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Impact of submerged culture different parameters on growth and naringinase activity of *Bacillus amyloliquefaciens* D1 isolated from citrus gardens of North-East India

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ABSTRACT

The study aims at screening, isolation, and characterization of naringinase enzyme producing bacterial strains from soil samples. In this study, 45 bacterial isolates were screened for naringinase activity, and among them, 20 bacterial isolates are found to be capable of naringinase production in liquid mineral medium. Bacterial isolate D1 exhibited the highest naringinase activity in liquid mineral medium. Based on the morphological, biochemical, fatty acid profile, and molecular characterization, bacterial isolate D1 was identified as *Bacillus amyloliquefaciens*. Impact of different submerged culture parameters were evaluated in different experimental conditions. The study revealed that 48 hrs of incubation periods, pH 6.0 and temperature 35^0C is optimal for naringinase activity when the culture medium is supplemented with starch and yeast extract as carbon and nitrogen sources. Thus, it can be concluded that due to the potential naringinase activity of the strain *Bacillus amyloliquefaciens* D1, further the identifying strain may be used in citrus processing and bioprocess industries upon large scale field applications.

Key word: *Bacteria, Bacillus amyloliquefaciens, Citrus, Naringinase, Optimization*

Introduction

Naringinase is one of the highly valued enzymes used in wide range of different biotechnological industries including debittering citrus technology, food industry and pharmaceutical industry (Kumar *et al.,* 2021). Sporadic works have been reported from across the world on the scope, isolation, and characterization of naringinase enzymes from various sources special reference to the microorganisms (Quinter *et al.,* 2023) The enzyme has a wide occurrence in nature and has been reported from animal tissues, plants, yeasts, fungi, and bacteria (Pegu *et al.*, 2021)

The North-eastern region of India being rich biodiversity zone harbors innumerable unexplored fauna and flora of economic importance. Many scientific reports are available on the use of bio-resources having industrial and pharmaceutical significance. However, major portions of the bio-resources available in the region have remained unexplored (Purkayastha, 2016).

Thus, the production of the enzyme at the industrial scale is expected to curtail a good amount of resources draining out from the country as well as for promoting the development of new industries related to the naringinase enzyme. This could be done 1) by screening and use of new more potential

naringinase producing microbial strains or 2) by optimizing the culture conditions of selected isolates for commercially viable naringinase production. Thus, this report deals with the isolation and screening of potential naringinase producing microbes from soil samples of Dibrugarh districts in upper Assam.

Materials and Methods

Soil samples

Soil samples collected in hygienic condition from different citrus gardens, citrus fruit markets, tea garden, kitchen outlet, and paddy field of Dibrugarh Districts, Assam as per the standard procedure describe by (Coale, 2009).

Isolation

One gram of soil sample serially diluted in sterile water and then inoculated into a selective medium having the composition (L^{-1}) NH_4NO_3 5.0 g, KCl 0.2 g, KH_2PO_4 0.4 g, $FeSO_47H_2O$ 0.01 g, $ZnSO_4$ 0.01 g, $MnSO_4$ 0.01 g, Agar 15.0 g and $MgSO_47H_2O$ 0.2 g, and Naringin 1.0 g (Sigma Aldrich, USA) and its initial pH was 6.0 at 30 ^{0}C. Distinct colonies were picked up and were transferred to nutrient agar plate. Naringinase producing isolates were maintained in nutrient agar medium infused with 0.01% naringin.

Assay for naringinase activity

The bacterial isolates were inoculated in liquid mineral medium with a composition (L-1) KCL 0.3 g, KH_2PO_4 0.5 g, $MgSO_4.7H_2O$ 0.2 g, NaCl 0.05 g, ZnSO4 0.1, $NaNO_3$ 5.0 g, 3.0 g, and naringin 2.0 g at pH 5.5. Inoculation was done from 24 h fresh culture in 100 ml mineral media contained in 250 ml Erlenmeyer flasks incubated at 30 ^{0}C in 180 rpm in a rotary shaker for one week. Then the culture broth was centrifuged at 10000×g for 150 RPM on 10 min at 4 ^{0}C and cell-free supernatants were collected and used for determination of naringinase activity. The naringinase enzyme activity of the isolates was done according to the (Davis 1947) utilizing naringin as a substrate. Then, 0.50 ml of 0.1% Naringin prepared in 0.01 M acetate buffer (pH 4.0) to react with 0.1 ml of naringinase enzyme for 50 min at 60 ^{0}C. From the reaction mixture, 0.2 ml was taken and mixed with 3 ml of 90% diethylene glycol and 0.1 ml of 1N NaOH. The assay mixture was kept for 10 min at room temperature and absorbance of resulted yellow color was recorded at 420 nm. The naringinase enzyme activity was estimated by calibrating with a standard curve prepared from the serial concentration of authentic naringin. One unit (IU) of naringinase enzyme activity was determined as the measure of an enzyme that could hydrolyze 1 µmol of naringin/ml in min at the assay conditions. Each treatment was replicated thrice and data were expressed as the mean value ± standard error.

