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Optimization of environmental parameters for enhancement of naringinase production of *Bacillus cereus-K1* a bacterial strain

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Abstract

The naringinase is one of the highly valued enzymes used in a wide range of industrial applications in food technology and pharmaceutical industries. Naringinase has become biotechnologically important due to its role in debittering of citrus fruit juices, in the manufacture of rhamnose, preparation of pruning, biotransformation of antibiotics and also to improve the properties and stability of wine. The present study investigates the optimization of environmental parameters for enhancement of naringinase production of *Bacillus cereus*-K1 bacterial strain. *Bacillus cereus*-K1 was used to produce extracellular naringinase in a basal medium. An initial medium pH of 6.0 and temperature 35^0C were optimal for enzyme production. Among various carbon and nitrogen sources used, starch and yeast extract was the most effective for enzyme yield.

Key word: *Bacillus*, basal, citrus, naringinase

1. Introduction

Microbial enzymes are widely used in various industrial processes nowadays. This makes them an issue of concern to researchers, especially when it comes to dealing samples from totally undiscovered areas because screening for enzyme activity of the microbes prevalent in the untapped virgin soil provides a lot of scope related to biological diversity and bio prospecting of that area. Most are derived from the soil microorganisms as they are a good source of enzyme production. In industrial Citrus fruit juices processing contain naringin (4,-5, 7-trihydroxyflavonone-7-rhamnoglucoside) that attributes bitterness to the juices (Thomas et al., 1958).Citrus fruit membrane major parts containing naringin, and when it is squeezed, the naringin is extracted into the juice. Naringin is abundant in immature fruit, but its concentration decreases during ripening. Natural grapefruit contains 0.017-0.025% naringin and the orange peel contains 0.036% naringin this reported shown to be responsible for making the juice too bitter (Yusof et al., 1990).). Naringin is a flavanone glycoside, a type of flavonoid. It is glycosylated by a disaccharide at position seven to give a flavanone glycoside. Since naringin is the main bitter component of kinnow juice, thus, its hydrolysis with a concomitant decrease in bitterness is of industrial importance. Presently most of the production goes for the fresh fruit market. It is notable that due to poor post-harvest infrastructure, wastage of citrus fruits is around 25-30% and that only a small percentage of the total production is processed due to its problem of bitterness. Naringinase is a debittering enzyme that is used in commercial production of grapefruit juice. The naringin level can be reduced by technologies such as adaptive debittering, chemical methods and treatments with polystyrene divinyl benzene styrene (DVB) resins, polyvinyl alcohol, supercritical carbon dioxide extraction, etc. (Tsai S-Y 1988; Mishra et al., 2003; Vila-Real et al., 2007; Ferreira et al., 2008;). A suitable bitterness can be achieved by treating the juice with an enzyme known as naringinase, which directly hydrolyzes naringin. Naringinase is commercially attractive due to its potential usefulness in pharmaceutical and food industries. It is of particular interest in the biotransformation of steroids, antibiotics, and mainly on glycosides hydrolysis. The deglycosylation of glycopeptide antibiotics, flavonoids, or glycolipids has been achieved successfully. Moreover, it has been used in Citrus juices debittering and wine industries (Marques et al., 2007; Ribeiro et al., 2008; Amaro et al., 2009; Saerens et al., 2009). Different natural glycosidase, which includes naringin, rutin, quercitrin, hesperidin, diosmin, and terphenyl glycosides containing terminal α-rhamnose and β-glucose can act as a substrate of naringinase. These substrates have great potential functional chemicals with important properties in the fields of healthcare, food, and agriculture. Biochemically, the naringin, the bitter component of certain fruits has sugar complexes

(ά-L-rhamnose and β-D-glucose) and an aglycone (Naringenin) part. The β-D-glucosidase (EC.3.2.1.23) and ά-L- rhamnosidase (EC.3.2.1.40) are collectively known as Naringinase. The enzyme ά-L- rhamnosidase acts on sugar complex, release prunin and rhamnose whereas the β-D-glucosidase acts on prunin to release naringenin and glucose.

