Original Research Article

Optimization of naringinase production by *Rhizophus stolonifer* in solid state fermentation media using paddy husk as support

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Abstract: The palmyrah (*Borassus flabellifer L*) tree represents a major socio economic factor in tropics. The economic value of the tree is reduced by fruit pulp, due to the bitter compound present in the fruit pulp but it can be hydrolyzed by the usage of naringinase enzyme. Hence this enzyme plays a key role in the food industry and pharmaceuticals for its debittering nature. Some microbes like bacteria and fungiare involved in the natural production of cost effective naringinase. The aim of this work was to optimize the cultivation conditions and the media to produce naringinase by solid state fermentation of *Rhizophus stolonifer*, using paddy husk as the support. The highest enzyme activity was obtained on the 7th day of incubation. Highest naringinase enzyme activity (57.97U/g DM) was obtained on the 7th day of incubation and after that activity started to decline. The enzyme activity (212.885U/g DM) obtained with 0.75% naringin concentration was significantly higher than that of other naringin concentrations tested. When the regular medium (0.5% glucose) was replaced with (w/v) 1.5% glucose and 1.5% sucrose, the enzyme activity obtained with 1.5% of glucose (484.96U/g DM) was significantly higher than that of 1.5% sucrose (238.88U/g DM). When naringinase activity was tested with different glucose concentrations, activity obtained with 1.5% of glucose concentration was significantly higher than that of other concentrations. Among the nitrogen sources tested, when 0.5 % ammonium nitrate was used significantly higher naringinase was produced by *Rhizophus stolonifer* than any other sources and their concentrations. The cost to produce 1073.97U/g DM was reduced by 20.15% when conditions and media composition are optimized when compared to the basal medium(57.97U/g DM). Therefore when naringinase was produced by *Rhizophus stolonifer* under optimized condition, the production cost was marginally reduced.
Keywords: *Rhizophus stolonifer*, Naringinase, Optimization, Palmyrah, Solid state fermentation

INTRODUCTION

The palmyrah (*Borassus flabellifer L*) contributes tremendously in arid and tropics of South-East America, East Africa, India, Sri lanka and South-East Asia for the socio economic life of the people [1]. In Sri lanka, palmyrah is widespread in North and East part. The economic value of the palmyrah tree is reduced by the underutilization of the fruit pulp. Onlya fractionof the total annual production is utilized but other fraction is wasted or used to animal feed due to the presence of bitter compound in the palmyrah fruit.The bitter compound of palmyrah fruit is flabelliferin ii (F ii) [2]. There are 2 types of bitterness named as higher and lower. The very high bitterness compound can be hydrolyzed by both naringinase and α-amylase but less bitter compound can only be hydrolyzed by naringinase [3-5].

Naringinase is a complex glycolytic enzyme that has α-L- rhamnosidase and β-D- glucosidase activities. The bitter compound naringin in citrus peel (flabelliferin in palmyrah fruit) can be hydrolyzed by naringinase into aglycone, rhamnose and glucose which are non bitter substances [6,7]. Hence the enzyme naringinase is popular in the debittering process in food industries. Naringinase enzyme is found in plants, yeast, fungi and diverse bacterial species [8]. The microbial production of naringinase enzyme is acceptable at commercial level due to the cost effectiveness and economical viability.

Studies on naringinase indicate that the filamentous fungi are good source of naringinase production but the naringinase production from bacterial source is limited. Among the filamentous fungus, the *Rhizophus stolonifer* has major role in the enzyme production at the commercial level due to its growth characteristics and ability to dissolve diverse groups of compounds [9, 10]. The enzyme α-rhamnosidase also can be produced by *Rhizophus*

nigricans [11]. Hence the naringinase enzyme production by *Rhizophus stolonifer* could be increased if the growing and media conditions are optimized. The *Rhizophus stolonifer* is common mold in bread and it is also important in the rotting of fruits and vegetables. The natural resourcessuch as fruits, sweets, decaying trees are considered as good source of organism that can produce large variety of enzymes [12]. Hence the decaying palmyrah fruit pulp was used as source of organism. The production of naringinase needs to be increased in order to recommend this organism. The production of naringinase enzyme could be increased by optimizing the cultural conditions and media composition. Carbon sources, nitrogen sources and minerals are the major factors that determine the growth and activities of microorganisms. The composition of the solid media such as carbon, nitrogen, vitamins and amount of water added, need to be changed on by one in order to get the highest growth and enzyme production.

