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Original Article

OPTIMIZATION OF NARINGINASE PRODUCTION AND ITS PURIFICATION FROM *MICROCOCCUS* SP.

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ABSTRACT

Objective: Isolation and purification of naringinase from *Mircoccus* sp.

Methods: The naringinase producing microorganism was isolated from the soil sample by the serial dilution method. The isolate was identified based on morphological, microscopic and biochemical characters. The physico-chemical parameters, effect of carbon and nitrogen source was carried out. The protein was purified using ultrafiltration, ammonium sulfate precipitation and Dialysis and detected by using SDS-PAGE.

Results: A total of 10 isolates were obtained among them isolate AMJVC-8 showed the highest activity and hence the same was selected for further studies. Based on morphological, microscopic and biochemical characters, the isolate tentatively identified as the member of *Micrococcus*. The optimum pH and temperature for nariginase production were found to be 6.0, 37°C respectively. The optimum inoculums size, agitation speed was found to be 6% and 160rpm respectively. The optimum carbon and nitrogen source for nariginase production were found to be 1.5%, 0.50% respectively. The molecular weight of the enzyme was found to 48 kDa.

Conclusion: Microbial enzymes are gaining special importance in the recent days due to cost effective production and economically viable process. Microbial naringinase has completely replaced the chemical methods of naringin reduction in industries. Production of naringinase has been very well studied in fungal sources, however, very limited reports are available on bacterial naringinase. There is a great scope for fermentation process development using new isolates, which would result in the commercially viable processes. Thus, isolation of new promising naringinase producers is the need of the hour.

Keywords: Naringinase, *Micrococcus* SP., Optimization of various parameters

INTRODUCTION

Microorganisms are present everywhere; their presence has not only seen in a normal environment, but also to extremity [1] hence we can say, microorganism are ubiquitous. They are used in various fields [2] especially in industries due to their ability to produce various enzymes; among various enzymes naringinase is of great importances. Naringinase (EC 3.2.1.40) is a hydrolytic enzyme containing both α – L - rhamnosidase and β -glucosidase activities, which hydrolyzes naringin to release L-rhamnose and naringenin [3]. The α - L - rhamnosidase activity of this enzyme is of particular interest to the structure determination of polysaccharides, glycosides, and glycolipids [4]. Naringin and its hydrolyzed product have several important industrial applications; one of them is in commercial citrus juices. The citrus juice has bitterness, especially in grapefruit juice, which is posing and serious problem. This bitterness is due to the presence of flavonone called naringin [5].

In the past, this bitter taste was decreased by the reduction of naringin content which was carried out using chemical methods, but this method had several drawbacks resulting in the inferior quality of fruit juice [3]. Recently microbial naringinases has completely replaced the chemical methods due to cost effective production and economically viable process [6]. Apart from de bettering of citrus juices, naringinase also finds applications in the production of glycopeptide antibiotic, deglycosylation of flavonoids, gellan depolymerisation and many more [3, 4].

Due to the wide application of naringinase the isolation of new promising naringinase producers is the need of the hour. Once new strains are isolated, identification of nutritional factors, such as carbon and nitrogen sources and their effect on the production needs attention. Hence with the increasing demand for microbial naringinases, the present work was undertaken with an aim of isolating a promising microbial strain for naringinase production. The present work attempts to characterize the microbial isolate, enhance the naringinase production by optimization of process parameters and partial purification of the enzyme.

MATERIALS AND METHODS

Collection of soil sample

Soil samples from Gulbarga region, Karnataka were collected in a screw cap bottles and used for the isolation of micro-organisms. 0.1 mL serially diluted samples were plated over Sabouraud's Dextrose agar (SDA) and Nutrient agar (NA) for growing fungi and bacteria respectively. The colonies were repeatedly streaked on the same media until they were pure and further they are preserved on agar slants at temperature 4° C for further work.

Screening of microbes in naringinase detection broth

Screening of fungi

The fungal colonies were inoculated into Naringin broth having composition (per L) 5g NH_4NO_3, 0.2g KCl, 0.4g KH_2PO_4, 0.01g $FeSO_4.7H_2O$, 0.01g $ZnSO_4$, 0.01g $MnSO_4$, 0.2g $MgSO_4.7H_2O$ and 1g naringin and its initial pH was 6 at 28° C incubated for 120 hrs on a rotatory shaker at 120rpm [7].