Naringinase has been isolated from various sources such as plants, animals, and microbes. However, the microbial sources only are practically suitable for the production of naringinase at a commercial level. So, industrial production of naringinase mostly come from microorganisms (Puri and Banerjee 2000; Ribeiro et al., 2010).

Thus, the production of the enzyme at industrial scale is expected to curtail good amount of resources draining out of the country as well as for promoting the development of new industries related to naringinase enzyme. The present study investigates the optimization of environmental parameters for enhancement of naringinase production of *Bacillus cereus*-K1 a bacterial strain

2. Material and method:

Naringin was obtained from Sigma, St. Louis, USA. Different growth factors and organic and inorganic nitrogen sources were obtained from Hi-Media, laboratories, Mumbai, India. All other reagents used were of analytical grade.

3. Culture medium and cultivation conditions

Bacillus cereus-K1 was isolated from soil samples of Dibrugarh Districts. The sequence has been submitted to Gen Bank (NCBI) and its accession number is MH938327.1. The strain was nutrient agar infused with 0.01% naringin as maintenance medium for naringinase producing isolates. The subculture of the pure isolates was done at every one month consequently. Naringinase production in basal medium composition in g/l: $NaNO_3$ 5, KCL 0.3, KH_2PO_4 0.5, $MgSO_4.7H_2O$ 0.2, NaCl 0.05, Fe Cl3, ZnSO4 0.001, Yeast extract 3, sucrose 5 and naringin 2. The pH of media is adjusted to 5.5 before sterilization.

4. Naringinase enzyme assay

The Assay of naringinase was carried out according to the method of the Davis method (1947) using naringin as substrate. Naringin (0.50 ml of 0.1%) in 0.01 M acetate buffer (pH-4.0) was made to react with 0.1 ml of naringinase enzyme for 50 min at 60°C. From the reaction mixture, 0.2 ml was taken and mixed with 5 ml of 90% diethylene glycol and 0.2 ml of 4N NaOH. The assay mixture was kept for 10 min at room temperature. The resulted yellow color developed after than measured at 420 nm. Determination of enzyme activity was calculated using naringin as a standard substrate. One unit (IU) of enzyme activity was calculated as the amount of enzyme that could hydrolyze 1µmol of naringin/ml in min at the assay conditions.

5. Optimization of environmental parameters of enhancement of naringinase producing most potentials isolates

The Erlenmeyer flasks (250 ml volume capacity) each containing 100 ml the basal medium are sterilized at 15 psi and 121^0C for 15 minutes. Optimization of enzyme production in shake flask in different incubation periods 12-120hr, pH range 4.0-9.0, temperature range 20^0-70^0C , different carbon sources (5%), diffenrt nitrogen sources, (3%) were added to the basal medium before autoclaving. 3% fresh inoculum are used in shake flask at orbital shaker (RIMI) 150rpm at 35^0C .

6. Statistical Analysis.

Data presented to the average of three replicates (±SE) are obtained from their experiments.

7. Results

7.1 Optimization of incubation periods

The duration of incubation plays an important role in the production of naringinase .To study the optimal incubation period for maximum naringinase production the flasks with a basal medium at pH 6 were inoculated and incubated at 35^0C at orbital shaker.

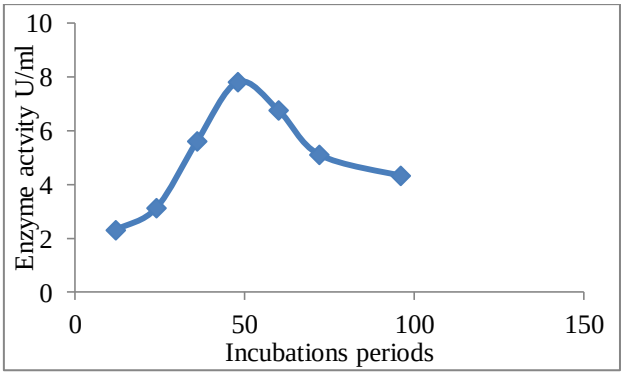

Fig. 1. Optimization of incubation periods by *Bacillus cereus*-K1 in basal medium

In this study best incubation periods was found 48h and maximum naringinase production (7.8 U/ml±0.08). Above 48h incubation periods than reduce the enzyme activity (Fig. 1). *Bacillus methylotrophicus*, a soil bacterium was naringinase production found in (12.05±0.03 U/L) 34 h incubation periods in bioreactor studies (Mukund et al., 2014). According to Saranya (et al., 2009) the protein concentration of the enzyme was

estimated and the maximum protein concentration and activity for naringin hydrolysis was observed high on Day 5 at the same condition with the present work.

