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PLANT PATHOLOGY

Screening and characterization of newly isolated thermotolerant and ethanogenic strain of *Pichia kudriavzevii*

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Abstract

Screening and characterization of thermotolerant and ethanogenic yeast with improved economics for ethanol production was attempted in present study. Eight thermotolerant and ethanogenic isolates of yeast were isolated from the diverge ecosystem. All the isolates grew at 40°C but EM12 and ST1 showed better growth than other six. Evaluation of ethanol tolerance showed that five isolates tolerate up to 12% ethanol. Enzyme profiling of these isolates revealed that they were found to possess β-galactosidase. The EM12 isolate was found to produce maximum of 49.21 IU/ml β-galactosidase amongst all. Isolate EM12 was most potential ethanogenic amongst all, with yield of 44.4% and fermentation efficiency of 86.38%. This isolate found to produce 38 g/l of ethanol. This thermotolerant and ethanogenic strain EM12 showed better economic for ethanol fermentation at elevated temperature in laboratory experiment. Therefore, it was selected for further thorough characterization. Based on the morphological, cultural and biochemical characterization followed by molecular analysis of 18S rDNA, EM12 isolate was identified as the strain of *Pichia kudriavzevii.*

Highlights

- Eight thermotolerant and ethanogenic yeast isolates were obtained after extensive screening from the diverge ecosystem.
- Further screening based on ethanol tolerancewas attempted and then selected five isolates were adapted to 12% ethanol concentration.
- Yeast isolates were finally evaluated for ethanol fermentation by shake flask studies.
- The results showed EM12 isolate as the most as most efficient ethanol produce with a yield of 44.4% and fermentation efficiency of 86.38%.
- This selected thermotolerate isolate found to 38 g/l of ethanol.
- Selected EM12 isolate is characterized as newly isolated strain of *Pichia kudriavzevii*.

Keywords: Thermotolerance, Ethanol tolerance, Ethanol yield, Molecular characterization

Energy crisis and environmental pollution from the use of fossil fuels has become a serious global threat. Therefore to move towards a sustainable development path and explore the use of biofuel as cheaper and cleaner alternate to fossil fuels is the need of present time. There has been increasing worldwide interest in alternative sources of energy (Lin and Tanaka 2006, Haberl *et al.* 2013). With rapid civilization the need for fossil fuel is increasing which has led to the consequences of limited fossil fuel and its impact on environment changes due to global warming. Production of ethanol (bioethanol) is one way to reduce both the consumption of crude oil and environmental pollution (Christopher and Molly 2005, Saboori *et al.* 2014).

Sugarcane (*Saccharum officinarum*) is a high biomass tropical crop and contains about 12–17% total sugars, of which 90% is sucrose (Wheals *et al.* 1999). Sugar concentration in cane juice depends upon the variety, maturity and the time of harvest. Sugarcane juice normally has sufficient organic nutrients and minerals in addition to fermentable sugars, and thus could be considered as an ideal substrate for ethanol production. Ethanol from sugarcane juice is being produced commercially in Brazil (Moreira 2000). India is the second largest producer of sugarcane after Brazil. In India, ethanol is produced from molasses, but limited availability and alternative uses of molasses has led to search for alternative substrates which can produce the bioethanol at competitive cost (Zyl *et al.* 2012, den Haan *et al.* 2013).

Cost economics of the bioethanol production is one of the limitations so far as its commercial exploitation. Many researchers recommended that the efforts should now be directed towards identifying thermostable, ethanol tolerance with ability of broad spectrum substrate utilization and a capability of producing substantial amounts of ethanol at elevated temperatures are the key features required for the selection of strain of microbes for fermentation (Rajendran *et al.* 2015). Looking to the present short supply of ethanol and further growth of ethanol demand at the rate of 10% annually needs to strengthen the existing technology to improve ethanol productivity per unit time. Also focused research on utilization of alternative cheap substrates, besides molasses for its bioconversion into ethanol need to be attempted. Present effort is to carry out the screening of thermotolerant and ethanogenic strains with potential to utilize diverge cheap sources of substrate for the bioethanol production.

