

Isolation and Molecular Characterization of the Naringinase Producing Micro-organisms for the Bio-transformation of Flavonoid

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Abstract

Naringin is a flavanone found in citrus fruits and vegetables which are responsible for the asperity, due to which citrus fruit are unable to consume and from which we are unable to get the valuable nutrition in our body. To overcome this asperity in citrus fruits, present investigation is carried out, which provides a detailed report of isolation and screening of naringinase producing micro-organisms from different samples collected from Kalyan -Karnataka region, India. A sum of around 50 strains were isolated from 20 different samples which showed the positive result for naringinase activity, among 50 strains obtained 42 strains are fungi and remaining 8 were bacteria which showed good to moderate results in preliminary screening by 1% FeCl₃ method, further 5 fungi isolates which showed a good naringinase activity in submerged fermentation were assayed by Spectrophotometric method. As a result, KLA-80 showed a maximum result with an activity of 559 U/ml. Further from the identification of the isolate KLA-80 by BLAST analysis found to be having similarity with *Aspergillus flavus* and the accession number of OQ152018 is obtained.

Keywords: *Aspergillus flavus*, Flavanone, Grape Fruit, Naringin, Naringinase and Naringenin

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INTRODUCTION

Citrus based fruits are popular all over the world with a production of over 100 million tons in every given year.¹ In India, North-eastern region is having a rich treasure of various citrus fruits, a numerous reservoir of citrus exists in wild and semi-wild form and it is dispersed in that region without any commercial value.² It stands at 3rd position after the production of mango and banana in India.³ The citrus fruit processing industries does not increased in India as compared to production due consumer awareness problems and the main problem is the bitterness of juices extracted from it.⁴ Citrus fruits have a compound flavanone neohesperidosides which is known to found in sour orange and other citrus varieties, which contain high level of naringin which is responsible for the bitterness of fruit,⁵ which is known to be present in grapefruits, buntan orange, natusdaidai.⁶

Naringin is a one of the major flavanone glycoside obtained from grapefruits and many more citrus fruits, it is relatable to almost all tasteless compounds like hesperidin which found in a sweet orange and lemons.⁷ The concentration of naringin flavanone in grape fruit is found to be around 0.017-0.025% and the orange peel has a concentration of 0.036% concentration of naringin which is accountable for the essence in making the juice bitter, about 50mg/l of naringin is responsible for imparting bitterness to the juice.⁸

The compound Naringin was first discovered by Devry (1857). It has a chemical formula $C_{15}H_{12}O_5$. Though at first it was considered to be a chalkonel it was finally proved to have the constitution of 5: 7: 4'-trihydroxyflavanone (I, R = H)⁹ (Figure 1).

Some conventional methods are used to decrease the concentration of naringin namely physical and chemical methods are applied for the removal of bitterness from the juices by Poly (styrene-co-divinylbenzene), resin, beta -D cyclodextrin bound polymer methods.¹⁰ The use of microbial enzymes in the textile and other chemical industries in processing is getting recognition worldwide because of their non-toxic and eco-friendly criterion.¹¹ Debittering the citrus juice using the naringinase enzyme is a budding approach, as it makes less damage to the nutritional quality and increases the palatability properties of the juices.

A large number of micro-organisms are isolated for the debittering of the citrus juices; specifically those micro-organisms have a naringinase enzyme which has the capability to degrade the naringin into a non-bitter compound although naringin is not like other common enzymes.¹²

Kishi (1955) has isolated 96 strains and it is found that *Aspergillus niger* can produce naringinase in significant quantity.¹³ Subsequently, other strains of *A. niger* were also identified for naringinase production such *A. niger* MTCC 1344,¹⁴ *A. niger* CECT 2088,¹⁵ *Bacillus cereus* K-1,¹⁶ *Aspergillus* sp. SK01,¹⁷ were reported as a good producer of naringinase enzyme. Further immobilization of naringinase by chitosan microsphere isolated from *Penicillium decumbens* was found to retain thermal stability of enzyme and also 88.1±28% of initial activity after 10runs of naringin hydrolysis from fresh grapefruit.¹⁸ While Thermogram analysis (TGA) showed that nano-encapsulated enzyme showed highest activity whereas the free enzyme loses its 92%