Morphological and biochemical characterization

Various morphological characterizations and Gram's reaction of the potential bacterial isolates were performed through microscopic examination, whereas biochemical tests were performed by using standard protocols of Bergey's Manual of Determinative Bacteriology (Buchanan *et al.,* 1970).

16S rDNA sequence analysis and Identification

Molecular identification of D1 bacterial isolate was carried out by 16S RNA sequencing and homology study analyzed and edited with Bio Edit (Hall, 1999). Isolation of DNA of the bacterial isolate D1 was done by standard methodology (Weisberg *et al.,* 1991). Naringinase producing bacterial genomic DNA was extracted from the potential isolates using QIAGEN (CA, USA) DNA extraction kit, and its concentration was determined using a UV–Vis spectrophotometer (NanoDrop 2000). The PCR product was purified using a QIAGEN PCR purification kit and sequenced using ABI 3730 capillary sequencer (16 capillary) with big-dye terminator reaction.

Phylogenetic tree analysis

The bacterial isolate D1 sequence files were checked for quality filtering and assembled using Codon Code Aligner (Richterich, 2004). The assembled sequence was identified using BLAST program against NCBI-nr/nt database and taxonomic profiling was done based on the identity percentage (> 97%). Sequence Alignment was performed using CLUSTAL W (Thompson *et al.,* 1994).The evolutionary relationship was determined by the neighbor-joining phylogenetic tree (Saitou *et al.,* 1987). The base substitution was calculated based on Juke-cantor one-parameter model with 1000 bootstrap values in MEGA 6.0 (Tamura *et al*., 2013). Identified sequences were compared with the reference sequence from the NCBI nucleotide database.

Fatty Acids profiling

The fatty acid methyl ester (FAME) profile was analyzed to identify the most potential naringinase producing isolate using the Sherlock-Midi system as per the methods of (Buyer, 2002). In preparation for FAME analysis, frozen bacterial isolates were streaked on solidified media, incubated 24-48 hr at 28 ^{0}C, and rechecked for purity. Identification and comparison were made to the Aerobe (TSBA version 3.9) database of the Sherlock Microbial Identification System. The Dendrogram program of the MIDI software package was used to produce un-weighted-pair matching based on fatty acid compositions.

Impact of submerged culture parameter on growth and naringinase activity potential isolates

The optimal incubation period to maximum naringinase production was estimated by incubation 12-96 hr of in liquid mineral medium. Study the effect of incubation temperature for maximum naringinase production was done by incubating the fresh bacterial culture flasks at various temperatures ranging from 25 ^{0}C to 60 ^{0}C. The impact of pH on naringinase activity was determined in different pH range from 3.0 to 9.0. The initial pH of the culture medium was adjusted by using 1N HCl or 1N NaOH by bacterial cultures were incubation in an orbital shaker (REMI). Different carbon sources glucose, fructose, sucrose, starch, sorbitol, and maltose were supplemented in the liquid mineral medium at 5.0 g L^{-1} concentration. The liquid mineral medium was supplemented with different nitrogen sources like yeast extract, peptone, urea, ammonium sulfate, casein meal, and soybean meal at 3.0 g l^{-1} concentration.

Statistical analysis

The arithmetic mean of three independent replications are calculated and tested for standard deviation. Determination of standard error of the result was carried out by the Statistical Analysis System (SAS) with the help of statistical software programme 'SPSS version 16'.