Screening of bacteria

The bacterial colonies were inoculated into Naringin broth having composition (per L) 5g NH_4NO_3, 0.2g KCl, 0.4g KH_2PO_4, 0.01g $FeSO_4.7H_2O$, 0.01g $ZnSO_4$, 0.01g $MnSO_4$, 0.2g $MgSO_4.7H_2O$ and 1g Naringin and its initial pH was 7.0 at 37° C incubated for 48 hrs on a rotatory shaker at 120rpm [8].

Naringinase assay

The fermented culture solution was centrifuged at 10000g for 10 min at 4°C and supernatant was collected for estimation of naringinase activity. 0.1 ml supernatant was mixed with 0.9 ml of 0.05% (w/v). Naringin dissolved in 0.1 M sodium citrate buffer (p^H 4.0). The mixture was stirred for 30 min at 50°C and the reaction was terminated with 0.5 ml Trichloroacetic acid (10%, w/v). Then, 0.1 mL of the reaction mixture was added to 5 ml of 90% (v/v)

diethylene glycol followed by the addition of 0.1 mL of 4N NaOH. This mixture was kept for colour development at room temperature at 10 min. The resulting yellow colour was measured at 420 nm. One unit (U) of naringinase activity was defined as the amount of enzyme that could hydrolyze 1 µmol of naringin/min at the assay conditions [8].

Identification of bacterial strain

The isolated microorganisms were identified based on morphological, microscopic and biochemical characters [9].

Optimization of physical parameters

Optimization of pH, temperature, inoculums size and agitation speed

At first set of experiment, the seed cultures were inoculated in a 250 ml Erlenmeyer flasks containing 50 ml of growth medium and incubated at different pHranging from 5.0 to 8.0 with an interval of 1.0 at 37° C, at 120 rpm for 48 hrs. In another set of experiments, the cultures were inoculated at pH 6.0 of the medium and incubated at different temperature ranging from 27° C to 42° C with an interval of 5° C at 120rpm for 48 hrs. In the next set of experiment, the inoculum size was varied from 2% to 8% with an interval of 2% with initial pH6.0. The flasks were incubated at temperature 37° C, at 120rpm for 48 hrs.

The fermentation was carried out at varying agitation speed. The medium with an initial pH6.0 at temperature 37° C inoculated with 6% culture incubated at different shaking speed ranging from 120-180rpm with an interval of 20 rpm for 48 hrs. All experiments were done in triplicates.

Optimization of nutritional parameters

Optimization of carbon and nitrogen source

The carbon source of the medium, i. e. naringin was varied from 0.5% (w/v) to 2% (w/v) with an interval of 0.5% (w/v). The initial pH6.0 of the medium inoculated with 6% (w/v) of culture and incubated at 37°-C, at 120 rpm for 48 hrs. The nitrogen source of the medium NH_4NO_3 was varied from 0.25% (w/v) to 1% (w/v) with an interval of 0.25% (w/v). The initial Ph 6.0 of the medium inoculated with 6% (w/v) of culture and incubated at37° C, at 120 rpm for 48 hrs.

Purification of naringinase enzyme

The purification was carried out using crude enzyme extract. The enzyme was purified by the following steps at 0-4°C, unless otherwise mentioned.

Ultrafiltration

Ultrafiltration was carried out by using membrane with molecular weight cut-off of 10 kDa. The concentrated retentate was used for ammonium sulfate precipitation.

Ammonium sulfate precipitation and dialysis

Finely powdered ammonium sulphate was added to the crude extract. The naringinase activity was associated with the fraction precipitated at 20-80% saturation. The precipitate was collected by centrifugation at 9,000g for 15 min, dissolved in 1M sodium citrare buffer pH 4.0 and dialyzed against the same buffer.

SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli, with a separating acrylamide gel of 10% and stacking gel 5% containing 0.1% SDS. The gel was stained with coomassie brilliant blue R250 and destained with a solution of methanol, acetic acid and water in the ratio of 4:1:5. The standard molecular weight markers used in our study were medium range produced from Merck, Bengaluru, India. The bands are of 97.4, 66, 43, 29, 20.1, and 14.3 kDa.

RESULTS AND DISCUSSION

Screening of naringinase producing microorganism

Total of 6 fungal and 4 bacterial strains were obtained by serial dilution methods. All strains were screened for naringinase activity in naringin broth (Table 1). Among the ten isolates, the isolate AMJVC-8 showed consistently high naringinase activity of 4.97IU. This isolate was chosen for further investigations and identified based on morphological, physiological and biochemical characteristics were studied [9].