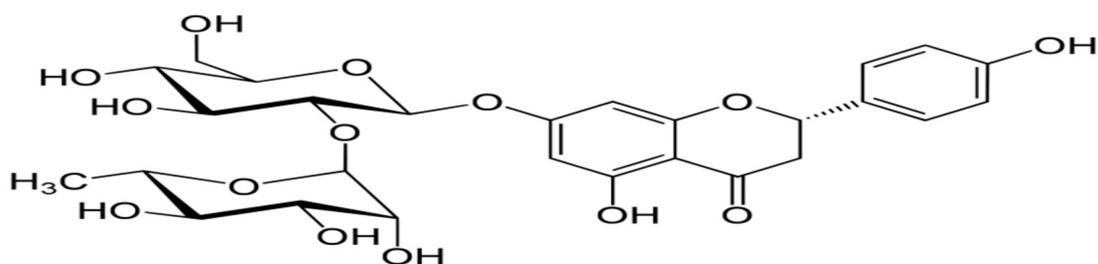


Figure 1. Structure of Naringin

of its weight at 110°C.¹⁹ From these studies it is found that immobilization of enzymes have gained importance nowadays in industries as it allows for the preservation enzymatic activity for a longer interval of time and ease in their duplicate uses.²⁰

Other than removal of bitterness from the citrus fruit, rhamnosidase activity of the enzyme in coalescence with arabinosidase and β -glucosidase was used for the enhancement of wine aroma.²¹ It also used in the metabolites bio-transformation such as antibiotics and steroids.²² Some studies also mentioned that naringinase have the capacity to produce specific fatty acids.²³ Rhamnosidase activity of the naringinase was found to have an important role in the softening of the viscous property of the gellan gum.²⁴

In the present investigation, isolation of naringinase producing micro-organisms from different types of samples and determination of the activity of the enzymes is checked along with the identification of the micro-organisms which have a highest activity of enzyme is determined and phylogenetic analysis of selected isolate is done.

MATERIALS AND METHODS

Chemicals and reagents

All the chemicals and reagents used in this experimental design were procured from HiMedia and SRL Ltd Mumbai, India.

Isolation of naringinase producing micro-organisms

A total of 20 different types of samples were collected from various regions of Kalyan-Karnataka. The samples were serially diluted using sterile distilled water. The medium used for the production of naringinase had the composition (g/l) sucrose, 30gm; Sodium nitrate, 4gm; Dipotassium hydrogen phosphate, 1gm; Magnesium sulphate, 0.50gm; Potassium chloride, 0.50gm; Ferrous sulphate, 0.010gm; Agar, 15gm and Naringin, 0.20gm pH at 6 at 30°C. The cultures showing good growth were transferred to selection media(g/l) NH_4NO_3 , 5gm; KCl, 0.2gm; KH_2PO_4 , 0.4gm; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01gm; ZnSO_4 , 0.01gm; MnSO_4 , 0.01gm; MgSO_4 , 0.2gm; Agar, 15gm; Naringin, 1gm; After three more transfers,

pure culture was obtained which were preserved at 4°C for future purpose.

Primary screening (qualitative assay)

Literature available on screening for naringinase producers is scanty. Kabyashree Phukan, et al.²⁵ has done primary screening by FeCl_3 method as prescribed; 24hrs old culture from the naringin PDB broth is used for screening i.e., 0.5ml 1% FeCl_3 was added in the 5 test tubes having 3ml of fresh liquid media and 0.5ml of 24hrs old culture i.e. inoculums. Once FeCl_3 is added to the media, the reddish brown color media changes to a light brown color, when it is collate with the control. Hence PDB which forms light brown color were showing positive results, and that cultures were used for the production of naringinase enzyme.