Materials and methods

Isolation of thermotolerant yeast

Samples for the isolation of yeast from diverge ecosystems such as muskmelon, honey, jaggery, soybean sauce, grape, sugarcane juice, sugarcane bagasse, *idli* batter and dairy effluent were collected. All samples collected were exposed to intense sunlight and fruits were allowed to ripen. These were enriched on YPD broth and incubated on orbital shaker at 40°C at 120 rpm for 24 h. After enrichment yeasts were isolatedusing on YPD agar plates. Each isolates then obtained were further purified on YPD agar plates and stored at low temperature in refrigerator on YPD slants.

Primary screening for thermotolerant yeast

The primary screening of isolated yeast was carried out on the basis of their thermotolerance. Each isolates were grown at four different temperatures in YPD broth on incubating shaker for 30 h. The temperature selected was 30°C, 35°C, 40°C and 45°C. After the course of incubation evidence of turbidity was measured spectrophotometrically at 600 nm. Viability of yeast cells incubated at 40°C was further reconfirmed by staining it with crystal violet followed by its microscopic observation (Kreger-Van Rij 1984).

Primary screening for ethanol tolerant yeast

Selected yeast strains showing temperature tolerance in primary screening were further evaluated for their ethanol tolerance. YPD broth containing different ethanol concentrations (6%, 8%, 10% and 12% v/v) was inoculated with each of the selected isolate of yeast, and incubated at 40°C for 48 h. The growth was estimated spectrophotometrically at 600 nm and compared with control where yeast strain was cultured in YPD broth without ethanol (Lee *et al.* 2011). Further the ethanol tolerance threshold of yeast under study was increased up to 12% by adaption. For the same the isolates was allowed to grow in higher concentration of ethanol at an increment of 0.5 v/v of ethanol in YPD broth for prolong period of time at 40°C. Viability and purity of these adapted yeast cells were confirmed by crystal violet staining during the study. Adapted culture was than centrifuged for 2-3 min at 10,000-12,000 rpm. Supernatant was discarded and pellet was streaked on YPD agar plates and incubated upto 4 days at 40°C.

Enzyme profiling of selected yeast isolates

Screening for Amylase activity: Selected isolates were studied for the presence of amylase. Distinct colony of each of the selected isolate was activated using YPD broth for 36 h at 40°C temperature. This activated culture was now spotted on starch agar plates. Then it was incubated at 40°C for 30

h. Amylase producing yeast strain found to show zone of starch hydrolysis surrounding to it. This was again reconfirmed by flooding the plates with iodine solution in which zone of hydrolysis appeared as colorlesswhereas rest of the plate appeared dark blue (Lee *et al.* 2011).

Screening for β-galactosidase activity: Selected yeast isolates were also studied for the presence of β-galactosidase. Colony from each of the isolate was inoculated in YPD broth and incubated on orbital shaker at 120 rpm at 40°C for 30 h for its activation. This activated culture was then streaked on lactose agar plates and incubated at 40°C for 2-3 days till their growth.

Quantitative estimation of β-galactosidase: Well grown colonies from lactose plate was inoculated in 2 ml of YPD medium and incubated at 40°C overnight using orbital shaking at 120 rpm. Then 1 ml sample was withdrawn and diluted in 9 ml same medium and optical density (OD) was determinedat 600 nm against blank as medium (Gupte and Nair 2010). From this 1 ml was transferred to an eppendorf tube and centrifuged at 14,000 rpm for 30 sec. The supernatant was discarded and cells were resuspended in 500 µl of Z buffer. To this 50 µl of 0.1% SDS was added and vortexed vigorously for 15 sec. Then 100 µl of ONPG (4 mg/ml) was added, vortexed and incubated at 40°C for 2-30 min till yellow colour developed. The reaction was quenched by addition of 500 µl of 1M Na_2CO_3. Then tubes were centrifuged at 14,000 rpm for 30 s. Supernatant was collected in a fresh tube and again OD was measured at 420 nm against a blank containing 500 µl of Z buffer, 100 µl of ONPG and 500 µl of 1M Na_2CO_3. The unit activity of β-galactosidase enzyme was calculated using following formula:

$$\text{Units of β-galactosidase activity} = \frac{1000 \times OD_{420}}{V \times t \times OD_{600}}$$

V= the volume of cells (ml); t = the incubation time (min)