Results and Discussion

Isolation

A total of 45 bacterial isolates were isolated from the soil samples in the selective medium in presence of naringin as the sole carbon source. Isolates were checked for purity and characterized morphologically as well as biochemically as per standard protocol. The quantitative assay of the isolates for naringinase activity was carried out in a liquid mineral medium. Among the isolates, twenty bacterial isolates were capable of producing the naringinase enzyme by hydrolyzing naringin in liquid mineral medium (Table 1). The bacterial isolate no. D1 showed consistently highest naringinase activity (14.17 U ml^{-1}) in comparisons to others, which was followed by isolate K1 (12.40 U ml$^{-1)}$ and D5 (5.09 U ml-1). Naringinase activity exhibited by the rest of the isolates was not significant, whereas minimum activity was found in isolate T1 (0.59 U ml^{-1}) and K3 (1.39 U ml^{-1}). Based on this quantitative efficiency, isolate no. D1 was selected for further study. This

Table 1. Naringinase activity of the bacterial isolates U mL^{-1}

S/N	Isolates code	Enzyme activity (U ml^{-1})	S/N	Isolates code	Enzyme activity (U ml^{-1})
1	D1	14.177 ± 0.02	11	KO2	5.09 ± 0.03
2	D5	5.09 ± 0.004	12	TK1	5.58 ± 0.01
3	K1	12.4 ± 0.003	13	TK2	3.63 ± 0.016
4	K3	1.39 ± 0.002	14	PD1	3.714 ± 0.017
5	K5	2.7 ± 0.003	15	PD2	3.54 ± 0.13
6	T1	0.59 ± 0.001	16	OC1	3.87 ± 0.033
7	T2	2.09 ± 0.024	17	OC 3	7.97 ± 0.033
8	B3	5.33 ± 0.006	18	TG1	2.7 ± 0.003
9	B4	3.57 ± 0.003	19	TG2	3.87 ± 0.033
10	KO1	2.99 ± 0.002	20	TG3	4.56 ± 0.013

Results represent the average ±SD of three replicates.
Sample code- D- Duliajan citrus garden soils, K- Khuwang soil, T- Tengakhat soil, B- Borborua soil, KO-Kitchen outlet, TK-Tingkang soil, PD-Paddy field soil, OC-Orchard garden, TG- Tea Garden Soil.

bacterial strain D1 was isolated from the soil sample collected from a citrus garden located at Duliajan, Assam.

Identification of isolate D1

The most potential naringinase producing isolate D1 was inoculated in Nutrient Agar (NA) medium at 30 ^{0}C for 24 hr and observed under in microscope following Gram staining procedure. It was a gram-positive, rod-shaped spore-forming bacterium, and the colony surface was found to be white and smooth. Isolate D1 is an aerobic bacterium that can grow up to a temperature range of 50 ^{0}C (Table 2). Based on biochemical characterization it was found to be positive in the hydrolysis of starch and production of catalase, nitrate reductase, oxidase, methyl red, gelatin liquefaction, fructose, D-glucose and sucrose whereas showed negative Indole test, citrate, Voges-Proskauer, urea hydrolysis, and phenylalanine.

Fatty acids profiling

Analysis of the whole-cell fatty acid profile of the bacterial isolate D1 showed the presence of different types of saturated and unsaturated fatty acids were documented (Table 3). The most dominant fatty acids detected in the strain were 12-Methyltetradecanoic acid (16.47%), Octadecadienoic (12.47%), 3-Hydroydecanoic acid (11.60%) and 14-Methylhexadecanoic acid (10.26%), which together comprises around 50% of total fatty acids. Some other minor fatty acids present in the strain are 8-Methyldecanoic acid (8.24%), 10-Methyldodecanoic acid (6.01%), Hexadecanoic acid (5.84%), 13-Methyltetradecanoic acid (4.16%), (9Z)-9 Octadecadienoic (4.07%) and 9z, 12, 12Z)-6, 9, 12-Octadecadienoic acid (3.99%). A similar kind of fatty acid profiling has been reported from different species of the genus *Bacillus* (Kaneda *et al.,* 1967) which indicates that naringinase producing bacterial isolate D1 belongs to the genus *Bacillus*.