The cultures which showing positive results for 1% FeCl_3 test (primary screening) were maintained in a pure culture of media containing 0.1% of naringin as a carbon source were maintained.

Selection of media for fermentation

There are 3 different types of media are used for the screening of naringinase with some changes in nitrogen sources to determine which media is suitable for naringinase production as mentioned in below Table 1.

Table 1. different media used for fermentation of naringinase

Media 1(g/l)	Media 2(g/l)	Media 3(g/l)
Naringin-1gm	Naringin-1gm	Naringin-1gm
NaNO_3 -2gm	Peptone-3gm	Yeast extract-2gm
MgSO_4 -0.5gm	MgSO_4 -0.5gm	MgSO_4 -0.5gm
KCl-0.5gm	KCl-0.5gm	FeSO_4 -0.5gm
K_2HPO_4 -1gm	K_2HPO_4 -1gm	NaCl-0.5gm
FeCl_3 -0.1gm		K_2HPO_4 -1gm

The 500ml of Erlenmeyer flask containing 100ml of production media was prepared with pH-6 and temperature was maintained at 35°C and it was inoculated with one ml of isolate. The inoculated flasks are kept in an incubator at shaking conditions of 120 rpm for up to 5 days. The samples are withdrawn aseptically at 24 hrs intervals to determine the activity of enzyme.

Inoculum preparation

5 days old culture of *Aspergillus flavus* KLA-80 was used for the preparation of the inoculum for the fermentation by adding 10ml of distilled water with 0.1% Tween-80 and suspending the spores with a help of sterile loop. The spore suspension was adjusted to desired range (i.e., 1×10^8) by using haemocytometer.²⁶

Enzyme assay

The naringinase assay was carried out by the method prescribed by Davis.²⁷ The fermented culture was centrifuged at 3,000rpm for 10min at 4°C from that centrifuged culture 0.1ml of supernatant is taken and that supernatant was made to react with 0.9ml of 0.05%(w/v) naringin

dissolved in 0.1M sodium acetate buffer(pH 4.0). The reaction mixture was then incubated at 50°C for 60min after that 5ml of 90% diethylene glycol is added followed by the addition of 0.1ml of 4N NaOH. The mixture was kept at ambient temperature for 10min for the colour development the resulting yellow colour was measured at 420nm. One unit (U) of naringinase activity was defined as the amount of enzyme that could hydrolyze 1 μ /mol of naringin/ min at the assay conditions.

HPLC analysis

HPLC analysis is performed for the both standard and the isolate. The HPLC system of an Shimadzu HPLC coupled with a RP C18 reverse