Secondary screening of selected yeast based on ethanol productivity

After primary screening and enzyme profiling, further secondary screening of selected yeast isolates was carried out based on their ethanol production ability. Each isolated colony from YPD agar plates was inoculated in 100 ml YPD broth as inoculum medium in (250 ml) flask which was incubated at 40°C on orbital shaker at 120 rpm for 30 h to initiate fermentation (Dhaliwal *et al.* 2011). These were then inoculated in 400 ml production medium prepared in (500 ml) amber colored glass bottle. These bottles were incubated at 40°C for 48 h and samples were withdrawn at an interval of 12 h and sugar utilized and ethanol produced was estimated. Conversion rate of sugar into ethanol (ethanol yield %) was calculated according to following formula:

$$\text{Yield (\%)} = \frac{\text{Ethanol produced (g)} \times 100}{\text{Sugar utilized (g)}}$$

The fermentation process efficiency was calculated for selected yeast was studied in the shake flake experiment as follow:

$$\text{Efficiency} = \frac{\text{Practical yield} \times 100}{\text{Theoretical yield}}$$

Analytical methods used for the evaluation of ethanol productivity

Yeast cell count was monitored with a haemocytometer and the cell viability was assessed by staining the cells with 0.1% methylene blue solution (Borzani and Vario 1958). Reducing sugars were determined with the dinitrosalicylic acid (DNS) method (Miller, 1959). Ethanol was estimation by gas chromatography. Standard solutions of ethanol were prepared in distilled water containing 1% v/v of n-butanol as an internal standard, extracted and injected.

Peak area ratios of the ethanol vs n-butanol were calculated and plotted against ethanol concentration (% v/v) to afford a calibration curve which served for ethanol quantification in the fermentation samples. Broth samples of 600 µL containing yeast cells were collected at the designated times through the rubber septum by a syringe equipped with an 18G needle. The broth was transferred to an Eppendorf tube and the tube was centrifuged (8,000 g for 2 min at 30°C) to sediment yeast cells. Afterward, 500 µL of the clear supernatant were transferred to a new tube without disturbing the cell pellet, and then 5 µl of n-butanol (as internal standard) were added and the tube was vortexed for 30 sec

at maximum speed. Then 1 ml of ethyl acetate was added, followed by 5 min of vortexing. Finally, the tubes were centrifuged to facilitate phase separation (5,000 g; 2 min at R.T.), and the organic phase (upper) was subjected to GC analysis (Gerchman, *et al.* 2012). Analysis of ethanol and butanol was conducted using Thermo Fisher Scientific Trace 1110 model, equipped with a 15 length × 0.32 inner diameter x 0.45 outer diameter, on-column injector and FID conditions: 250°C; H_2, 80 PSI, equivalent to 80 ml/min; air, 80 PSI, equivalent to 100 ml/min and N_2, 80 PSI. The GC was also equipped with an internal air compressor and hydrogen generator. N_2 was used as carrier gas with pressure control (24 psi constant). The GC was connected to a computer and data was analyzed using Chrom Card Simple software. Oven temperature (and hence column and injector temperature) was initially set at 150°C and then elevated at the rate of 5°C/min to 200°C, thus giving a total run time of 10 min. Furthermore, 2 µL sample was injected manually at time 0, using a 5 µl Hamilton syringe and temperature cycle was started. Syringe was thoroughly washed with ethyl acetate between injections to avoid cross-contamination. Ethanol routinely came out at retention time equivalent to 65°C.

Characterization of selected yeast isolate

Morphological, Cultural and Biochemical characterization: Selected EM12 isolate was further studied for its morphological, cultural, biochemical and molecular characterization. Vegetative cell, ascospore and colony morphologies of the isolated yeasts grown on YM agar and 5% malt extract agar were observed and compared with type strain as described in The Yeast: a taxonomic study, 4th ed (Kurtzman and Fell 1998). Sugar utilization was studied using various sugars such as D-glucose, D-raffinose, D-sucrose, D-trehalose and D-lactose. The nutrient sugar (g/l00 ml) contains: 10 ml (10%) respected test sugar solution; 90 ml 1% peptone water; 1 ml Andrade's indicator. Broth was inoculated with loopful EM12 as a test culture and incubated at 40°C for 24 h. The tubes for acid and gas production were observed.

