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ENRICHMENT AND CHARACTERIZATION OF LIMONIN DEGRADING MICROORGANISMS ISOLATED FROM KINNOW ORCHARD SOIL, PEEL AND WASTE SITE

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ABSTRACT

The present research was aimed to screen and characterize limonin degrading microorganisms and assess their efficiency in limonin reduction. Eight bacterial isolates from kinnow orchard soil and two yeast isolates from soft kinnow peel and kinnow waste site (Abohar, Punjab) were isolated. All the isolates were evaluated for their limonin utilizing ability by swarm plate assay. Two bacterial isolates (S1 and S2) and both yeast isolates (PY1 and AY1) showed considerable zone of TTC reduction. All the four selected isolates were studied for their growth profile and rate of limonin degradation in minimal media broth with limonin (28 ppm) as a sole carbon source. Bacterial isolate S1 showed maximum limonin degradation (52.9%), followed by yeast isolates PY1 (48.5%), AY1 (31.1%) and bacterial isolate S2 (26.4%). Ca-alginate immobilized cells of PY1 yeast and S1 bacteria were also studied for their limonin degrading ability which respectively showed 94.8 and 98% limonin reduction. The two microbial isolates were molecularly identified as *Bacillus megaterium* strain KU570370.1. on 16S rDNA basis and *Meyerozyma caribbica* isolate KP860076.1. on 18S rDNA basis These microbial isolates could be exploited for the production of enzyme limonin dehydrogenase for possible use in debittering kinnow juice.

Key words: *Bacillus megaterium*, degradation, immobilized cells, limonin, *Meyerozyma caribbica*

INTRODUCTION

Limonin is a bitter compound present in citrus juices, formed as a result of enzymatic (hydrolase) conversion of a non-bitter compound, limonoate-A-ring lactone (LARL). Limonin hinders processing of kinnow juice; and several approaches have been used to either avoid limonin formation or reduce limonoid in citrus juices to a permissible limit of 6 ppm (Hasegawa and Maier, 1983). Debittering enzymes of microbial origin have some inherent advantages such as a) single step hydrolysis, b) operation under mild conditions, c) cost-effectiveness and energy saving, and d) preservation of natural qualities of juice such as flavour, colour, vitamins and organoleptic compounds. These advantages have prompted many workers to isolate such microorganisms and purify their enzyme(s) for debittering of citrus fruit juices (Nagra and Puri, 2010). Limonoate dehydrogenase (LDase), enzyme is detected in several microbes and it can prevent limonin production by catalyzing the oxidation of LARL to the corresponding 17-dehydrolimonoate, a non-bitter derivative. This enzyme has been isolated from *Rhodococcus fascians, Arthrobacter globiformis* and *Pseudomonas* sp. strain 321- 18 and used for the degradation of limonin from citrus

fruit juices (Humanes *et al.*, 1997; Puri *et al.*, 2002). Limonin degradation by whole cell immobilization has also been achieved using acrylamide gel immobilized *Corynebacterium fascians* cells (Hasegawa *et al.*, 1974; 1985), calcium alginate immobilized cells of *Pseudomonas putida* G7 (Malik and Ghosh, 2012) and k-carrageenan immobilized *Rhodococcus fascians* cells (Iborra *et al.*, 1994).

Kinnow, a hybrid of *Citrus mobilis* and *Citrus deliciosa*, is presently the primary citrus crop of Punjab with annual production of 1.1 million tonnes from an area of 48000 ha (Punjab Horticulture Mission, 2014-15). In order to utilize the high production of kinnow, a viable alternative is to increase its shelf life by fruit processing, but kinnow processing industry has not progressed due to inadequate debittering technology. Earlier efforts on fermentation of kinnow juice ameliorated date juice by MTCC 11815 resulted in 9.4% alcohol production (Dua and Kocher, 2017). Puri *et al.* (2002) reported successful degradation of limonin (66%) in kinnow mandarin juice at pH 8 with partially purified limonoate dehydrogenase; however, they noticed relatively small degradation of limonin at lower pH (3-4). In this communication, enrichment of limonin degrading yeasts and bacteria from kinnow fruit, kinnow waste and kinnow orchard soil and their characterization for limonin degrading potential is reported. Limonin degrading yeasts have not been reported to the best of our knowledge which opens up a window for the processing of kinnow juice into yeast fermented drinks such as wine, beverages, etc.