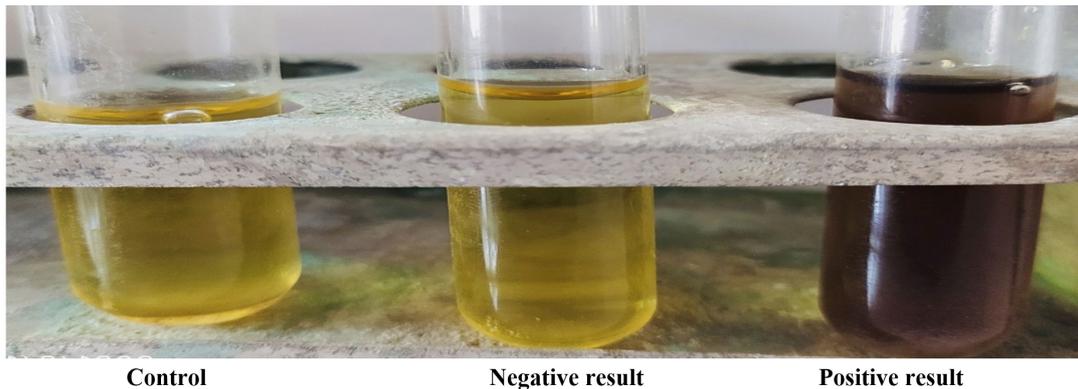


Figure 2. Primary screening of naringinase

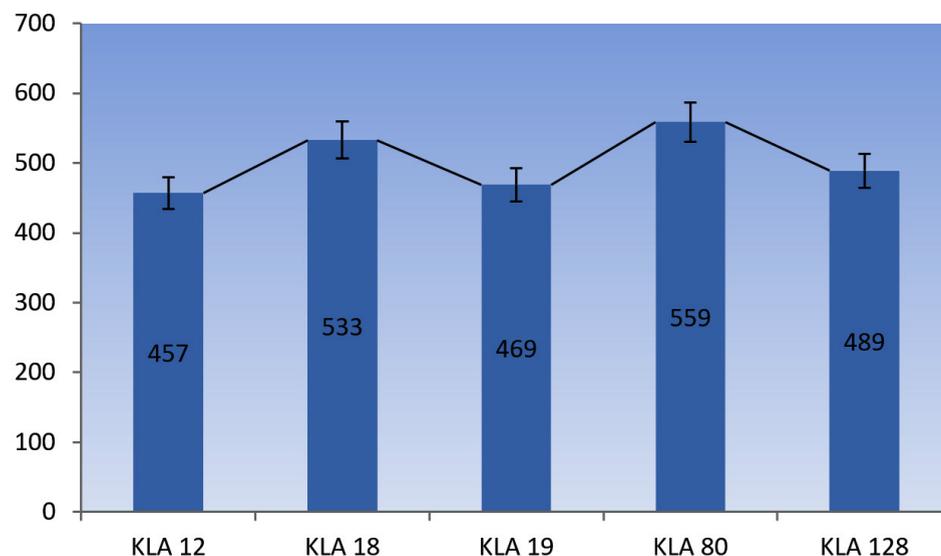


Figure 3. Microbial isolates showing highest enzyme activity

Table 2. Different isolates showing naringinase activity

Fungi		
No.	Isolate	Activity (u/mL)
1.	KLA 5	376 U/mL
2.	KLA 12	457 U/mL
3.	KLA 18	533 U/mL
4.	KLA 19	469U/mL
5.	KLA 13	386 U/mL
6.	KLA 28	427 U/mL
7.	KLA 33	386 U/mL
8.	KLA 36	438 U/mL
9.	KLA 37	345 U/mL
10.	KLA 38	251 U/mL
11.	KLA 45	298 U/mL
12.	KLA 49	202 U/mL
13.	KLA 57	318 U/mL
14.	KLA 59	298 U/mL
15.	KLA 63	274 U/mL
16.	KLA 65	201 U/mL
17.	KLA 72	380 U/mL
18.	KLA 80	559 U/mL
19.	KLA 81	379 U/mL
20.	KLA 88	315 U/mL
21.	KLA 92	387 U/mL
22.	KLA 96	284 U/mL
23.	KLA 84	217 U/mL
24.	KLA 93	284 U/mL
25.	KLA 103	216 U/mL
26.	KLA 113	317 U/mL
27.	KLA 120	368 U/mL
28.	KLA 123	400 U/mL
29.	KLA 128	489 U/mL
30.	KLA 138	298 U/mL
31.	KLA 140	285 U/mL
32.	KLA 141	326 U/mL
33.	KLA 158	373 U/mL
34.	KLA 160	368 U/mL
35.	KLA 163	391 U/mL
36.	KLA 166	287 U/mL
37.	KLA 169	239 U/mL
38.	KLA 173	261 U/mL
39.	KLA 177	374 U/mL
40.	KLA 197	315 U/mL
41.	KLA 198	254 U/mL
42.	KLA 183	267 U/mL
Bacteria		
No.	Isolate	Activity (u/mL)
1.	KLA 53	102 U/mL
2.	KLA 78	80 U/mL
3.	KLA 109	122 U/mL
4.	KLA 118	108 U/mL
5.	KLA 135	124 U/mL
6.	KLA 143	198 U/mL
7.	KLA 185	87 U/mL
8.	KLA 186	124 U/mL

column (2.5x 150mm, 3.5µm) and a photo diode array detector was used for the analysis with an wavelength set at 280nm. The flow rate of 0.3ml/min was programmed and 5µl of the volume was injected. Naringin in the sample is identified by comparing the retention time of sample with standard.

