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Batch Fermentation of Underutilized Pear Fruits using Newly Isolated Stress Tolerant Ethanogenic Yeast *Pichia kudriavezeii* BGY1

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ABSTRACT

To explore high efficacy native stress tolerant yeast strain for better ethanol yield as compared to *Saccharomyces cerevisiae,* five isolates were selected by plate assay and quantified spectrophotometrically for ethanol production. The yeast cultures varied in their ethanogenic capabilities and ethanol tolerance limits. The isolate BGY1 showing better *in vitro* ethanol production (7.1 g/l) and alcohol dehydrogenase (ADH) activity (9.13 U/ml) in YPD broth in 48 h along with better tolerance to higher ethanol concentration (20%) as compared to *S. cerevisiae* and other strains, was found superior. Except for *S. cerevisiae*, rest of the four yeast isolates showed ethanol production during growth on xylose as carbon source with maximum growth and ethanol yield shown by BGY1. All the five cultures were tested for their alcohol producing efficacies using diluted unprocessed whole fruit pulp of inferior quality pear *(Pyrus communis)* under liquid state fermentation (LSF) conditions. Batch fermentation with native yeast isolate BGY1 resulted in higher ethanol yield (38.7 g/l) as compared to that with *S.cerevisiae* (20.1 g/l) in 48 h. Ability to grow in YPD broth with higher (30%) glucose concentration and at higher temperature (44^0C) indicated better osmo and thermo tolerance of the isolate BGY1 as compared to *S. cerevisiae* and other cultures. The isolate BGY1, comprizing of elongated cells with pseudohyphal structures as revealed by phase contrast microscopy and SEM was identified as *Pichia kudriavezeii* BGY1 based on 5.8 SITS rRNA gene sequence analysis.

Keywords *Pichia* sp., Stress tolerance, Alcohol dehydrogenase, Ethanol production.

INTRODUCTION

Increasing emissions of noxious gases in the environment upon burning of hydrocarbon based fossil fuels has lead to an increase in global warming (Dhaliwal et al. 2011). Bioethanol, is biologically generated fuel that can replace the fossil fuels in the form of gasohol (Isono et al. 2012). Bioethanol can be produced either from sugary substrates or lignocellulosic feedstocks (Ma et al. 2017). The bioconversion of sugar from these substrates into bioethanol is carried out by the enzyme alcohol dehydrogenase (ADH) that catalyzes the forward reaction converting acetaldehyde to

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ethanol and is synthesized by some bacteria such as *Zymomonas mobilis* and the yeasts such as *Saccharomyces cerevisiae, Kluyveromyces* sp. and *Pichia kudriavezeii* (Fonseca et al. 2008). *Saccharomyces cerevisiae,* most widely employed for industrial ethanol production has certain disadvantages due to some of its limitations like sensitivity towards the nature and concentration of fermenting sugar, ethanol and temperature. It has narrow range of substrate, preferably it uses glucose for ethanol production but is unably to ferment C_5 sugars (e.g. xylose) into ethanol (Hashem et al. 2014). In assessing an yeast strain for industrial use, specific physiological properties are required such as tolerance to temperature, sugar and ethanol concentrations (Yuangsaard et al. 2013). Moreover, the alcoholic fermentation is an exothermic reaction, therefore, the strain used should also be tolerant to higher temperature (thermotolerant) so as to achieve the higher product yield making the process more cost effective (Zhao and Bai 2009). *Pichia* sp., having more tolerance to sugar and alcohol concentrations has been reported as more promising fermenting yeast as compared to *S. cerevisiae* for alcohol industry and is also capable of utilizing both hexose and pentose sugars with higher ethanol yield (Ngoc et al. 2013).

Availability of feed stock for alcohol industry is also a major concern limiting ethanol production. India is well known for its high diversified fruit production. Pear (*Pyrus communis*), a highly perishable and nutritive fruit, is a major fruit crop of Kumaon and Garwal hilly regions but around 20 to 30% of total fruit produce is left unutilized and goes waste due to lack of transportation, proper post-harvest processing technologies and knowledge about its nutritive values. Pear fruit is a potential energy source for ethanol production (Kishor et al. 2016). Considering the need for search of a more efficient stress tolerant yeast strain for industrial bioethanol production together with the sustainable feed stock for ethanol industry, attempts were made to identify a high efficacy novel yeast strain during present investigation. Emerging yeast *Pichia kudriavezeii* isolated during this study can combat these stresses and constitute capability to withstand high sugar and heat ranges. The newly isolated *Pichia* strain was further evaluated for its efficacy for bioconversion of whole per fruit pulp into bioethanol and compared with *S. cerevisiae* during batch fermentation.