16S rRNA gene analysis and homology

The 16S rRNA gene (1452 bp) of the strain D1 was amplified and sequenced using respective primers through the courtesy of Agrigenome Labs Pvt. Ltd., Kerela. A comparison of these test sequences against the NCBI GenBank database was performed with BLAST. BLAST result showed that the 16S rDNA sequence of isolate D1 has got the highest degree of homology with *Bacillus amyloliquefaciens* strain BCRC 11601 (98.56%), *Bacillus amyloliquefaciens* strain NBRC 15535 (98.56%), and *Bacillus licheniformis* strain ATCC14580 (96.77%). Subsequently, 16S rDNA gene sequence was aligned and the phylogenetic tree was constructed, which shows the relationship between the isolate D1 and the closely homologous group of bacteria (Fig. 1). Based on the 16S rDNA homology to *B. amyloliquefaciens*, the potential naringinase producing bacterial isolate D1 was referred to as *Bacillus amyloliquefaciens* strain D1 with NCBI Gene Bank accession number MK334656.1

Impact of culture parameters on enzyme activity

Incubation periods

The optimum incubation period for the highest growth and naringinase enzyme activity was determined. The broth cultures were incubated up to 96 hr and growth as well as enzymatic activity was assessed in every 12 h. Incubation up to 48 hr was found to be optimum for growth (measured as OD) as well as naringinase activity (9.86 U ml^{-1}) in mineral medium (Fig. 2). Maximum growth and naringinase activity were recorded just after the isolate D1 reached its stationary phase and remaining almost constant up to 72 hr and then activity re-

Table 2. Biochemical characterization of potential bacterial isolate D1

Test	Result	Test	Result
Aerobic	+	Voges-Proskaue	-
Growth temperature	20-50 ^{0}C	Citrate utilization	+
Catalase	+	Fructose	+
Nitrate reduction	-	D-glucose	+
Oxidase	+	Gelatin liquefaction	+
Starch hydrolysis	+	Urea hydrolysis	-
Indole	-	Sucrose	+
Methyl red	+	Phenylalanine	+

'+': positive activity '-': negative activity

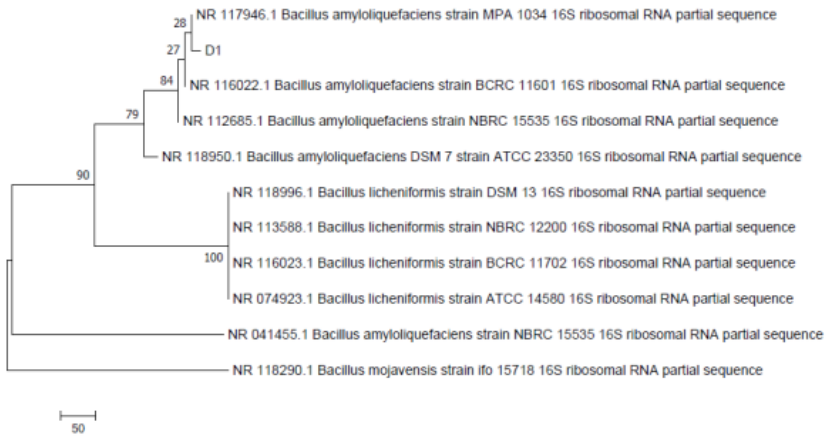

Fig. 1. Phylogenetic relationship of 16S rDNA sequence of the strain *B. amyloliquefaciens* D1

duces.

Effect of initial pH

Variation in the initial pH of the medium exhibited significant effects on growth and naringinase activity by *B. amyloliquefaciens* strain D1. The maximum rate of naringinase activity (9.34 U mL-1) and growth (0.620) obtained at pH 6.0 (Fig. 3). However, naringinase activity found at par in slightly acidic pH like 5.5 (8.64 U mL-1) and 5.0 (7.92 U mL-1). Neutral pH also supports the growth (0.365) and naringinase production (5.4 U ml-1) of the strain D1. No growth was observed at pH <3.0 and pH >11.0. Below or above this pH, there was a radical reduc-