Solid state fermentation (SSF) can be defined as the microbial cultivation process in the absence or near absence of free water in the substrate. Although naringinase productions were performed using submerged systems, solid state fermentation is more economical due to the cheap and abundant availability of agricultural wastes which can be used as substrates. At that time when this research was going on, we were experiencing an irregular power cut. Therefore the solid state fermentation method was recommended. The literature survey has shown that so far no investigations were made on the naringinase production by *Bacillus pumilus*, in SSF. The objective of the study was to optimize the fermentation media to produce naringinase by Solid state fermentation using *Rhizophus stolonifer*, with paddy husk as support.

MATERIALS AND METHODS

Chemical

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

Microorganism for enzyme production

Rhizophus stolonifer used in this study was previously isolated from Palmyrah fruit pulp.

Effect of time on enzyme production using basal medium

Solid state fermentation was carried out to extract the crude naringinase enzyme. The solid state fermentation medium was adjusted to the ratio of 2:10 with paddy husk and liquid broth. The cleaned paddy husk of 20g was taken with 100ml liquid broth. The liquid broth was prepared with (w/v) 0.2% naringin, 0.5% glucose, 0.5% 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% $FeSO_4.7H_2O$. The pH of the medium was adjusted to 6. The paddy husk and liquid broth were sterilized under autoclave at 121^oC 15psi for 20 minutes separately. The paddy husk was transferred to liquid broth under aseptic condition. 6 days old culture of *Rhizophus stolonifer* from naringin agar medium was obtained to make inoculum with sterilized 0.85% NaCl solution and spore size was adjusted upto 10^8spores/ml. The experiment was carried out to three replicates. 1ml of inoculum was inoculated into the medium under aseptic condition and it was allowed to ferment for 9 days. After 3 days of incubation, the culture filtrate was withdrawn at one day regular interval until 9th day for estimation of naringinase enzyme activity.

Effect of inducer on the naringinase production

Naringin was used as inducer in this experiment. The concentration of naringin was only changed in liquid broth of control medium and the concentration ranges from (w/v) 0.25% to 1.25% with 0.25% interval. The pH of the medium and all the other conditions for the fermentation were maintained same as basal medium. Fermentation was allowed for nine days and the optimum enzyme activity was obtained on the 7th day at room temperature.

Effect of different carbon sources on enzyme production

The carbon sources of glucose (monosaccharide) and sucrose (disaccharide) were used to determine the best carbon source for optimum enzyme production. The glucose and sucrose were used at the concentration of 1% and 1.5% and replaced the carbon source (glucose) of the basal medium and naringin in the basal medium was also used at the optimum concentration (0.75%). All the other conditions were maintained same as the basal medium.

Optimization of concentration of carbon source

From the above study, glucose was selected and it was used as carbon source for further studies. The concentration of glucose ranges from (w/v) 0.5% to 2.5 % with 0.5% interval were used in the media and the optimum naringinase production was determined. With the optimum concentration of naringin, different glucose concentrations were replaced in the basal medium while other conditions of SSF were maintained as for basal medium.

Effect of different nitrogen source

The effect of different nitrogen sources and its concentration for maximum enzyme production were also determined. The nitrogen sources such as peptone (organic nitrogen source from animal origin), soya bean (organic nitrogen source from plant origin) and NH_4NO_3 (inorganic nitrogen source) were used at two different concentration ((w/v) 0.5% and 1%). The optimum concentration of inducer (0.75%), suitable carbon source with optimum concentration (1.5% glucose) were used in determining the nitrogen source first and then its concentrations and they were used to replace naringin, glucose and peptone respectively as in

their initial non-optimized amount used in the basal media. The pH of the medium was kept at 6.0 and all the other conditions for fermentation were maintained as basal medium.