MATERIALS AND METHODS

The work was conducted in the Department of Microbiology, PAU, Ludhiana (India) in the year 2014-2016. Limonin was extracted from kinnow seeds (local variety) using petroleum ether and chloroform as per the method of Vaks and Lifshitz, (1981).

Isolation of limonin degrading microorganisms

Bacterial and yeast cultures were enriched from kinnow orchard soil, kinnow peel and kinnow waste site of Punjab Agro Juices, Abohar (Punjab, India). For the isolation of bacteria, 50 g kinnow orchard soil was first mixed with 250 mL phosphate buffer (pH 7), shaken for 1 h at 120 rpm on orbital shaker (MAC, MSW132) and then allowed to settle. The cell suspension formed was centrifuged in a cooling centrifuge (REMI, C24) at 10,000 rpm for 10 min. The pellet was collected and re-suspended in phosphate buffer. The suspension was serially diluted and 10^4-10^8 dilutions were pour-plated with minimal salt agar medium [KH_2PO_4 - 0.20 g, $Fe_2(SO_4)_3.6H_2O$ - 0.054 g, K_2HPO_4 - 0.5 g, $(NH_4)P(Mo_3O_{10})_4$ - 0.024 g, $NaH_2PO_4.H_2O$ - 2.0 g, $ZnSO_4.H_2O$ - 0.05 mg, Na_2HPO_4 - 1.5 g, $CuSO_4.5H_2O$ - 0.0025 mg, NH_4NO_3 - 0.6 g, $MnSO_4$ - 0.0055 mg, $NaNO_3$ - 3.8 g, H_2BO_3 - 0.057 mg, $MgSO_4.7H_2O$ - 0.3 g, H_2O – 1000 mL] containing limonin (10 ppm) as sole carbon source. For the isolation of yeasts, glucose yeast extract medium (glucose - 5 g, peptone - 3 g, yeast extract - 3 g, H_2O - 1000 mL) supplemented with a piece of soft kinnow peel and kinnow pulp waste separately in 250 mL flasks. The flasks were incubated at 28^0C under shaking conditions in an incubator shaker (MAC, MSW132) and streaked on petriplates containing media with limonin as sole carbon source. Inoculated Petri-plates were observed for 4-7 days for microbial growth. Bacterial and yeast colonies were differentiated from each other on the basis of their morphology and microscopic studies.

Screening of isolates by swarm plate assay

Limonin utilizing ability of all the isolates was evaluated by swarm plate assay (Jain, 2003). Plates with minimal media (without any carbon source) containing limonin (0.2%), TTC (0.05%) and buffer in semisolid agar (0.15%) were prepared. Log phase culture (1 mL) was harvested by centrifugation. Pellets were washed and resuspended (at cell density of 0.8 OD 600) in carbon-free

minimal media. One hundred microliter of each culture suspension was placed at the center of each set of plates (3 plates/set). Plates were incubated at 30^0C for 24 h. The reduction of TTC and peripheral movement in semisolid medium containing limonin (chemotaxis) was observed in quintuplicates and area of TTC reduction zone was calculated by measuring the diameter of zone with a measuring scale followed by calculating its area (πr^2).

Growth kinetics and limonin degradation

The isolates screened from swarm plate assay were analyzed for their growth kinetics and limonin degrading ability. The selected isolates were inoculated in minimal salt media having three pH conditions (4, 5 and 7) with nutrient broth - 0.1%, yeast extract - 1% and limonin (28 ppm) as the sole carbon source. The flasks were incubated at 28^0C under shaking at 120 rpm and samples taken (every day till the end of stationary phase) to study growth spectrophotometrically (Systronic 106) at 600 nm. Limonin degrading ability was determined by measuring residual limonin at different time of growth spectrophotometrically at 503 nm (Vaks and Lifshitz, 1981).