Molecular Characterization: 18 S rDNA sequencing and phylogenetic analysis of the EM12 isolate was carried out. Yeast DNA was extracted by method of Manitis *et al.* (1982). The rDNA was amplified using universal fungal forward primer, 1F-5'-CTGGTGCCAGCAGCCGCGGYAA-3' and 4R-5'-CKRAGGGCATYACWGACCTGTTAT-3' as reverse primer, (Machida, 2012). Amplicon purified by Gel/PCR DNA Fragment Extraction Kit (Geneaid Biotech Ltd., Taipei, Taiwan) was directly sequenced by ABI PrismTM BigDyeTM terminator cycle sequence ready reaction kit (Applied Biosystems, Stafford, USA) according to manufacturer's instruction. The resultant sequenced file were processed, trimmed and developed the contigs by using Chromas and BioEdit software. Developed contig were used for the identification of isolate at species level by using online tool BLAST of NCBI .Contigs were processed by offline tool sequin and resulted sequin processed files were submitted to genebank for accession number. Phylogenetic tree was constructed by using online BLAST tool.

Results and discussion

Isolation of thermotolerant yeast strain

Yeasts were isolated from the fruits, honey, idli batter and dairy effluent. Yeasts are the normal flora of ripened fruits (Eghafona *et al.* 1999). Osmotolerant yeast were found to be present in honey (Snowdon and Cliver 1996). Also amylolytic yeast are present in fermented foods (Ameh *et al.* 1989). Dairy effluent rich in whey may have possibility of lactose utilizing yeast. Keeping this in view, isolation of yeast from above mentioned diverse sources was attempted in present study. From each of this sample isolation was carried out after its enrichment for the yeast and different isolates were obtained as shown in Table 1. Total of eleven isolates were obtained during present study.

Table 1: Isolates of yeast with their coding

Source	*Yeast isolates obtained*
Muskmelon	MT1 and EM12
Grape	GT1 and GT2
Honey	HT1
Soybean sauce	ST1
Dairy effluent	DT1
Jaggery	JT1
Sugarcane juice	SJT1
Sugarcane bagasse	SBT1
Idli batter	IT1

All isolates showing distinct cultural characteristics are as shown. Isolate JT1 was producing large and dry colony on YPD agar plate. The isolate ST1 and EM12 found to produce medium size colony and the isolate GT1 and GT2 was found to produce small and shining colony.

Primary screening for thermotolerant yeast

Primary screening of 11 isolates obtained was attempted based on their thermotolerance. The ability to grow at different temperatures (30°C, 35°C, 40°C, 45°C), was determined. The result shows that eight isolates were found to grow at temperature above 40°C as shown in Fig. 1. Growth of isolates SBT1, SJT1 and IT1 was significantly affected as the temperature increased to 35°C from 30°C. At 40°C temperature, growth of these 3 isolates was severely affected while rest of the 8 isolates was found to grow at similar rate upto 40°C (Fig. 1).

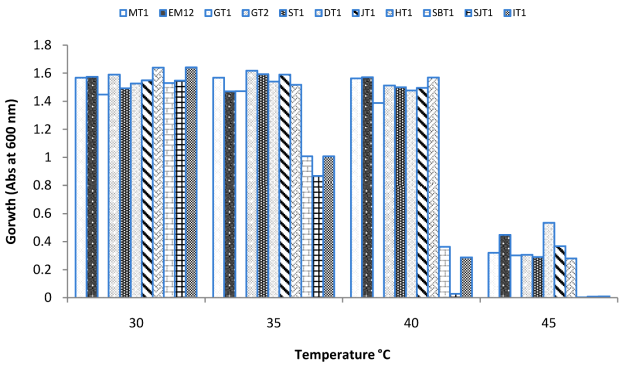

Fig. 1: Screening for thermotolerance of isolated yeast

Further increase in temperature there was significant decrease in the growth of all the isolates under study. MT1, EM12 and HT1 found to show same growth upto 40°C temperature. The isolates SBT1, SJT1 and IT1 were not able to grow at 45°C. (Lee *et al.* 1980) in his data reported that the *S. uvarum*was able to show its optimum growth at temperature 33°C.Based on this studies the 8 isolates which shows the thermotoleranceupto 40°C temperature were selected for their further evaluation for ethanol tolerance.