MATERIALS AND METHODS

Yeast cultures and growth conditions

Twenty five different cultures of yeast were isolated from diverse rotten fruit sources using yeast extract peptons dextrose agar (YPD) medium containing (%, w/v)) Yeast extract 1.0, peptone 2.0, glucose 2.0, agar 2.0 (Gupta et al. 2009). *Saccharomyces cerevisiae,* ATCC 9763 procured from MTCC, Chandigarh, India was used as standard culture. The cultures were grown on YPD medium at 28 ± 2 ^{0}C for 48 h. The isolates were routinely sub-cultured at an interval of 15 d and maintained on the same medium.

Screening of yeast isolates for ethanol production

Preliminary selection of ethanol producing cultures was done by qualitative plate assay using cerric ammonium nitrate reagent with slight modifications (Pinyou et al. 2011). Wells made in YPD agar plates by removing agar plugs, were filled together with active yeast cultures and cerric ammonium nitrate reagent and visualized for the development of intense red color. Based on the plate assay, five isolates (BGY1, BGY2, DPY2 and DPY1) and *S. cerevisiae* showing intensely red color were selected. All five cultures were further quantified for *in vitro* ethanol production following the method given by Pinyou et al. (2011) with slight modifications. YPD broth (containing 2% glucose) inoculated with active culture (OD: 0.8) at the rate of 10% (v/v) was incubated at 28 ± 2 ^{0}C for 96 h. The samples were withdrawn periodically at an interval of 24 h and color was developed by adding acidic cerric ammonium nitrate reagent. The intensity of color was measured spectrophotometrically at 525 nm and ethanol was estimated using standard curve.

Determination of ethanol tolerance

For determination of *in vitro* ethanol tolerance of yeast cultures, YPD broth containing different concentration of ethanol (0 to 20%, v/v) was inoculated with active yeast cultures at the rate of 10%, v/v individually and incubated for 48 h at 28 ± 2 ^{0}C. Cultures were

then evaluated for cell viability by measuring optical density at 600 nm by UV visible spectrophotometer. The maximum ethanol concentration upto which the culture could grow was determined in each case.

Estimation of intracellular alcohol dehydrogenase (ADH) activity

The selected isolates and *S. cerevisiae* cultures were evaluated for the activity of the enzyme alcohol dehydrogenase responsible for ethanol production. ADH enzyme preparations were made following the method given by Gupta et al. (2009). YPD grown active broth cultures (48 h old) were centrifuged at 8000 rpm for 10 minutes (4^0C) and cell pellets washed twice with 0.1 M phosphate buffer (pH 7.5). The cell extracts were prepared by sonicating cell preparations with glass beads (0.7 mm diameter) at 0^0 C for 2 min at an interval of 30 s with LABSONIC U-sonicator (133V, 0.5 repeating cycles per seconds). Unbroken cells and debris were removed by centrifugation at 4^0C for 10 min at 12000 rpm. Purified cell extracts were used as crude enzymes and for estimation of protein. Crude enzyme preparations from all the selected five yeast cultures were analyzed for ADH enzyme activities. Enzyme activity was determined following the method described earlier. The reaction mixture contained 0.1 M sodium pyrophosphate buffer, pH 9.2, 1.5 ml, 2.0 M ethanol 0.5 ml, 0.025 M NAD +1.0 ml and crude enzyme (0.8 ml). The increase in absorbance at 340 nm for 3-4 min at room temperature (25^0C) was recorded. Δ Absorbance (340 nm per min) was calculated from the initial linear portion of the curve. One unit of enzyme activity is the amount of enzyme required to reduce one micromole of NAD^+ per min at 25 ^{0} C under specified conditions. Specific ADH activity was calculated using the formula given below:

$$\text{ADH units/mg protein} = \frac{\text{A340/min}}{6.22 \times \text{mg protein/ml reaction mixture}}$$

Xylose (C_5) assimilation for *in vitro* growth and ethanol production

All the five yeast cultures were evaluated for *in vitro* xylose assimilation and ethanol production during growth in YPD broth supplemented with 2% xylose (pentose sugar) as the only carbon source. For growth determination and ethanol production from xylose, YPD broth containing xylose (2%) was inoculated with active culture at the rate of 10% (v/v) and incubated at 28 ± 2 ^{0}C for 72 h in each case. Aliquots were withdrawn after 48 and 72 h and analyzed spectrophotometrically for cell density by measuring optical density at 600 nm. Subsequently, concentration of ethanol produced in xylose fermenting broth was also checked spectrophotometrically at 525 nm using cerric ammonium nitrate reagent as describe by Pinyou et al. (2011).