Whole cell immobilization and limonin degradation

The cells of selected microbial isolates were immobilized in calcium alginate beads by entrapment method (Chibata and Tosa, 1977). Microbial cells were grown in minimal media containing limonin as sole carbon source at 28˚C under shaking conditions (120 rpm). The 72-96 h old microbial cells (stage of maximum limonin degradation rate) were harvested by centrifugation at 8000 rpm for 15 min, suspended in 4% sodium alginate solution and filled in a hypodermal syringe. The same was dropped in 0.2M ice cold solution of anhydrous $CaCl_2$ to prepare beads. The beads so formed (2 mm) were stored in a refrigerator till use.

The immobilized cells of yeast and bacteria in calcium alginate beads were packed separately in a glass column of 2 cm diameter and 15 cm length. Minimal medium containing limonin (50 ppm) as sole carbon source was passed repeatedly through these columns @ 100 mL h^{-1}, at 30˚C to study limonin degradation. Residual limonin was estimated spectrophotometrically at 503 nm (Vaks and Lifshitz, 1981) after every passaging through the column.

Morpho-biochemical characterization of selected isolates

Yeast (PY1) and bacteria (S1) were characterized with respect to their morphological, biochemical and molecular characteristics:

Morphological characterization: The bacterial and yeast isolates were grown on nutrient agar (beef extract - 3 g, peptone - 5 g, NaCl - 5 g, H_2O - 1000 mL) and glucose yeast agar medium by incubating at 28±2˚C for 2-3 days and their morphology was examined microscopically. The following features of isolates were recorded: texture, colour, shape, pigmentation and surface of colonies (Kreger-van Rij, 1984, Kurtzman *et al.*, 2011).

Biochemical characterization: The identification of the selected bacterial and yeast isolates was determined on the basis of biochemical tests (Van der Walt and Yarrow, 1984, Barnett *et al.*, 2000; Holt *et al.*, 1994). For bacteria, classical approach of identification involving preliminary microscopic examination of Gram- and endospore-stained cells, followed by biochemical tests like carbohydrate fermentation (D-glucose, D-galactose, D-fructose, D-trehalose, D-maltose, D-lactose, D-raffinose, D-xylose, cellobiose and sucrose), catalase activity, starch hydrolysis, gelatin hydrolysis, casein hydrolysis, indole production, methyl-red test, Voges-Proskauer test and citrate utilization (iMViC test) [Holt *et al.*, 1994]. For yeast identification, the biochemical tests included fermentative growth on specific carbon sources (D-glucose, D-galactose, D-fructose, D-trehalose, D-maltose, D-lactose, D-raffinose, D-xylose, cellobiose and sucrose, and other tests like growth on lysine agar medium, flocculation test, starch hydrolysis, catalase test and tolerance to 1% acetic acid (Kurtzman *et al.*, 2011, Yarrow, 2000).

Molecular identification of selected isolates: For molecular study, the microbial isolates were selected on the basis of their limonin degradation ability. Molecular identification of selected

isolates was outsourced from Eurofins Genomics India Pvt. Ltd, Bangalore (India) using 18s rDNA gene sequencing for yeast and 16S rDNA for bacteria (Maniatis *et al.,* 1982, Tamura *et al.,* 2007). The respective rDNA was evaluated on 1.0% agarose gel, which was amplified by PCR. The forward and reverse DNA sequencing reaction of PCR amplicon was carried out with ITS1 and ITS4 primers for yeast and 27F and 1115R primers for bacteria (Bangalore Genei, India) using BDT v3.1 Cycle sequencing kit (Thermo Fisher Scientific, 310 Genetic Analyzer) on ABI 3730xl Genetic Analyzer. Consensus sequence of rDNA gene was generated from forward and reverse sequence data using aligner software. The rDNA gene sequence was used to carry out BLAST with the database of NCBI Genebank Database. Based on maximum identity score the first 10 sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and phylogenetic tree constructed using MEGA 4 (Kimura, 1980). The gene sequences retrieved were submitted to National Center for Biotechnology Information (NCBI) and their accession numbers obtained. The sequences were matched on GenBank and phylogenetic trees were drawn.