Primary screening for ethanol tolerant yeast

Eight selected yeast isolates which shows thermotolerance upto 40°C, were further screened for its ability to tolerate ethanol at various concentrations (6%, 8%, 10% and 12% v/v). The ethanol tolerance profile of isolates under study is depicted in Fig. 2.

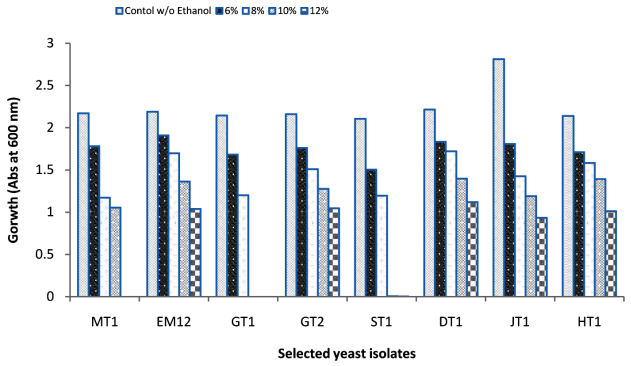

Fig. 2: Screening of selected yeast isolates based on ethanol tolerance

Ethanol was found to be inhibitory at the all concentration selected in the study. All isolates were able to grow upto 8% concentration of ethanol. However their growth was ceased. But at 10% concentration ST1 isolate were not grew. As the concentration of ethanol was further increased to 12%, the isolates MT1 and GT1 were not survived. Isolates EM12, GT2, DT1, JT1 and HT1 were able to grow upto 12% concentration (Fig. 2) although their growth was inhibited to some extent. These were allowed to adapt by repeating subculture into fresh 12% ethanol containing YPD medium until their growth was not affected significantly (Osho *et al.* 2005) reported in its paper that *S. cerevisiae* and *S. uvarum* showed growth in medium containing 9% ethanol. In same context, these isolates were shown 12% threshold for ethanol tolerance. These adapted isolates were selected for further studied.

Enzyme profiling of selected yeast isolates

Five selected thermotolerant and ethanol tolerant isolates were further studied for the presence of amylase and lactase. Amani *et al.* (2003) have reported that potatoes contain about 72% of starch. The transformation of these tuber wastes with high level of starch into microbial biomass requires amylolytic yeast strains with strong potential to hydrolyse starchy substrates to produce ethanol. Rogosa *et al.* (1947) reported that yeast could be able to ferment whey lactose to ethanol and overcome the problems related to bioremediation. Looking to the importance of these two enzymes in conversion of various substrates into fermentable sugar which will easily fermented to ethanol, this study was undertaken.

Screening for amylase activity: The amylase producing ability of the selected isolates was assessed by starch agar plate assay. Amylase was not produced by these isolates.

Screening for β-galactosidase activity: Selected isolates were also evaluated for their ability to utilize lactose. Results shows that all selected isolates were able to utilize lactose. The quantitative production of β-galactosidase by these yeast isolates was also studied. The results are summarizedin Table 2.

Table 2: β-galactosidase production by yeast isolates

Isolate	*β-galactosidase productivity (IU/ml)*
EM12	49.21
GT2	41.39
HT1	42.07
JT1	36.51
DT1	15.38

All yeast isolates were producing significant amount of β-galactosidase. EM12 and HT1 were found to be superior, as they produce 49.21 and 42.07 IU/ml of β-galactosidase. This ability of yeast under study to produce β-galactosidase reveals their potential to direct conversion of dairy processing waste into ethanol at elevated temperature has reported to use whey to produce ethanol (Guimaraes *et al.* 2010). Since the β-galactosidase can split the whey lactose into glucose and galactose out of which glucose can undergo fermentation by the yeast isolates under study, EM12 and HT1 could able to ferment whey lactose into ethanol.

Secondary screening of selected yeast based on ethanol productivity

Secondary screening of these five potential thermotolerant isolates was attempted based on their ability to produce ethanol in laboratory scale fermentation studies. The 20% inoculum was used to inoculate the 400 ml production media. 10% sucrose was used as the carbon source. The fermentation was monitored for sugar and ethanol production at 12 h interval for 48 h.