Table 3. Fatty acids profiling of *B. amyloliquefaciens* D1

Fatty acids	IUPAC/Systematic Name	D1 Concentration (%)
11:0 ANTEISO	8-Methyldecanoic acid	8.24
10:0 2OH	2-Hydroydecanoic acid	3.3
10:0 3OH	3-Hydroydecanoic acid	11.60
12:0 ANTEISO	9-Methylundecanoic acid	1.88
12:0	Dodecanoic acid	1.18
13:0 ANTEISO	10-Methyldodecanoic acid	6.01
14:0 ISO	12-Methyltridecanoic acid	1.14
14.0	11-Methyltridecanoic acid	1.82
15:1 ISO W9C	5(Z)-13-Methyl-5-Tetradaecenoic acid	2.07
15:0 ISO	13-M ethyltetradecanoic acid	4.16
15:0 ANTEISO	12- M ethyltetradecanoic acid	16.47
15:1W5C	(10z)-10-Pentadecenoic	1.32
16:1W7C ALCOHOL	(9Z)-9-Hexadecen -1-ol	0.68
16:0 Aldehyde	Palmitaldehyde	0.82
16:0 ISO	14-Methylpentadecanoic acid	2.12
16:0 ANTEISO	13- Methylpentadecanoic acid	0.61
16:0 1WIIC	(11Z)-112-Hexadecenoic acid	0.33
16:0	Hexaecanoic acid	5.84
15:0	Pentadecanal	1.12
17:1 ANTEISO W9C	(8Z)-8-Hepatadecenoic acid	1.42
17:0 ISO	15-Methyhexadecanoic acid	3.06
17:0 ANTESIO	14- Methyhexadecanoic acid	10.26
18:3 W6C	(9z,12,12Z)-6,9,12-Octadecadienoic acid	3.99
18:1W9C	(9Z)-9 Octadecadienoic	4.07
18:0	Octadecadienoic	12.47
19:0	Nanadecanoic	1.21
20.1 W4C	(16Z)-16-Icosenoic acid	1.45

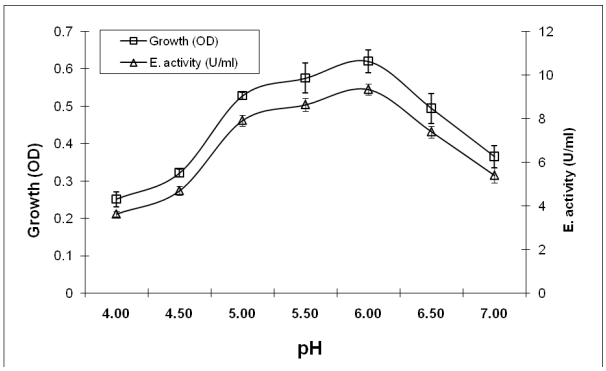

Fig. 2. Effect of incubation period on growth and naringinase activity of *B. amyloliquefaciens* strain D1

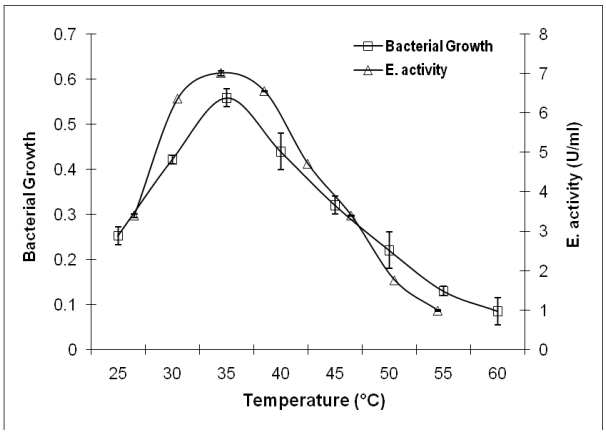

Fig. 3. Effect of pH on growth and naringinase activity of *B. amyloliquefaciens* strain D1

tion in naringinase activity. Therefore, medium pH is very important in nutrient absorption, growth of bacteria, biological functions and encouragement of enzyme production.

Effect of temperature

The temperature required for optimum naringinase activity was recorded ranging from 25 ^{0}C to 60 ^{0}C. Maximum growth (4) and naringinase activity (7.01 U mL-1) by *B. amyloliquefacien*s strain D1 was recorded at incubation temperature 35^{0}C (±2) and it was followed by 30 ^{0}C, 40 ^{0}C, respectively (Fig. 4). An exponential growth pattern was recorded at a temperature between 10 ^{0}C to 35 ^{0}C, while growth ceased at < 10^{0}C and > 45^{0}C. The study proved that low temperature may cease the metabolic activity of the bacteria and high temperature kills the bacterial cells. An increase in temperature thereafter caused a reduction in enzyme activity. However, optimum temperatures for longer incubation periods are favorable so as to reduce the cost of enzyme production.