7.2 Optimization of temperature:

Optimization of various temperatures in *Bacillus cereus*-K1 we have found 35^0C was (8.54±0.05 U/ml) (fig 2) maximum naringinase production. Enzyme activity if the temperature increases, initially the enzyme activity increased while above 35^0C enzyme productivity was decrease at higher temperatures at the same time. The decrease in enzyme activity may be the deactivation of enzyme due to the weakening of non-covalent interactions of the protein structure. Optimum temperatures of naringinase production in *Aspergillus niger* 30^0 (Puri et al., 2010), *Penicillium decumbens* 30^0 (Ribeiro and Ribeiro 2008) are reported. Above 35^0 C of temperatures in media bacterial cells are disrupt the shape of the active site of the enzyme, which will decrease its activity, or stop it from working. Then enzyme will have been denatured.

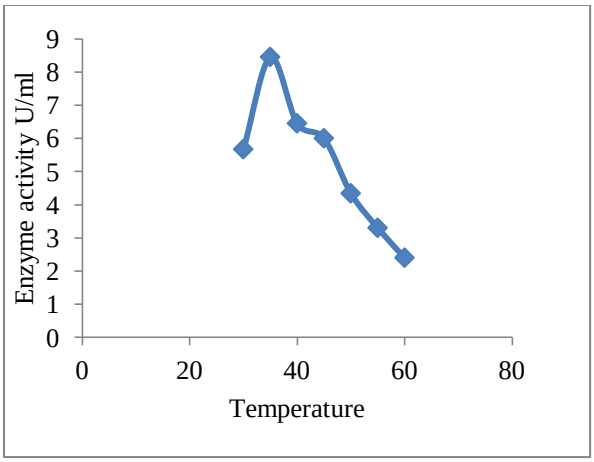

Fig. 2 Optimization of temperature by *Bacillus cereus*-K1 in basal medium

7.3 Optimization of pH

The effect of pH on naringinase activity was determined in different pH values ranging from 4.0 to 9.0. Production naringinase in pH was significant effect of *Bacillus cereus*-K1 in shake flask at orbital shaker. We have observed pH 6.0 was maximum naringinase production (9.2±0.05U/ml) in a basal medium. In the present study, increase in pH from 4 to 9 was go with by increase in naringinase activity elsewhere which the naringinase activity decreased making 9 the optimum pH for naringinase activity as seen in the fig (3). Similar results obtained maximum naringinase activity of was observed in pH 5.5 *Staphylococcus xylosus* MAK2 (Puri et al., 2010).

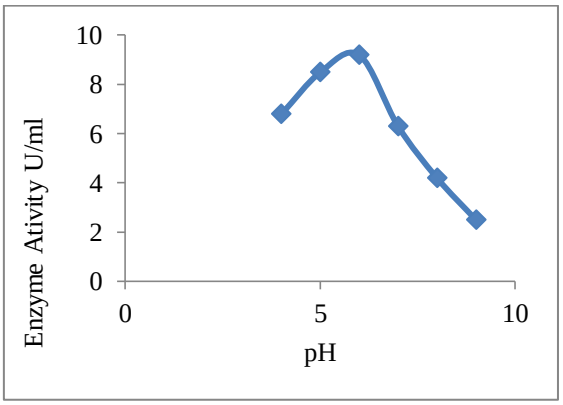

Fig. 3. Optimization of pH by *Bacillus cereus*-K1 in basal medium

7.4 Optimization of carbon source:

Different carbon sources (5%) were used in a basal medium for naringinase production. The maximum naringinase (10.56 ±0.02U/ml) was found when starch as used carbon source as compared to control. In this study fructose is low activity of enzyme production as compared to control. Various authors reported Corn steep liquor, (Bram et al., 1965) Molasses (Puri et al., 2005), Rhamnose (Thammawat et al., 2008**)**, Glucose and Sucrose (Mukund et al., 2014), as best carbon source in their medium for enzyme production.