Sugar estimation of fermentation samples: The initial production medium consisted of 10% sucrose, after inoculation it was in the range of 8.45 to 9.45 g% as shown in the Table 3. The sugar standard curve is depicted in the Fig. 3, which was used to estimate the total sugar. The sugar utilization pattern was shown in Fig. 3.

Table 3: Evaluation of ethanol productivity by selected isolates

Culture	*Initial Sugar (%)*	*Sugar consumed (%)*	*Ethanol produced (%)*	*Ethanol yield (%)*	*Ethanol fermentation efficiency(%)*
EM12	9.45	8.55	3.8	44.44	86.38
GT2	9.75	8.10	3.0	37.00	71.23
HT1	8.85	7.65	2.5	32.68	63.59
JT1	9.15	7.80	2.3	29.49	57.14
DT1	8.55	6.90	2.2	31.88	61.66

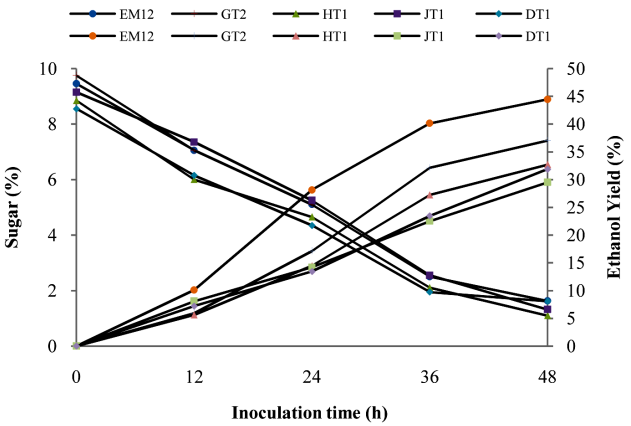

Fig. 3: Sugar utilization and ethanol production profile byselected isolate

After 48 h of fermentation, EM12 isolate was found to be most efficient in terms of sugar utilization. The isolates EM12, GT2, HT1, JT1 and DT1 were found to consume 90.5, 83.1, 86.4, 85.3 and 80.7 % of sugar respectively at the time of termination of fermentation (Table 3 and Fig. 3).

Ethanol estimation using GC: Standard ethanol was used to determine the qualitative analysis by knowing its retention time and co-relating it with retention time of fermented broths. The single peak was observed and the 0.6 min. retention time of samples and reference ethanol confirm the ethanol as sole fermentation product. The standard curve of known ethanol concentration vs peak area is obtained with r^2=0.99. Percent ethanol obtained by the selected isolates is shown in Table 3. Results show that EM12 isolate produced 38 g/l ethanol which is highest than other isolates under study. The isolate GT2 was also producing 30 g/l of ethanol which was also significant. While the other isolates HT1, JT1 and DT1 were found to produce 25 g/l,

23 g/l and 22 g/l ethanol respectively (Table 3 and Fig. 3).

Overall fermentation data shown in Table 3 reveals EM12 isolate as most efficient ethanol producer with ethanol yield of 44.44% with fermentation process efficiency of 86.38%. Jutakanoke *et al.* 2014 reported yeast spp. which was able to produce 0.22 g/g ethanol at elevated temperature. While the EM12 isolate found to produced ethanol production with efficacy of 44.44% at 40°C. The yield of EM12 isolate was also superior amongst other isolate GT2, HT1, JT1 and DT1 i.e. 37.0, 32.68, 29.49 and 31.88% respectively (Table 3). EM12 was found to be the most ethanogenic isolate in the fermentation studies. Beside ethanogenic it was also thermotolerant and producing highest β-galactosidase (49.21 IU/ml). Considering overall screening data, the EM12 was selected as most efficient for the ethanol fermentation. Looking to the better prospects of the isolate EM12, it was further studied for the thorough characterization.

Characterization of selected yeast isolate EM12

Morphological characterization: Morphology and biochemical characterization of highest ethanol producing strain EM12 were examined. The isolate EM12 cells are ovoid to elongate and occur in singly or in pairs few cells showed budding which is represented by marking arrows in Fig. 4. B and Table 4.