RESULTS AND DISCUSSION

Isolation and screening of limonin degrading microorganisms

Eight bacterial isolates were enriched from kinnow orchard soil and named as isolate S1 to S8. Two yeast cultures were enriched one each from soft kinnow peel and kinnow pulp waste site of Abohar and named as PY1and AY1, respectively.

All the ten isolated cultures were assayed for limonin degrading ability by swarm plate assay (Jain, 2003). The bacterial isolates, S1 and S2 and yeast isolates PY1and AY1 showed remarkable zone of TTC reduction and chemotaxis. Maximum chemotaxis and TTC reduction zone (2.01 cm^2) was in S1 bacterial strain, followed by PY1 yeast strain (1.77 cm^2) and S2 bacteria strain (0.64 cm^2) and minimum TTC reduction was shown by AY1 yeast (0.28 cm^2).

Tetrazolium chloride-dehydrogenase activity (TTC-DHA) method is recommended as a very sensitive and simple technique to determine sludge activity (Lazarova and Manem, 1995). TTC is a kind of colorless soluble dye, which serves as terminal acceptor in biochemical reactions. Therefore, TTC-DHA method was used in the study of biological activity of microbial cells growing in limonin containing media. Reduction of TTC which causes the appearance of red colour from the centre of plate towards the edge, indicates the microbial movement in the same direction. This suggests that cells are utilizing limonin as carbon source were moving from a zone where carbon compound was used up to another zone where carbon compound was available for growth. So. as it moved it reduced 2, 3, 5 triphenyl tetrazolium chloride to a red colored water insoluble formazon indicating that it is a physiologically active system. Chang *et al* (1999) also reported that the dehydrogenase of respiratory system in bacteria is responsible for this reduction.

Growth kinetics

The four microbial isolates *viz*. PY1 yeast, AY1 yeast, S1 bacteria and S2 bacteria were studied for their growth profile and limonin degradation in minimal salt medium with limonin as a sole carbon source. The study was carried out for 7 days. With increase in microbial growth in minimal salt media there was decrease in limonin content. The comparative analysis of growth and limonin degradation is shown in Fig. 1. The yeast strains PY1, AY1 and bacterial strains S1 and S2 showed a continuous increase in OD_{600nm} till 5-6 days and then declined till 7 days of incubation under shaking (120 rpm) conditions. More optical density is observed in both yeast strains at pH 4 and 5, owing this to its optimum pH for growth. Maximum reduction in limonin content was observed at pH 7 than pH 4 and 5 for both the yeast isolates. At pH 7, PY1 yeast, AY1 yeast, S1 and S2 showed 48.5, 31.1, 52.9 and 26.4% reduction in limonin content, respectively, within 7 days. Maximum