Naringinase enzyme assay

Enzyme assay was done based on the Miller [13] method that was modified by Roy and Uddin. The glucose standard curve was plotted with 0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml of glucose at 550nm. The amount of glucose hydrolyzed by crude naringinase was estimated from standard curve. The culture filtrate was centrifuged at 10000rpm for 20minutes and supernatant was taken as crude naringinase enzyme. The substrate for enzyme reaction was prepared with 1% naringin in pH 4 citrate buffer which was sterilized in autoclave at 121^{o}C 15psi for 20 minutes. 0.25ml of supernatant and 0.25ml of substrate were pre incubated at 35^{o}C for 3minutes separately and after it was allowed to reaction at 65^{o}C for 10 minutes. Then 0.5ml of DNS solution was added and it was allowed to boil for 5minutes. 5ml of sterilized distilled water was added to test after cooling and it was stirred well. Tests were carried as 3 replicates. The control was also prepared for each test and in control, 0.25ml of substrate was allowed to pre incubation at 35^{o}C for 3minutes and 0.5ml of DNS solution was added. Then it was stirred well and 0.25ml of supernatant was added. The mixture was allowed to boil upto 5minuute and it was cooled. 5ml of sterilized distilled water was added and stirred well. The reading was taken at 550nm when the control was taken as zero.

Unit of crude naringinase (U/ g DM): Amount of naringin (µ mole) which can be hydrolyzed by 1ml of crude enzyme naringinase in 1 minute and it was converted to base on dry solid substrate (µ mole/ gram of dry substrate).

Statistical analysis

Data were analyzed by Completely Randomized Design (CRD) using the SAS 8 statistical package. The significant difference was compared using Least Significant Different (LSD).

RESULTS

Effect of time on naringinase production

The figure 1 shows how naringinaseenzyme activity varies with time in the basal medium. Highest enzyme activity was obtainedon the 7th day of incubation and after that activity (57.97U/g DM) started to decline.

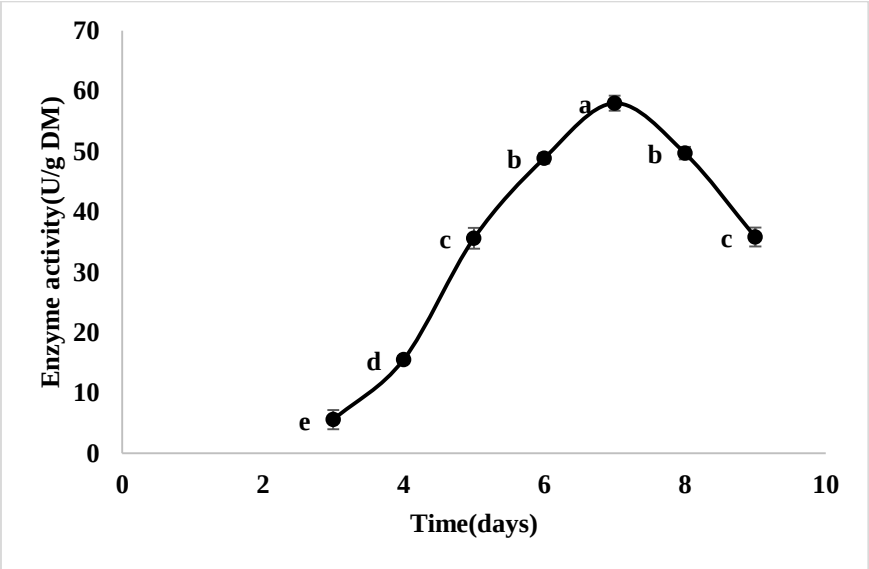

Fig-1: Naringinase enzyme production by *Rhizophus stolonifer* with time in a basal medium at room temperature

Effect of concentration of naringin

The concentration of naringin was only changed in the basal medium with ranges from (w/v) 0.25 to 1.25% with 0.25% interval. The enzyme activity increased upto 0.75% and then started to decline (Figure 2). The enzyme activity (212.885U/g DM)obtained at 0.75% naringin concentration was significantly higher than that of other naringin concentrations tested. Therefore, 0.75% of naringin was chosen in the basal medium for further studies.