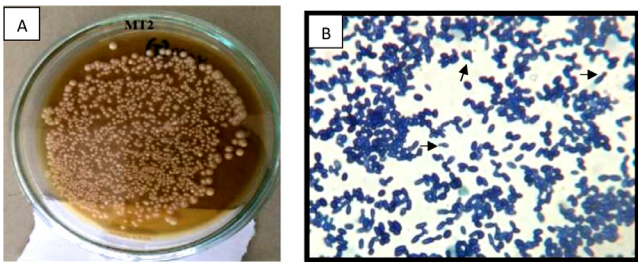

Fig. 4: Characteristics of selected EM12 isolate: (A) cultural and (B) morphological

Cultural and Biochemical characterization: The isolate EM12 formed butyrous and light-cream coloured colonies as shown in Fig. 4. A. The cultural characteristics are shown in Table 4. The sugar utilization test showed that EM12 found to utilize D-glucose, D-sucrose and D-lactose (Table 4). But other sugars like D-Raffinose and D-Trehalose were not utilised by the isolate EM12.

Molecular characterization: The thermotolerant yeast strain EM12 was genetically characterized using 18S rDNA analysis. Genomic DNA isolation was carried out. A single intact band of DNA was confirmed on agarose gel electrophoresis. Quality of DNA was further confirmed by taking A_{260}/A_{280} ratio, which was obtained 1.74. The concentration of DNA was adjusted to 50 ng/µl for further work. PCR amplification of 18 SrRNA genes was carried out by using conserved primer, after amplification it was purified by QIAquick PCR purification kit. Quality of the product was checked by gel electrophoresis and nanodrop. Cycle sequencing was performed from the cleaned products by using Applied Biosystem cycle sequencing kit. Which was further purified and Purified product was sequenced by capillary sequencer. Raw data files generated which was further converted in to FASTA format. An 809 nucleotides sequence fragment generated by aligning both the sequence by using SeqScape software.

Table 4: Characterization of selected EM12 isolate

Morphological characteristics	
Size	Small
Shape	Ovoid to elongate
Arrangement	Cells appeared in form of single or cluster
Cultural characteristics	
Size	Medium
Shape	Round
Margin	Undulate
Texture	Powdery
Elevation	Umbonate
Opacity	Opaque
Colour	Light cream
Biochemical characteristics	
Assimilation of sugar;	
Glucose	+
Raffinose	-
Sucrose	+
Trehalose	-
Lactose	+

This sequence compared with the available sequences in the databank with help of BLAST homology search. Data analysis using online BLAST tool of NCBI, ETGS1 has shown very close homology (99%) with the *Pichia kudriavzevii* (JF274497.1) confers that

the isolate belong to species level similarity with *P. kudriavzevii*. It also produced significant amount of β-galactosidase enzyme with value of 49.21 IU/ml. *P. kudriavzevii* cells appeared to be ovoid to elongate and occur in singly or in pair of few. The colonies were found to be butyrous and light-cream coloured on growth agar plates which matches with the characteristic of *P. kudriavzevii* isolated from juices (Jutakanoke *et al.* 2014). This selected isolate EM12 was characterized based on morphological, cultural, biochemical and molecular analysis as the new isolate of *P. kudriavzevii*. Gallardo *et al.* (2011) reported in that *P. kudriavzevii* cannot utilize lactose sugar. While our strain EM12isolated from source musk melon, identified as *P. kudriavzevii* found to utilize lactose, with ability to produce49.21 IU/ml of β-galactosidase. This revels that EM12 isolate is the new strain of *P. kudriavzevii.* Sequence of *P. kudriavzevii* strain was further processed by sequin stand-alone software and sequin generated file submitted to Genebank. A KM588183 accession number has been allotted from Genebank to this newly ethanogenic thermo tolerant isolate of *P. kudriavzevii*.

Conclusion

Extensive screening for the ethanogenic and thermotolerant yeast with improved process kinetics having ability to convert the diverge substrates into bioethanol is undertaken in the present study. Primary and secondary screening of these isolates revealed EM12 as the most efficient ethanol producer which has thermotolerance up to 40˚C and ethanol tolerance threshold is 12% ethanol. It was also found to produce β-galactosidasewhich confers the ability of this isolate to direct bio convert the whey based lactose waste into ethanol. In fermentation studies, it was found to produce 38 g/l ethanol with fermentation efficiency of 86.38%. Overall prospects of the screened out strain of *P. kudriavzevii* was showing better potential for its exploitation for the ethanol production and elevated temperature.

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