Table 1: Isolation of naringinase producing microbes

S. No.	Micro-organisms	Naringinase Activity (IU)
	Fungi	
1	AMJVC-1	1.07
2	AMJVC-2	4.17
3	AMJVC-3	0.96
4	AMJVC-4	2.28
5	AMJVC-5	4.38
6	AMJVC-6	1.82
	Bacteria	
7	AMJVC-7	2.03
8	**AMJVC-8**	**4.97**
9	AMJVC-9	4.02
10	AMJVC-10	3.57

Identification of bacterial strain (AMJVC-8)

The strain AMJVC-8 was identified as Gram positive coccus, non-motile, non-spore former. The strain was found to be negative for mannitol salt fermentation test, anaerobic growth test and positive for catalyse test, glucose fermentation test (Table 2). Based on the said results, the strain was presumptively identified as *Micrococcus* sp.

Table 2: The Morphological, Physiological and Biochemical characteristics used to identify AMJVC8

Characters	Results
Colony margin	Entire
Colony elevation	Convex
Colony surface	Smooth
Gram staining	Positive
Pigmentation	Creamish yellow
Cell shape	Cocci
Motility	Non-motile
Spore	Absent
catalase	Positive
Mannitol fermentation	Negative
Glucose fermentation	Positive
Anaerobic growth	Negative

Optimization of physical parameter

Optimization of pH, temperature, inoculums size and agitation speed

Optimization of pH for naringinase production revealed that a pH of 6.0 was found to be best for enzyme production by bacterial strain. The enzyme production was increased from pH 5.0 to 6.0 and thereafter production was decreased. Therefore, pH 6.0 was found to be best for enzyme production and the naringinase activity was 5.54 IU. The results were depicted in fig. 1.

Our results are in close agreement with that of the previous report. This observation was contrary for bacterial α-L-rhamnosidases, for which neutral and alkaline pH optima have been found [10-12].

Optimization of temperature was carried out for naringinase production at different temperatures ranging from 27° C to 42° C with an interval of 5° C. The result shows that the naringinase production was increased with increase in temperature from 27° C to 37°C thereafter the naringinase production was decreased. Hence a temperature of 37°C was found to be best for enzyme production showing an activity of 6.32 IU. The results were depicted in fig. 2.

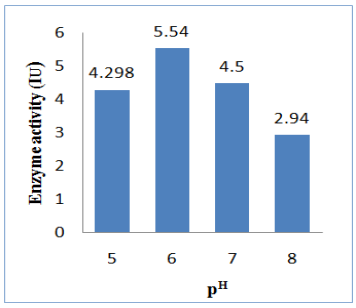

Fig. 1: Optimization of pH

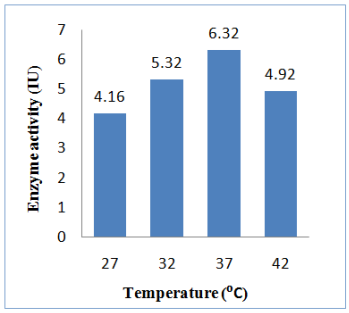

Fig. 2: Optimization of temperature

It is important to provide an optimum inocula level in the fermentation process. At a suitable inoculums size, the nutrient and oxygen level are enough to sufficient growth of bacteria and therefore enhance naringinase production. If the inoculums size is too small, insufficient biomass will lead to reduced levels of secreted naringinase. High inoculums size can result in the lack of oxygen and nutrient depletion in the culture media resulting in poor product yield. There was a significant effect of size of inoculum on naringinase production. Increased naringinase production was observed with increase in inoculums size from 2% (v/v) to 6% (v/v). Further increase in inoculum size 8% (v/v) resulted decrease in enzyme synthesis, probably due to nutrient limitation. At the end of fermentation, the naringinase yield obtained at 6% (v/v) inoculum size was 7.83IU which was higher than 2%, 4% and 8% (v/v) inoculum size (the results were depicted in Fig. 3.) In the previous studies Puri et al. [13] used an inoculum level of 1% (v/v) to 7% (v/v) in the cultivation medium to establish the effect of inoculum size on the enzyme production by *Staphylococcus xylosus* MAK2. A 5% (v/v) inoculum was optimal. With 2-4% (v/v) inoculum size, the lag phase did not reduce significantly, and maximal enzyme production was obtained at a longer incubation time of 60 hours.

Optimization of agitation speed for the naringinase production was carried out at various agitation speeds ranging from 120rpm to 180rpm. The result shows that the naringinase production was increased with increase in agitation speed from 120 rpm to 160 rpm thereafter the naringinase production was decreased. Therefore 160rpm was found to best for enzyme production and the enzyme activity was 8.02 IU. The results were depicted in fig. 4.