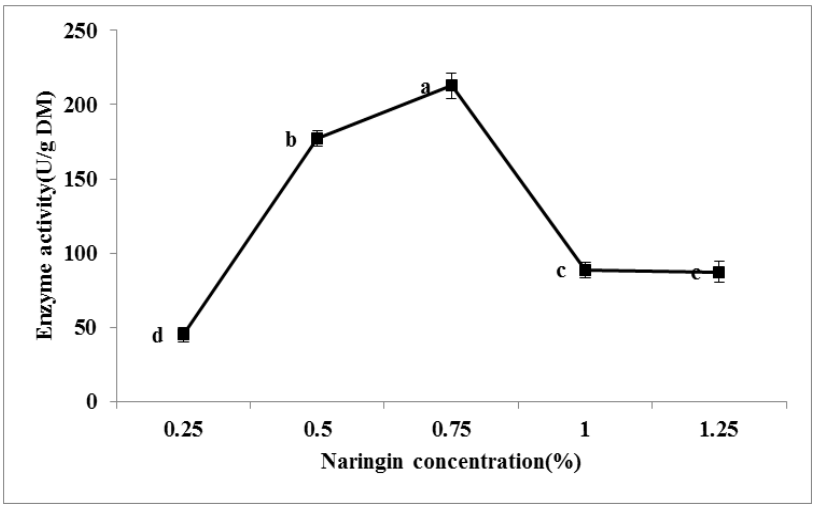

Fig-2:Naringinase enzyme production by *Rhizophus stolonifer* in basal medium with different concentrations of naringin at room temperature

Effect of different carbon sources on enzyme activity

The carbon sources glucose(monosaccharide) and sucrose(disaccharide) were used to select the best carbon source for the maximum naringinase production by *Rhizophus stolonifer*. There was no significant difference in the naringinase activities when the medium containing (w/v) 1% sucrose (387.60U/g DM) and 1% glucose(369.26U/g DM) were separately used as the carbon sources, respectively (Figure 3). When the regular medium(0.5% glucose) was replaced with (w/v) 1.5% glucose and 1.5% sucrose, the enzyme activity obtained with 1.5% of glucose (484.96U/g DM) was significantly higher than that of 1.5% sucrose(238.88U/g DM). Hence 1.5% of glucose was chosen in the medium for further studies.

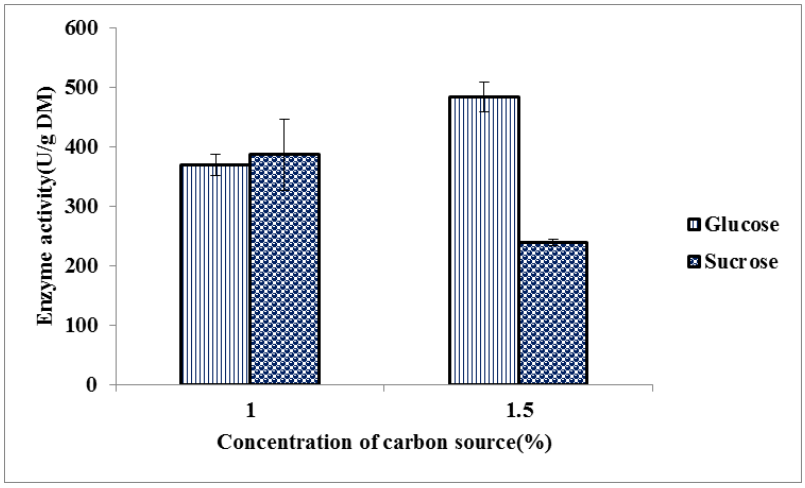

Fig- 3:Naringinase enzyme produced by *Rhizophus stolonifer*with different carbon sources such as glucose and sucrose with different concentrations ((w/v) 1% and 1.5%) at 7th day at room temperature

Effect of concentration of glucose

When the basal medium containing naringin ((w/v)0.75%) was replaced by glucose with the concentrations ranging from (w/v)0.5% to 2.5% with 0.5% interval, were used to see how naringinase enzyme production changes with different substrate. Highest enzyme activity (490.369U/g DM) was obtained at 1.5% of glucose concentration(Figure 4). Naringinase activity obtained with 1.5% of glucose concentration was significantly higher than that of other glucose concentrations (Figure 4). Hence 1.5% of glucose concentration was chosen for further studies.