Molecular characterization of the isolated strain

The selected isolate KLA-80 was undergone identification by lacto-phenol cotton blue test and *Aspergillus* differentiate media is done to determine the type of fungi. The genomic DNA extracted from the potential isolate KLA-80 using DNA miniprep kit. ITS-rDNA partial gene was successfully amplified using primers ITS4 & ITS5. The sequencing PCR was set up with ABI-BigDye® Terminatorv3.1 Cycle Sequencing Kit.

Phylogenetic tree analysis

The sequence analysis was carried out using Bioinformatics tool BLAST of NCBI. Multiple sequences is aligned used Clustal W. The phylogenetic tree was constructed by neighbour-joining method (NJ) using Mega11 software.²⁸ The obtained sequences are compared to the sequences obtained in the gene bank using BLAST program on the NCBI website. Some of the closely related sequences from the gene bank are used to prepare Phylogenetic tree.

RESULTS

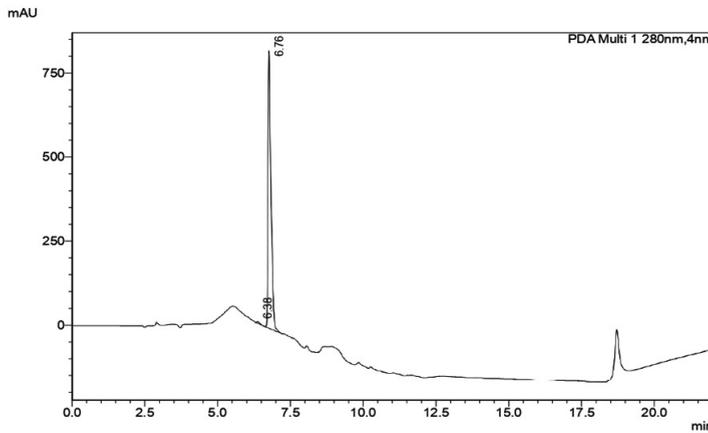
Isolation of the micro-organism

Isolation of micro-organisms from different types of samples were done by serial dilution method, i.e., 1gm of the samples is serially diluted using sterile distilled water , for the bacterial isolation 10^{-5} and 10^{-6} is taken so as for fungi isolation 10^{-3} and 10^{-4} should be taken. 0.1ml from the dilution test tube is taken on above mentioned media which is supplement with 0.1% of Naringin. The culture which showing good results are transferred to the fresh media after 3 more transfers, the pure culture of the micro-organism is obtained. The pure culture was maintained in the Naringin Agar media for the further investigation.

Primary screening

As mentioned above primary screening was done using Naringin PDB media i.e., the isolated colonies on the selected media is transferred to Naringin PDB media and allowed it to grown for 3 days and primary screening method

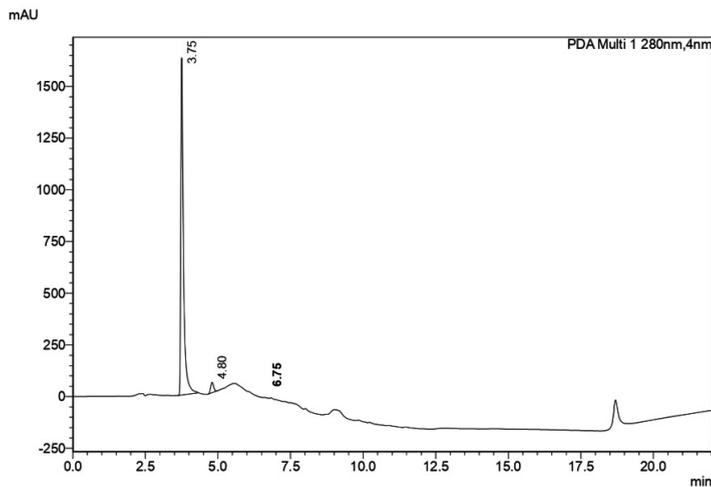
was performed by using 1%FeCl₃ method i.e., if the strain produces naringin enzyme then it hydrolyses naringin present in the media and the end product of the reaction is naringenin (Figure 2). Naringenin reacts with FeCl₃ and gives a reddish brown colour. Therefore, the strain which shows reddish brown