Batch fermentation of pear fruit-pulp and estimation of ethanol

The underutilized or rotten/damaged pear fruits *(Pyrus communis)* were collected from local vendors of Champawat district (Uttarakhand) and whole fruits used without any pre-treatment or processing for ethanol production. The fruits after thorough washing with distilled water were chopped and grinded in mixer grinder to make a slurry. The whole fruit pulp was diluted with sterile distilled water in 1 : 9 (w/v) ratio and pH was adjusted to 5.0. The samples were sterilized by autoclaving at 15 lb psi for 20 min and inoculated with active cultures (at the rate of 10%, v/v) of the 4 yeast isolates and *S. cerevisiae* individually. Inoculated samples were incubated for first 16 h under oxic conditions in an incubator shaker (120 rpm) at 28 ± 2^0C followed by incubation under static conditions upto 72 h. The fermented samples were centrifuged at 8000 rpm for 10 min and the supernatant analyzed for ethanol content. The ethanol was recovered from the fermented fruit samples by distillation in separate graduated dry tubes immerzed in ice cooled water. The distillates were analyzed for ethanol contents by Nucon gas chromatograh model 5700 using flame ionization detector.

Determination of stress (osmo-and thermo-) tolerance

The potential yeast isolate BGY1 and *S. cerevisiae* were further evaluated for their *in vitro* sugar and

temperature tolerance limits (Yuangsaard et al. 2013). For heat stress determination, YPD broth inoculated with active culture (OD : 0.8) at the rate of 10% (v/v) was incubated for 48 h at different temperature range of 28 to 44 ^{0}C. Osmo- tolerance of the cultures was determined by growing the cultures in YPD broth supplemented with different concentrations of glucose (2 to 26%, w/v) at 28 ^{0}C for 48 h. Growth of the cultures under both the stress conditions was then determined spectrophotometrically by measuring optical density at 600 nm. The glucose concentration and the higher temperature that completely suppressed the growth was determined.

Characterization and identification of the potential isolate BGY1

The selected wild type strain was identified on the basis of phenotypic and genotypic characters. Live unstained cell preparations were visualized by phase contrast and scanning electron microscopy. Molecular idenfification was done on the basis of 5.8S ITS rRNA gene sequence analysis.Genomic DNA for molecular identification was extracted using a chromous Genomic DNA isolation kit. A PCR amplification of 5.8 S ITS rRNA gene was carried out using forward primer ITS-F; (5' GRAAGNAHADGTVGKAAYAWSG-3') and the reverse primer ITS-R: (5'-TCCTNCGYTKATKGVTADGH-3'). The PCR amplification was done under the following conditions 95 ^{0}C, 5 min ; 35 cycles of 94 ^{0}C, 30 sec; 52 ^{0}C, 30 s and 72 ^{0}C, 45 s; l cycle of 72 ^{0}C, 7 min. PCR amplified products were then purified, sequenced and compared with sequences available in nucleotide data base (NCBI) using the BLAST algorithm. Multiple sequence alignment was carried out with CLUSTAL W. Phylogenetic analysis was carried out by neighbor joining with MEGA version 5 program.

Statistical analysis of data

Analysis of variance (ANOVA) was done with statistical software using the program Stpr 2 and Stpr 3. All the experiments were conducted in triplicates and the results have been reported in terms of critical difference (CD).

RESULTS AND DISCUSSION

Selection of potential native ethanogenic yeasts

For selection of an indigenous high efficacy alcohol fermenting yeast, a total of 25 cultures were isolated from diverse rotten fruit samples viz. black grapes, banana, apple, chiku and date palm using the pure culture techniques and tested for their *in vitro* ethanol producing capabilities. Although, all the 25 isolates showed ethanol production during qualitative testing but, only 4 isolates along with the standard culture of *Saccharomyces cerevisiae* were selected based on their higher potentiality. During qualittative plate assay, the reagent cerric ammonium nitrate is used that reacts with ethanol and forms red color. Intensity of the color produce is used as a qualiative measure for selection of ethanogenic strains (Pinyou et al. 2011). Thus, the four newly isolated yeast cultures (DPY1, BGY2, DPY2, BGY1) and *S. cerevisiae* producing intense red color were selected. The ethanol production in YPD broth was quantified spectrophotometrically after 48 h in batch cultures. The *in vitro* ethanol yield for various cultures varied from 4.6 to 7.1 g/l. Comparative evaluation of the selected cultures for ethanol producing capabilities clearly revealed their variable potential for ethanol production. The isolate BGY1 showed maximum ethanol production that was significantly higher than that for *S.cerevisiae* and rest of the cultures. The study revealed that natural fruits especially black grapes and date palm harbor a variety of yeasts and can be used as rich source for isolating high efficacy ethanogenic strains.