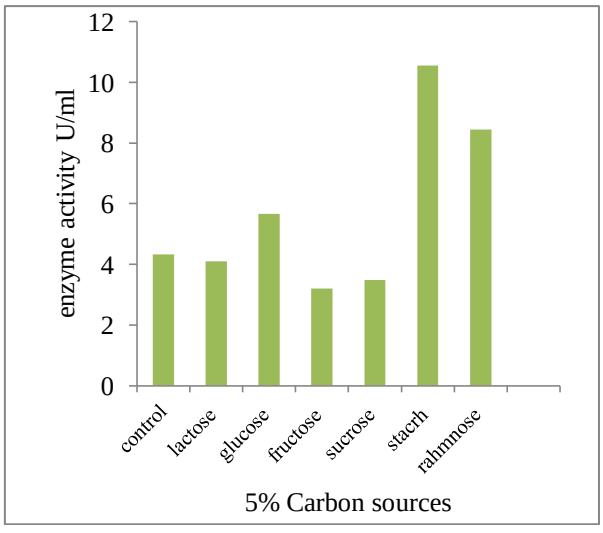

Fig.4.Optimization of carbon sources by *Bacillus cereus*-K1 in basal medium

7.5 Optimization of nitrogen sources

Nine different of nitrogen sources were used in production medium, in this study we have found yeast extract as a best nitrogen sources was found (9.4±0.04 U/ml) maximum naringinase activity in production medium. Other nitrogen sources also good amount of naringinase activity as compared to control (fig5). Similar results reported the (Bram et al., 1965) yeast extract was best nitrogen sources in shake flask method in production of naringinase. Previously various authors reported the optimization nitrogen sources they establish peptone (Puri et al., 2005), Soya peptone (Thammawat et al., 2008).

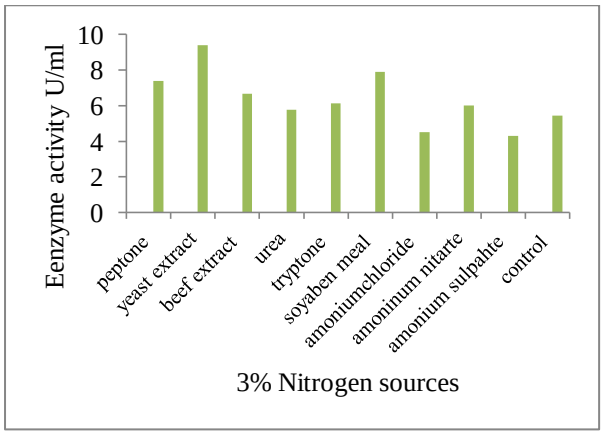

Fig 5.Optimization of various nitrogen sources by *Bacillus cereus*-K1 in basal medium

8.Conclusion

This *Bacillus creeus*-K1 bacterial strain was produces a significant amount of extracellular naringinase in the basal medium. The various environmental parameters were optimized in shake flasks for enhancement of naringinase production in basal medium. During investigation, we observed the 48 hours incubation periods were best time of production of naringinase in basal medium. Optimum pH 6 and temperature 35^0 C were of optimal for naringinase enzyme production in medium. Among the various carbon and nitrogen sources were found to enhance enzyme production. During the investigation starch and yeast extract were the most effective for naringinase enzyme production in shake flask method. This isolated could be potential biotechnological applications in citrus processing and bioprocess industries. The enhanced productivity of naringinase from potential isolate *Bacillus cereus*-K1 strain could be development of cost-effective, environmentally friendly as well as healthy technology for optimum utilization of local bio resources will enhance the commercial value of the low-cost of substrate locally available in the region. This enzyme-based technology may emerge as an easier and cost effective process for profitable business among the country. Further work of purification and characterization of enhancing naringinase production are going on.

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