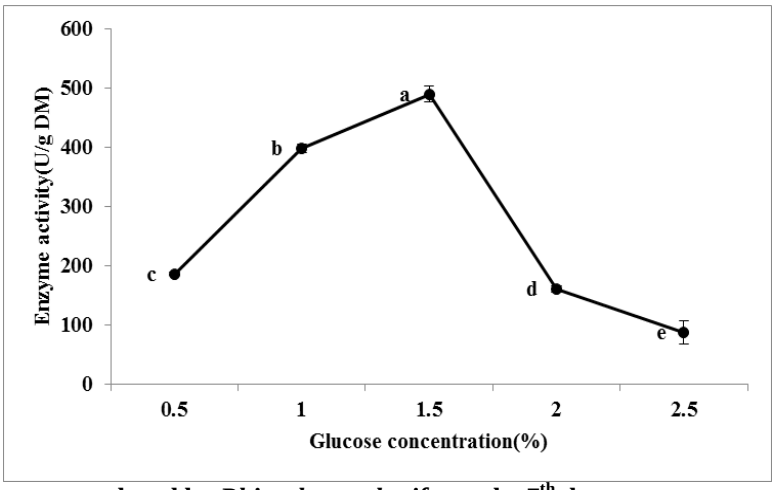

Fig-4: Naringinase enzyme produced by *Rhizophus stolonifer*on the 7th day at room temperature with different concentration of glucose

Effect of nitrogen sources on naringinase activity

The different nitrogen sources of organic(soya bean, peptone) and inorganic (NH_4NO_3) origins were used with same concentrations to identify the best nitrogen source for naringinase production by *Rhizophus stolonifer*. In this experiment, the maximum enzyme activity was obtainedwhen (w/v) 0.5% of NH_4NO_3 was used(1073.97U/g DM). There was no significant difference in the enzyme activities obtained when (w/v)0.5% and 1% of peptone and soya bean were used. Naringinase activity obtained with (w/v) 0.5% NH_4NO_3 was significantly higher than that of (w/v) 1.0 % NH_4NO_3 (Figure 5). Among the nitrogen sources tested, soya bean yielded very low naringinase production by *Rhizophus stolonifer*. Hence 0.5 % NH_4NO_3 was used as nitrogen source for further studies.

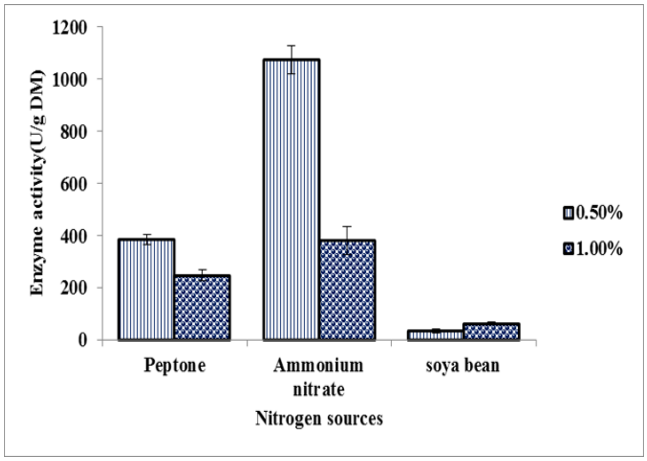

Fig-5:Naringinase enzyme production by *Rhizophus stolonifer* with different nitrogen sources, such as peptone, ammonium nitrate and soya bean in different concentrations (0.5 % and 1.0 %), on the 7th day at room temperature.

Cost analysis for optimized medium

When the basal medium was replaced by (w/v) 0.75% naringin, 1.5% glucose as carbon sources and 0.5% NH_4NO_3as nitrogen source, production of naringinase by *Rhizophus stolonifer* was significantly increased. The enzyme activity increase was from 57.97U/g DM to 1073.97U/g DM. The cost analysis for medium optimization was detailed in Table1. When (w/v) 0.75% of naringin and 1.5% of glucose were used to replace the basal medium containing 0.2% naringin and 0.5% glucose, the cost of the medium was increased by 73.33% and 66.67% respectively. The cost of the optimized medium was reduced by 99.2%, when (w/v) 0.5% ammonium nitrate was used to replace the basal medium containing 0.5% peptone. The total cost for higher naringinase production (1073.97U/g DM) at optimized level was reduced up to 20.15% of the initial cost of the basal medium. It clearly indicates that NH_4NO_3 was a suitable nitrogen source compared to peptone based on the higher enzyme activity and very low production cost.