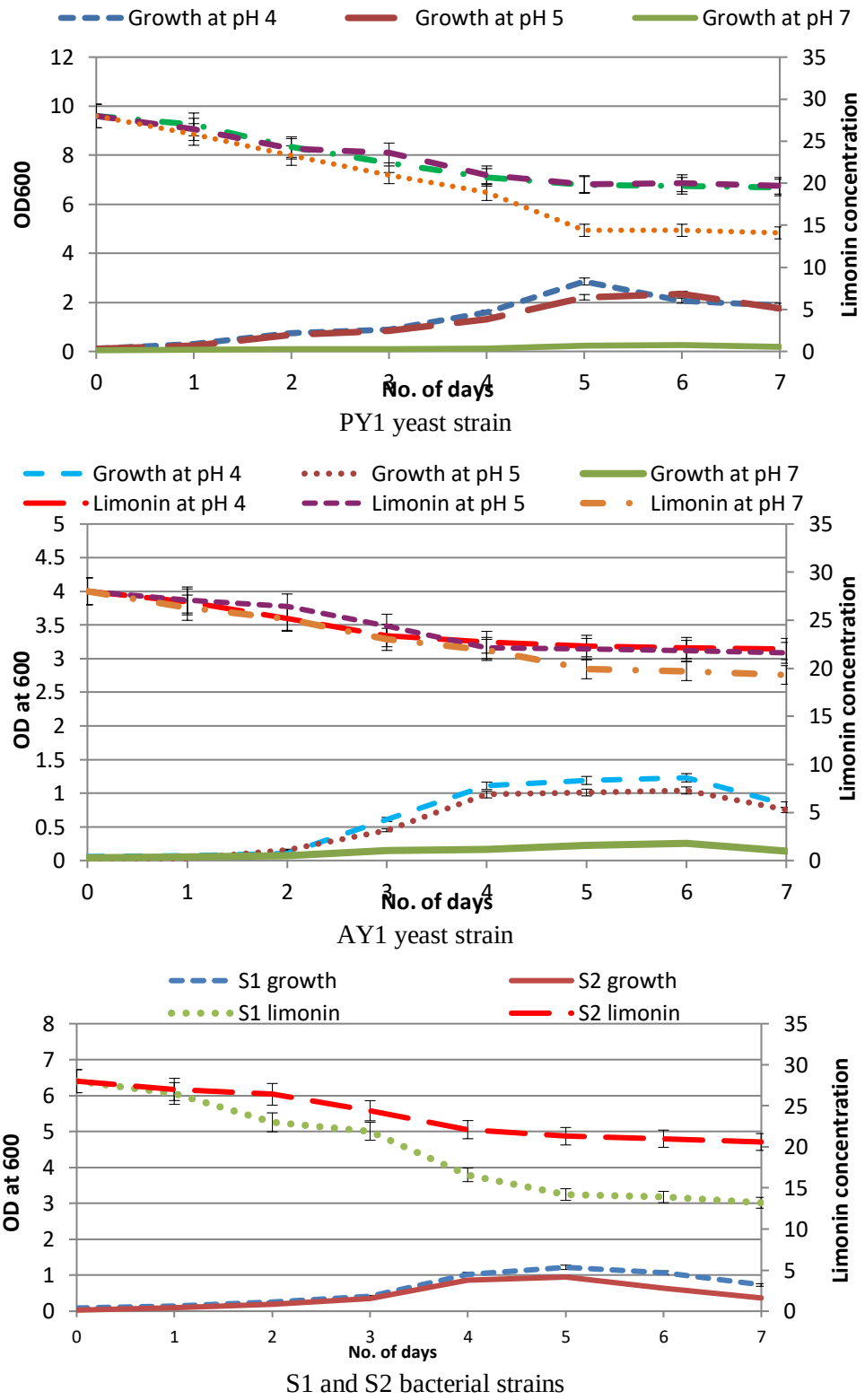


Fig. 1: Growth and limonin degradation of selected isolates in minimal media containing limonin as sole carbon source

limonin reduction at pH 7 might be due to more activity of enzyme limonin dehydrogenase at alkaline pH than acidic pH conditions. Puri *et al.* (2002) reported optimum enzyme activity of

limonin dehydrogenase at pH 8 while Verma *et al.* (2010) reported pH 8.5to be the optimum pH value for enzyme limonin dehydrogenase isolated from *Pseudomonas putida.*

Limonin reduction was proportionate with the growth of culture and maximum limonin reduction occurred during log phase of microbial growth No significant change in limonin amount occurred during stationary phase which might be due to the formation of other inhibitory secondary metabolites which rendered microbe unable to utilize limonin further (Moat *et al* 2002). Therefore, complete removal of limonin was not achieved in culture supernatant. All the four microbial isolates showed long lag phases which might be due to the additional time taken by cultures to adapt to new culture conditions having limonin as a sole carbon source.

Whole cell immobilization and limonin degradation

Minimal media containing limonin (50 ppm) as sole carbon source was repeatedly passed through two columns containing immobilized cells of PY1 yeast and S1 bacterial strain separately. Residual limonin was observed after every passaging (Fig. 2). In columns containing immobilized cells of PY1 yeast strain and S1 bacterial strain, limonin reduction of 94.8% in 2 h 30 min and 98% in 1 h 45 minutes after 7 and 4 passages, respectively.