Ethanol tolerance limits of selected strains

Since higher concentration of ethanol becomes inhibitory for microbial growth, therefore, it is essential to determine the ethanol tolerance limits of the fermenting yeast. During present study, therefore, the *in vitro* ethanol tolerance limits of the selected ethanogenic strains was determined by growing the cultures in YPD broth containing varying concentration (0-20%, w/v) of ethanol. Growth of all the cultures decreased gradually with increasing concentration of ethanol in the broth. The isolate BGY1 showed much higher tolerance to ethanol (showing growth upto 20% ethanol) as compared to that of *S.*

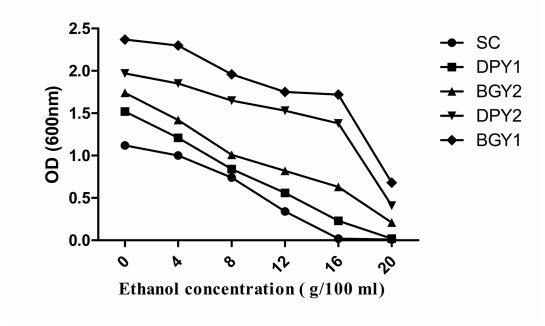


Fig. 1. Growth capabilities *(in vitro)* of selected cultures in YPD medium containing different concentrations of ethanol at 48 h.

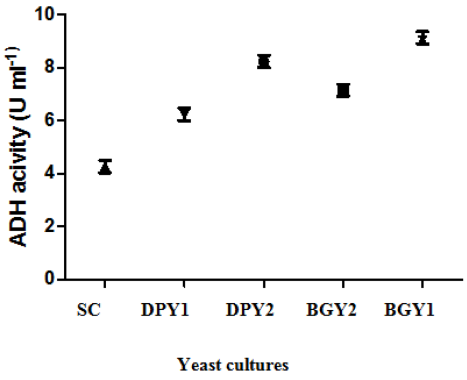


Fig. 3. Intracellular alcohol dehydrogenase activity of YPD grown yeast cultures at 48 h.

cerevisiae that could grow only upto 14% ethnol concentration (Fig.1). Ethanol inhibits microbial growth by inactivating cellular proteins, altering membrane permeability due to dissolution of lipids, ereating porosity in membrane, damaging mitochondrial DNA and causing inactivation of enzymes such as hexokinases and alcohol dehydrogenases in yeast cells. Higher concentrations of ethanol become toxic to the living cells of even ethanol producing strains, thereby limiting their growth, metabolic activity and in turn the ethanol yield as is also evident in the present study. A direct correlation between ethanol tolerance limits of yeast cultures and ethanol yield in broth cultures was therefore, observed invariably in all the cases; the isolates showing higher ethanol tolerance also depicted higher ethanol yield during *in vitro* alcoholic fermentation. The study shows clearly the diversity of alcohol fermenting yeast from

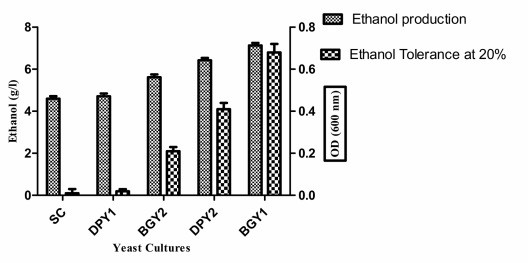


Fig. 2. *In vitro* ethanol production and tolerance (at 20% concentration) of newly isolated yeast cultures in comparision to *S. cerevisiae* in YPD broth.

diverse natural sources with variable potential for ethanol production as well as for ethanol tolerance. The black grape isolate BGY1 with maximum ethanol tolerance at 20% concentration, also showed highest ethanol yield (7.1 g/l) in the medium (Fig. 2). The isolate BGY1 was thus found superior among all the newly isolated cultures as well as the commonly used alcohol fermenting yeast *S. cerevisiae*. Higher alcohol tolerance limits of yeast observed in present study has also been well reported (Gupta et al. 2009).