Peak Table

Peak#	Ret. Time	Area	Area%
1	6.38	35836	0.606
2	6.76	5881968	99.394
Total		5917804	100.000

Standard Naringin



Peak Table

Peak#	Ret. Time	Area	Area%
1	3.75	10311595	96.243
2	4.80	206002	1.360
3	6.75	358547	2.397
Total		10670144	100.000

Isolate KLA-80

Figure 4. HPLC analysis of standard Naringin and the isolate KLA-80

colour appearance was determined as naringinase producing strains and which does not develop brown color is considered as negative result for naringinase production. In the primary screening procedure 50 strains are showed vibrant results in which 42 strains are fungi and remaining 8 isolates are bacteria. As there is no proper evidences are available to confirm these isolates as naringinase producers so further secondary screening is carried out for all the 50 isolates (Table 2).

Screening of selection media

Three different types of media are used to determine the activity of the enzyme out of which Media-1 have NaNO_3 acts as a in-organic nitrogen source along with FeCl_3 which helps in the oxidation-reduction process for the production of enzyme, whereas in the Media-2 Peptone and in Media-3 Yeast extract is used as nitrogen source along with naringin as a sole carbon source in all the 3 media. After fermentation Media-1 has showed highest activity when compared other two

media. So Media-1 is used for the fermentation of naringinase.

Production of naringinase under sub-merged fermentation

Submerged fermentation was done for the all 50 isolates in the media-1, which is found to be good naringinase producer among the 3 different media. The media- 1 contains a composition of Naringin, 1g; NaNO_3 , 2g; KH_2PO_4 , 1g; KCl, 0.5g; MgSO_4 , 0.5g; FeCl_3 , 0.1g. Among all 50 isolates 5 isolates showed good results. Out of which KLA-80 showed a highest activity of 559 u/ml as shown in Figure 3. The activity of enzyme is expressed in u/ml. Further identification and sequencing and biochemical properties of KLA-80 have been done to identify the specific micro-organism (Figure 3).