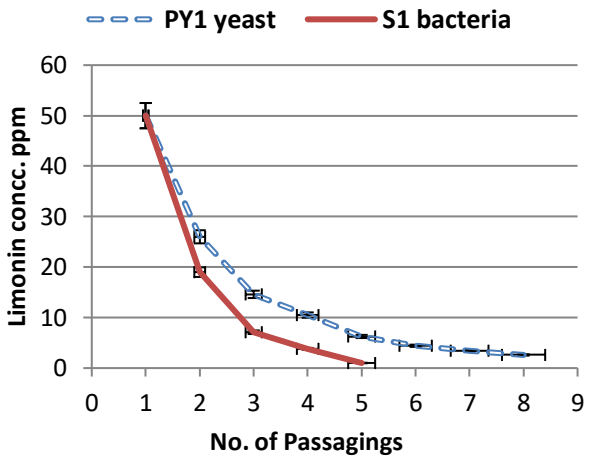


Fig. 2: Limonin degradation by immobilized whole cells of yeast isolate PY1 and bacterium isolate S1

Malik and Ghosh (2012) optimized the immobilization parameters for *Pseudomonas putida* G7 in calcium alginate beads for biotransformation of limonin and reported 58% limonin bio-transformation. A continuous process of limonin degradation by *Rhodococcus fascians* cells entrapped in K-carrageenan in a continuous stirred tank reactor was done by Iborra *et al* (1994) where they reported that immobilized cells were able to debitter limonin-containing media. Hasegawa *et al* (1983) reported reduction of limonin bitterness in navel orange juice serum with *Arthrobacter globiformis* cells immobilized in acrylamide gel. They treated 30 mL serum (10-27 ppm limonin) on a 1.5 cm diameter column packed with 1.6 g immobilized cells (16 mL bed volume) and reported 70% limonin reduction.

Vaks and Lifshitz (1981) entrapped the bacterium, *Acinetobacter sp.*, in a dialysis bag and used it for debittering of early season orange juice. They reported that it was possible to reduce the bitterness of juice by transferring same dialysis bag from batch to batch. They further added that 120mg of bacteria could convert 1L of bitter orange juice to a portable one. The two microbial isolates (S1 and PY1) were found promising for their limonin degrading ability, so they were identified on the basis of 16S and18S rDNA, respectively.

Morphological and biochemical characterization of isolates

Morphological and biochemical characterization was carried out as given in material and methods, which revealed the bacterial isolate to be Gram positive, endospore containing with white, circular colonies on nutrient agar medium (Table 1). The strain was found to be positive for catalse test, starch, gelatin and casein hydrolysis, citrate utilization and methyl red test. The carbon assimilation test results showed that the isolate was able to utilize all the sugars mentioned in material and methods. Presumptive identification of the strain on the basis of its morphological and biochemical characteristics, revealed it to be a *Bacillus sp.* as per Holt *et al.* (1994).

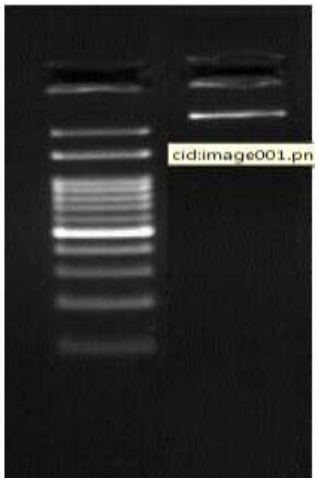
Table 1: Morpho-biochemical characterization of bacterial isolate S1 and yeast isolate PY1

Characteristics	Bacteria S1	Yeast PY1
Morphological characteristics		
Colony colour	White colour	Creamish-white
Colony surface	Smooth/ dry	Smooth/slimy
Colony shape	Circular	Circular
Pigmentation	-ve	-ve
Margin	Entire	Entire
Cells shape	Rod	Oval
Gram staining	+ve	NA
Endospore staining	+ve	NA
Motility	+ve	NA
Biochemical characteristics		
Sugar utilization: D-glucose, galactose, fructose sucrose, xylose, maltose, lactose, trehalose and raffinose	+ve	+ve
Cellobiose utilization	+ve	-ve
Catalase	+ve	-ve
Starch hydrolysis	+ve	+ve
Gelatin hydrolysis	+ve	NA
Casein hydrolysis	+ve	NA
iMViC	+ve	NA
Tolerance to 1% acetic acid	NA	-ve
Flocculation	NA	Bottom yeast
Growth on lysine agar medium	NA	-ve
Growth on vitamin free medium	NA	+ve
Ethanol tolerance	NA	13%