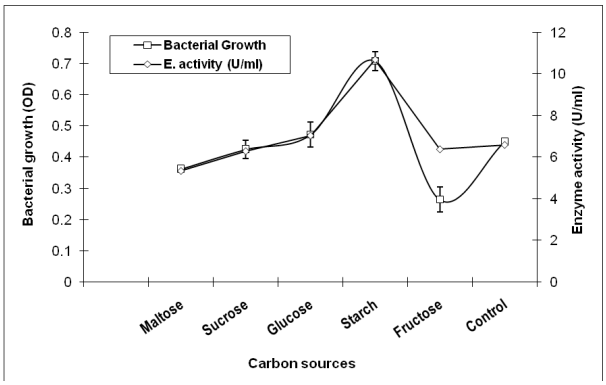

Fig. 4. Effect of temperature on growth and naringinase activity of *B. amyloliquefaciens* strain D1

Effect of carbon (C) sources

A statistically significant higher growth and naringinase production (10.7 U ml^{-1}) at 48 hr were exhibited when starch (5 g l^1) was supplemented as sole carbon source. Other carbon sources, like fructose (6.39 U mL^{-1}) and Glucose (7.05 U ml-^{1}) also supported naringinase production, whereas minimum naringinase activity was observed when maltose and sucrose were supplemented as carbon source (Fig. 5).

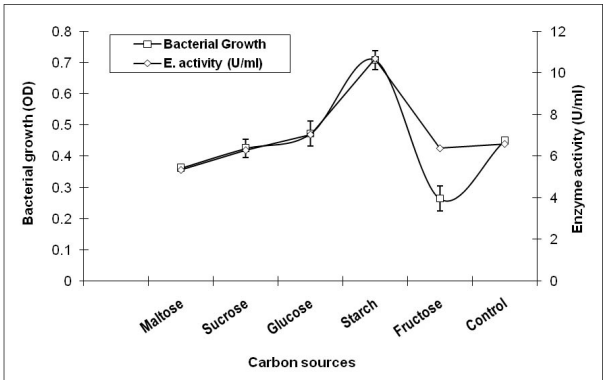

Fig. 5. Effect of carbon source on growth and naringinase production of *B. amyloliquefaciens* strain D1

Effect of nitrogen sources

Nitrogen is secondary energy sources which play an important role in the growth and metabolic activity of microorganism. Yeast extract and peptone exhibited the highest naringinase activity (11.43 and 9.3 U ml-1) after 48 hr fermentation (Fig. 6). The lowest naringinase activity was recorded in ammonium nitrate in the medium. The enzyme production

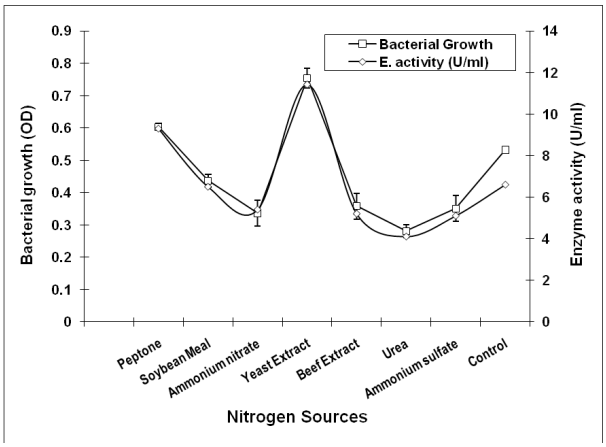

Fig. 6. Effect of nitrogen source on growth and naringinase production of *B. amyloliquefaciens* strain D1

reached the maximum when yeast extract was used as the nitrogen source. This may be due to other nutrients and growth enhancers present in the liquid mineral medium.

Discussion

In this study, 45 bacterial isolates were screened for naringinase activity, and among them, 20 bacterial isolates were capable of naringinase production in liquid mineral medium. Bacterial isolate D1 exhibited the highest naringinase activity in liquid mineral medium. Based on the morphological, biochemical, fatty acid profile and molecular characterization of bacterial isolate D1 was identified as *Bacillus amyloliquefaciens* strain D1. Bacterial isolate no. D1 showed consistently highest naringinase activity (14.17U ml-1) compared to other bacterial isolates. Previously different kind of microbial strains naringinase production were reported by *Bacillus methylotrophicus*- PMY (3.44 U mL-1) (P. Mukund *et al*., 2014), ***Bacillus*** *subtilis* (7.09 ± 0.09 U ml-1) (Selim *et al*., 2023), *Aspergillus flavus* (5.55 U ml-1) (Sindhe *et al.*, 2023), *Serratia marcescens* C10 (30.81 U/ml) (Chen *et al.,* 2023). *Bacillus* is generally considered dynamic microorganism that can survive in antagonistic environmental conditions and grow easily to very high densities. *Bacillus* has been recognized for its biotechnological applications on a large scale (Elshaghabe *et al*., 2017; Zhu *et al*., 2017)