HPLC analysis

The HPLC analysis was done for the determination of naringinase activity in the

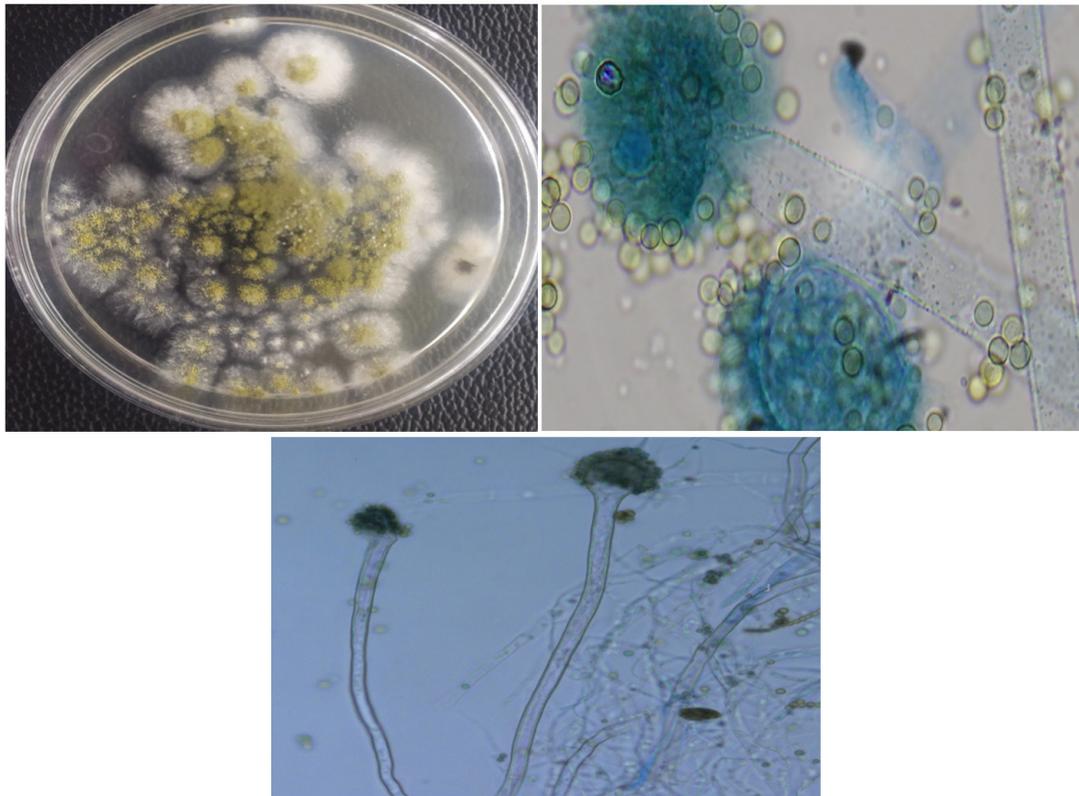


Figure 5. a) KLA-80 grow on PDA media containing 0.1% Naringin, b) Lacto-phenol cotton staining of KLA-80 (Radical Microscope), c) Digital Microscopic image of KLA-80

micro-organisms. The sample along with standard was analyzed for the presence of Naringin in the sample. Both the sample and the standard were detected at 280nm and the results were analyzed. In which it is found that sample retention time 3.75 was decreased in compare to standard retention time of 6.76. From this it is found that the isolate KLA-80 has a naringinase activity (Figure 4).

Identification of microbial isolate

The microbial isolate KLA-80 was described as a fungus on the basis of cultural characteristics and microscopic observations. Colonies on MEA

at 25°C citrine green, velvety, fast growing, reverse dull olive green. Conidial heads loose on in compact columnar, splitted into columns. Conidiophores straight, colourless, smooth walled, width gradually increasing towards length, foot cell present, upto 675.4× 14.0µm. Vesicles sub-globose to flask shaped, olivaceous from, fertile all over, upto 32.8×31.3 µm. Sterigmata uni to biseriate, primaries ampulliform, smooth walled, olivaceous brown, upto 15.6×5.5 µm, Secondary-ampulliform, olivaceous brown, smooth walled, upto 6.3×3.4 µm. Conidia produced in long chain, globose to subglobose, sub-olivaceous too livaceous, smooth