NA = Not available

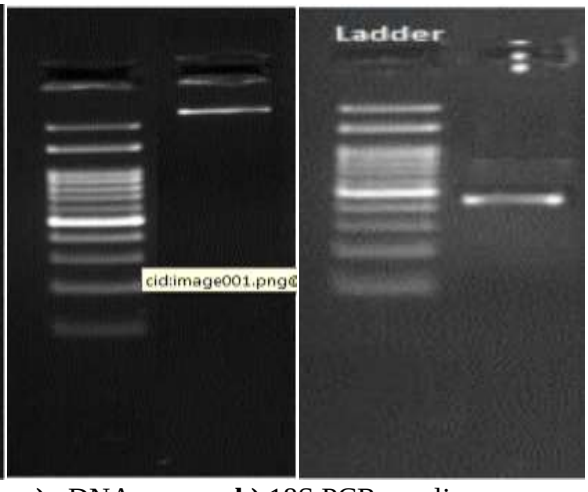
The oval shaped cells of yeast isolate had creamish white circular colonies with smooth surface. The yeast was found to be positive for starch hydrolysis, growth on vitamin-free medium and carbohydrate utilization, except for cellobiose. The culture was observed to be a bottom fermenting yeast which could tolerate ethanol upto 13%. The culture did not grow on lysine agar medium indicating it to be a *Saccharomyces* strain.

The sequence analysis of phylogeny for microbial taxo-nomy is a more accurate method for determining inter- and intra-specific relationships because traditional identification methods, depending on phenotypes, usually lead to uncertain and inaccurate interpretations of species inter-action (Kurtzman *et al.*, 2011). For identification upto species level molecular characterization of isolates was done through outsourcing from Eurofins Genomics Lab, Bangalore.



gDNA 18S PCR amplicon

Fig 3: gDNA and amplicon QC data (2nd lane)



a) gDNA **b)** 18S PCR amplicon

Fig. 4: gDNA and amplicon QC data (2nd lane)

Molecular characterization of isolates

Two isolates, *viz.*, S1 bacterial strain and PY1 yeast strain were subjected to molecular identification outsourced from Eurofins Genomics lab, Bangalore on the basis of DNA sequencing.

Isolate S1: A single band of high-molecular weight DNA was observed (Fig. 3a & b) which on amplification showed a 1100 bp band. The 16S rDNA gene sequence was used to carry out BLAST