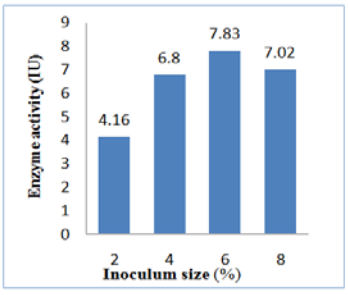

Fig. 3: Optimization of inoculum size

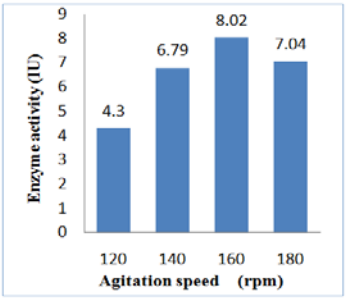

Fig. 4: Optimization of agitation speed

Optimization of nutritional parameters

Optimization of carbon and nitrogen source

The addition of different concentration of carbon source (naringin) to the synthetic medium had a positive effect on naringinase production. Increased naringinase production was observed with increase in naringin concentration from 0.5% (w/v) to 1.5% (w/v), further increase in naringin concentration 2% (w/v) resulted decrease enzyme synthesis. The results were depicted in fig. 5.

The results showed that the maximum naringinase activity was 8.76IU when added naringin at the concentration of 15 gm/L in to the medium. According to Puri M. et al. [13] Sucrose and molasses (1%, w/v) exhibited the highest naringinase production (5.7 IU ml^{-1}) after 36 hours of fermentation. Other carbon sources, for example, lactose (4.9 IU ml^{-1}) and maltose (4.9 IU ml^{-1}) also supported naringinase production, but the yield was lower when compared to that obtained with sucrose. However, naringinase production was very low using either glucose (2.25 I U ml^{-1}) or fructose (2.7 IU ml^{-1}). Suppression of naringinase activity by rhamnose, citrate, and some other carbon sources has been reported, although these carbon sources supported excellent growth [14].

Although molasses as a carbon source resulted in high naringinase production, it was not selected as the main carbon source, as it also produced a thick brown color, which hindered the estimation of microbial growth during fermentation runs. For all subsequent experiments, sucrose was used as the primary carbon source. In order to determine the optimum concentration of sucrose for enzyme production, different concentrations (5–30 gl^{-1}) of sucrose were used in the medium. With increased sucrose concentration, naringinase production increased (5.7 IU ml^{-1}) upto15gl^{-1}sucrosein the medium and thereafter declined. In one of the studies, rhamnose has been used as the sole carbon source in the induction of microbial rhamnosidase by *Burkholderia SP.* [15]

According to Pavithra et al. [8] glucose, Rhamnose and sucrose were the 3 different carbon sources studied. Inclusion of glucose in the medium resulted in the peak production of 5 U/L after 36 h of fermentation with 4 g/L biomass production. Sucrose showed a moderate naringinase yield (3.44 U/L) with high biomass production (5.6 g/L) after 36 h of fermentation. Rhamnose inclusion resulted in the reduction of enzyme productivity.

Optimization of nitrogen source of the medium i. e. NH_4NO_3 is varied at different concentration. The result shows that the enzyme production was increased with increase in NH_4NO_3 concentration from 0.25% (w/v) to 0.50% (w/v); thereafter the enzyme production was decreased. Therefore, 0.50% (w/v) of NH_4NO_3 concentration shows maximum naringinase production and the naringinase activity was 9.7 IU. The results were depicted in fig. 6.

Purification of Naringinase enzyme

The protein concentration of the culture supernatant was estimated by Lowry method (Lowry et al. 1951) [16]. The enzyme was purified by using ultrafiltration, Ammonium sulfate precipitation and Dialysis and detected by using SDS-PAGE and has been found that the molecular weight of the enzyme was 48 kDa.

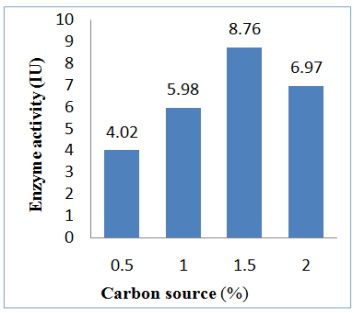

Fig: 5. Optimization of carbon source

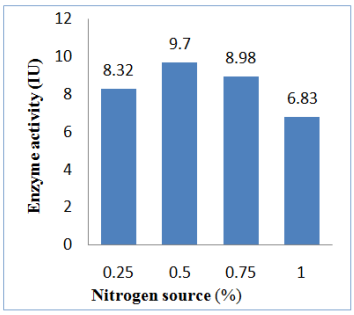

Fig: 6. Optimization of nitrogen source

CONFLICT OF INTERESTS

Declared None

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