Figure 6. *Aspergillus* differentiate media

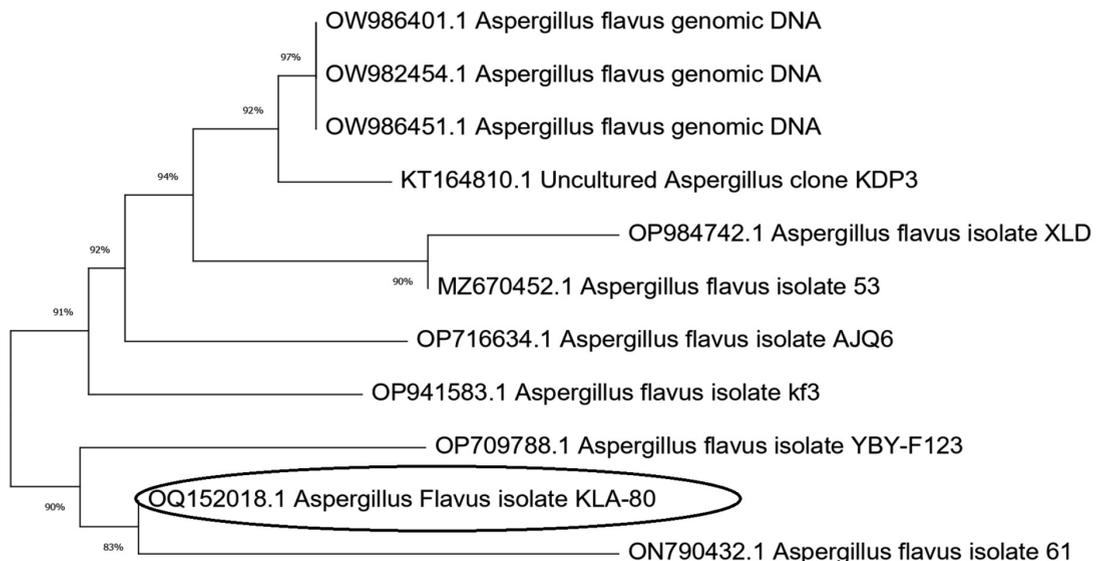


Figure 7. Phylogenetic relationship of the sequence of the *Aspergillus flavus*, KLA-80 strain with relation to other fungi

to roughed walled, up to $6.8 \times 6.6 \mu\text{m}$. Sclerotia, Chlamydospores absent and Teleomorph absent (Figure 5).

Aspergillus differentiate media is done to determine the type of *Aspergillus* based on their growth recommended by Dehghan et al.²⁹ The *Aspergillus flavus* (KLA-80) appears green on the upper layer and orange on the reverse whereas *Aspergillus niger* (KLA-33) shows black on the upper layer and on the reverse also, from this we can easily differentiate *Aspergillus flavus* from *Aspergillus niger* (Figure 6).

Phylogenetic tree

The phylogenetic analyses of the selected naringinase producing fungi are closely related to the *Aspergillus* genera. In this study the isolated culture showed 100% sequence similarity with *Aspergillus flavus* based on nucleotide homology and analysis and accession number of OQ152018 is obtained for the given culture (Figure 7).

DISCUSSION

Enzymes extracted from the micro-organisms are gaining interest because of their widespread uses in the industries due to their catalytic activity, stability, and ease in the production and optimization than plants and animal enzymes.³⁰ Submerged fermentation is a technique which is quite fit for bacteria and fungi as they requires a high moisture content due to which purification of product is simpler in Smf method.³¹ The present research was aimed to produce naringinase using sub-merged fermentation was employed in which 5 fungal isolates showed a good naringinase activity as shown in Figure 3. There are numerous work was done on the naringinase activity isolated from the different sources. *Aspergillus flavus* isolated from citrus fruit showed a highest activity of 449.58ug/dry matter on the eighth day at 50mg/L concentration of naringin and increase in concentration results in the decrease in enzyme activity.³² Under solid state fermentation. *A. oryzae* JMU316 which was isolated from a decayed 'Guanxi pomelo' has showed activity in the presence peptone as a nitrogen source with a activity of 408.28IU/ml.³³ Fungi at 30°C showed a

growth with an initial pH of 7 using rutin and diammonium hydrogen phosphate as carbon and nitrogen sources 8.0 and 9.0 g/l, respectively.³⁴ In the present study, there were 5 fungal isolates are obtained from the soil which showed a good naringinase activity under Smf. From this research it is found that soil is a good source of *Aspergillus flavus*, which is a best source for naringinase production. Fungi is known to be one of the best sources for naringinase production as compared to bacteria, various sources such as soil, peels of citrus fruit are found to be a home for fungi as they have a compatible environment for their growth.

CONCLUSION

From the above investigation it was found that the isolate KLA-80 is showing good naringinase activity; from this, it is concluded that *Aspergillus flavus* is found to be a good naringinase producer and suitable for industrial production of naringinase when compared to other strains, as it showed a good naringinase activity in submerged fermentation. Further, this isolate is used for the optimization process to enhance the activity of the naringinase enzyme.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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