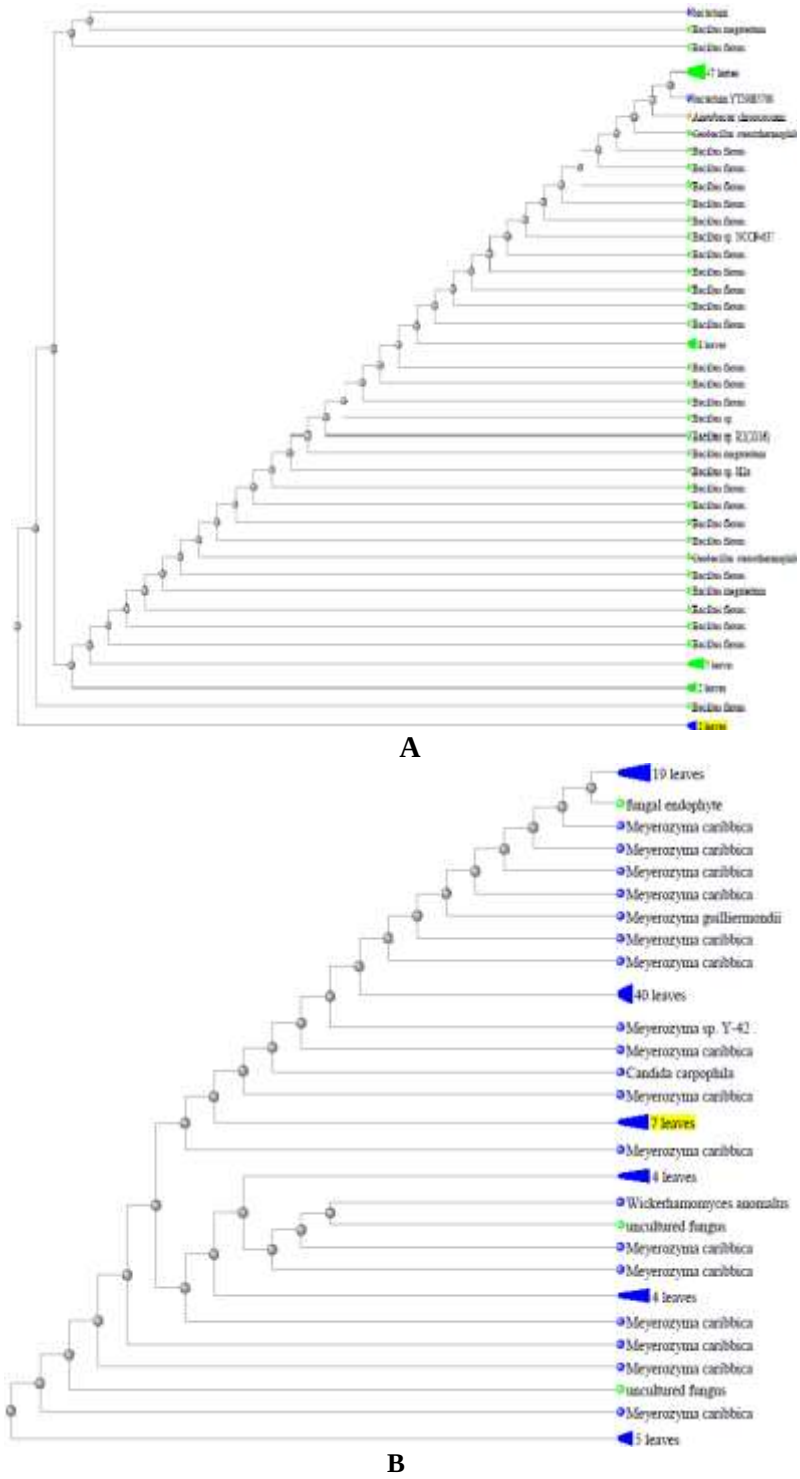


Fig. 5: The phylogenetic tree of bacterial isolates S1, *Bacillus megaterium* (A) and PY1 yeast, *Meyerozyma caribbica (B*)

with the database of NCBI Genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree (Fig. 4) was constructed using using BLAST pairwise alignments. The results revealed a 99% match of S1 with *Bacillus megaterium* KU570370.1. The S1, identified as *Bacillus megaterium*, can be accessed from NCBI database as *Bacillus megaterium* MF623895.1 *(https:// www.ncbi.nlm.nih.gov/nuccore/ 122 9396582*).

Isolate PY1: A single band of high-molecular weight DNA was observed (Fig 4a & b) which on amplification showed a 500 bp band. The 18S rDNA gene sequence was used to carry out BLAST with the database of NCBI Genbank database. Based on maximum identity score, the first 10 sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and phylo-genetic tree was constructed using BLAST pairwise alignments (Fig. 5). The results revealed a 99% match of PY1 with *Meyerozyma caribbica* KP860076.1. PY1 yeast, identified as *M. caribbica*, can be accessed from NCBI database as *Meyerozyma caribbica* MF623892.1 *(https:// www.ncbi.nlm.nih.gov/nuccore/MF6 23892.1)*.

Bacillus megaterium strain KU570370.1 and *Meyerozyma caribbica* strain KP860076.1 have the potential to degrade limonin in kinnow juice. These strains can be explored further for extraction of limonin degrading enzymes and processing of kinnow juice. The isolation of a

fermenting and limonin degrading yeast has in fact encouraged for the processing of kinnow juice into fermented beverages and wines.

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