Here we have 48 hr incubation periods showed for naringinase growth and production in minerals medium (Fig. 2). The reduction in production time is important because it decreases the fermentation costs and contamination with opportunistic microorganisms during the upscale of the process ((Chen *et al*., 2023)). Previously *Aspergillus niger* MTCC 1344 was found 72 hr old seed culture when used as inoculum age was, gave maximum naringinase production in the fermentation medium (Puri *et al.* 2005). Another author reported the *Bacillus methylotrophic* strain was 48hr incubation periods were gave better naringinase productivity during fermentation periods (Mukund *et al.,* 2014). Microbial naringinase production has been influenced by temperature. From (Fig. 4) it was found maximum naringinase production was recorded the 35^{0}C by this D1 strain. Similar result found *Aspergillus foetidus, Aspergillus niger* was 35^{0}C (Mendoza-Cal *et al.,* 2010). Optimum temperatures for naringinase production for others microbes was noted at temperature are *Staphylococcus xylosus* MAK2, (30 ^{0}C) (Puri *et al.,* 2011), *Aspergillus niger* VB07 (28 ^{0}C) (Pavithra et al. 2012), *Micrococcus sp.* (37 ^{0}C) (Amena *et al.,* 2015). Changes in pH have an influence on enzymes. The most favorable pH value is known as the optimum pH. It is the point that the enzyme is most active. A variety of amino acid residues as well as the carboxyl and amide termini of proteins has different pH ranges. As a result, a change in pH can protonate or deprotonate a side group thereby changing its chemical features (Saranya *et al*., 2009). Among different carbon, sources are enhancement of naringinase activity in liquid minerals medium. Starch was the best carbon sources are for naringinase production by this D1 strain (Fig. 5). This is could be reflective of the various physiological and biochemical parameters are variations of the bacteria *Bacillus sp.* and that the enzyme activities could be different with time and the available energy sources (Seiboth *et al*., 2002). Previously carbon compounds such as rhamnose and molasses exhibit high in the medium of naringinase activity (Pedro *et al.,* 2007). Repression of naringinase activity is exhibited by carbon sources such as glucose, sucrose, and citrate (Puri *et al.,* 2005). The production of naringinase is sensitive to the nitrogen source in the minerals liquid medium. The production medium was incorporated with different nitrogen sources to determine a suitable nitrogen source for naringinase production. In this study, yeast extract and peptone exhibited highest naringinase activity (11.43 and 9.3 U ml^{-1}) after 48 hr fermentation (Fig. 6).

In a similar study reported the yeast extract yeast

extract (Mukund *et al*., 2014) were able to increase the production of naringinase enzyme. Although peptone was the excellent source naringinase biosynthesis from *Aspergillus oryzae* JMU316 (Chen *et al*., 2010), *Aspergillus niger* MTCC 1344 (Puri *et al.,* 2005), *Aspergillus niger* VB07 (Kumar and Babu 2010).

One such resource is the enormously diverse microbial species with industrial applications. In Assam huge quantity of citrus fruits is produced from plants grown in semi-wild condition and goes spoiled due to lack of adequate provision for storage or sometimes also neglected due to the low quality of taste. In many cases, the poor taste of these citrus fruits is due to the presence of naringin, a flavonoid that imparts the bitter taste of the fruit juice. The enhanced productivity of naringinase from potential microbial isolate could be a cost-effective, environmental friendly as well as healthy technology for optimum utilization of local bio-resources will enhance the commercial value of the low-cost semi-wild Citrus fruits locally available in the region. Since there is ample scope for the small-scale citrus beverage industry in the North-eastern part of the country, this enzyme-based technology may emerge as an easier and cost-effective process for profitable business among the youth.

Conclusion

The bacteria as enzyme sources have many advantages that, the naringinase enzymes produced are normally extracellular, making easier production for the downstream process. Further, the development of economically feasible technologies for naringinase production and for the enzymatic hydrolysis of naringinase materials will enable to utilize the large quantities of the substrate such as the residues of both food industries and agriculture.

Declaration

Financial interests
The authors declare they have no financial inter-ests.

Conflicts of interest

The authors declare no conflict of interest

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