Table-1: The cost analysis for basal and optimized medium for naringinase production by *Rhizophus stolonifer*

Components	Basal medium (price in %)	Optimized medium (price in %)
Naringin	26.67	100
Glucose	33.33	100
Nitrogen source	100	0.8
Total components	100	79.85

DISCUSSIONS

The experiment was done to increase the naringinase production and using the low cost agro-wastes, plant products, machinery, equipments, raw materials and also labour. The solid state fermentation (SSF) might b a good solution for this than the submerged fermentation system [14]. The solid support should be optimum surface area for oxygen diffusion, nutrient absorption and assimilation. The paddy husk functions as a good support for solid state fermentation by the bacteria [15, 16]. The filamentous fungi were used to produce naringinase in SSF using grape fruit rind and orange rind as support but the grape fruit rind functioned as a best support [17]. The possibilities are found to produce naringinase enzyme by solid state fermentation using *Aspergillus niger* MTCC 1344 which is capable of producing high levels of naringinase enzyme [18]. *Aspergillus terreus* CECT 2663 produced α-L-rhamnosidase in SSF system [21]. Cellulose-free alkaline xylanase was produced by *Rhizophus stolonifer* in SSF system using wheat bran as a support [19]. Limited amount of studies are available for *Rhizophus stolonifer* in SSF system and this study involves in the application of SSF using paddy husk as support to produce naringinase enzyme from *Rhizophus stolonifer*.

Inducers increased the naringinase production by *Aspergillus niger* MTCC 1344 and this study indicates that naringin could be one of the best inducer among rhamnose, naringenin, rutin and hesperidin. The activity of naringinase from *Aspergillus niger* MTCC 1344 was highest (9.68U/ml) on the 7th day under shake flask experiment [22]. Another study with *Aspergillus niger* VB07 introduces the time when the inducer such as naringin, naringenin, rutin and hesperidin for naringinase production should be used by shake flask experiment. Highest naringinase activity was obtained from *Aspergillus niger* VB07 with different inducers on the 7th day [20]. Effect of concentration of naringin was studied using *Aspergillus niger* and this study concluded that stepwise addition of smaller concentration of naringin to the medium was more effective than the addition of higher amount of naringin at initial [23]. Hence the concentration of naringin should be considered during naringinase production from microbes. The enzyme activity of naringinase produced by *Streptomycetes* was high at low concentrations of naringin and the activity of naringinase increased from 7th day to 14th day [24]. Hence inducer is important for the microorganism to produce appropriate enzyme. In the present study (w/v) 0.75% of naringin gave highest naringinase activity.

The effect of carbon source is important in order to produce higher amount of naringinase enzyme. Carbon source will act as a source for energy and carbon. The glucose is widely utilized as carbon source but other carbon sources like sucrose, maltose, lactose, rhamnoseetc have been used. The glucose gave prominent naringinase enzyme production than sucrose and rhamnose [24]. Another study indicates that sucrose and molasses can exhibit maximum nariginase production but maltose and lactose produce low level of naringinase production [25]. The study of naringinase production by *Micrococcus* gave the result as when increasing concentration of surose from 0.25% to 0.5%, the naringinase enzyme was increased and then after declined [26]. Sucrose was best carbon source for naringinase production by *Streptomycetes* than glucose [27]. The present study indicates that glucose at 1.5% of concentration is the best carbon source than sucrose but at 1% of glucose and sucrose gave same results.

NH_4NO_3 was used as nitrogen source with varies concentration which provided positive effect, when increasing concentration from 0.25% to 0.5% the activity was increased and after 0.5%, the activity was decreased [26]. The naringinase production by *Aspergillus oryzae* JMU316 proved that peptone was one of the best nitrogen source [28]. Xylanase enzyme production by *Bacillus pumilus* was also increased by the addition of soya bean powder in solid state fermentation using paddy husk as support [29]. The present study was conducted to identify the best nitrogen source to produce naringinase enzyme and the result was NH_4NO_3 at 0.5% was a suitable nitrogen source with reasonable production cost.

CONCLUSION

The naringinase production by *Rhizophus stolonifer* could be increased by optimizing the media composition. After the optimization of the media, naringinase activity produced by *Rhizophus stolonifer* incrasd from 57.97U/g DM to 1073.97U/g DM. The SSF system using paddy husk as support would be a good cost effective method for the production of naringinase based on the fact that paddy husk is one of the cheapest and abundant agro waste. Naringinase production cost was reduced by 20.15% when conditions and media composition were optimized.

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