Intracellular alcohol dehydrogenase activity of yeast cultures

Alcohol dehydrogenase (ADH) is the key enzyme responsible for ethanol production in ethanogenic organism and is produced intracellulary by most of the yeasts. Therefore, ADH activity is considered as a measure for determining the ethanol fermenting capability of the organism; higher is the ADH activity more will be the ethanol production. Crude intracellular enzyme preparations of YPD grown yeast cultures for 48 h showed significant differences in ADH enzyme activities that varied from (4.27 to 9.13 Uml^{-1}) (Fig. 3). Maximum ADH activity (9.1 Uml^{-1}) recorded for BGY1, was significantly higher than the ADH activities recorded for other cultures. The wild type strains isolated from natural fruit sources during present study showed better alcohol dehydrogenase activities as compared to *S. cerevisiae* that showed minimum values (4.27 Uml^{-1}). Earlier studies have

also reported the lower intracellular ADH activity (4.135 Uml^{-1}) for *S. cerevisiae* cells during alcoholic fermentation of sugarcane juice (Gupta et al. 2009). The *in vitro* ethanol production in YPD broth containing 2% glucose gradually increased upto 48 h but thereafter a gradual decline was noted in all the cases; this gradual decline in ethanol production might be due to the inhibition of alcohol dehydrogenase enzyme activity in yeast cultures with increasing ethanol concentration. The activity of the enzyme alcohol dehydrogenase is regulated by feedback inhibition, i.e. the end product ethanol represses the activity of the enzyme (ADH) responsible for its own synthesis.

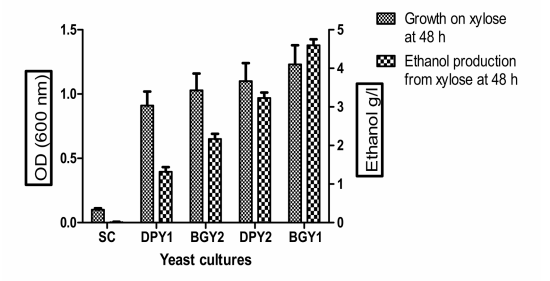


Fig. 4. Xylose (C_5 sugar) assimilation capability along with ethanol production during *in vitro* fermentation of YPD broth containing xylose as a carbon source.

Xylose (C_5 sugar) fermenting ability

Utilization of different sugars is one of the most common restrictions for majority of yeasts. These yeasts appear to have limited value for ethanol production because of lack of wide substrate utilizing ability. Utilization of narrow substrate range for alcoholic fermentation by *S. cerevisiae,* the most commonly used yeast for industrial ethanol production, is also one of the major constraints limiting ethanol production. It can utilize mainly hexose sugar, glucose, as carbon source for ethanol production. Better ethanol yield can be achieved if the fermenting organism has the ability to utilize pentose (xylose) as well as hexose (glucose) sugars for ethanol production from lignocellulosic biomass which are comprised of both C_5 and C_6 sugars. Only few yeasts, such as *Candida* sp., *Metschnikowia* sp. and *Pichia* sp. are reported to have potential to ferment xylose into ethanol. Several species of *Candida* and *Pichia* have emerged as good fermentors of D-xylose (Kim et al. 2011). Attempts were, therefore, made during present investigation to identify the newly isolated wild type ethanogenic yeasts for *in vitro* xylose assimilation and fermentation efficacies and to explore the possibility of broad spectrum utilization of sugars from green biomass with enhanced alcohol yield. All the four isolates showed active growth in YPD broth (containing 2% xylose) except for *S. cerevisiae* after 48 h of incubation at 28 ± 2^0C. Maximum growth was observed for BGY1 followed by DPY2, BGY1 and DPY1 at 600 nm indicating xylose assimilation during growth. Fermenting xylose as carbon source, the isolate BGY1 showed maximum ethanol yield (4.6 g/l) among all the other cultures (Fig. 4). Minimum ethanol production was recorded for DPY1 (1.32 g/l). In comparison to yeast isolates, *S. cerevisiae* showed a very little growth with no ethanol production at all utilizing xylose as carbon source in YPD broth after 48 h at 28 ^{0}C ± 2. Ability of yeasts to ferment xylose depends upon the presence of three enzymes xylose reductase, xylitol dehydrogenase and xyulokinase which function with same coenzyme system under anaerobic conditions. Enzyme xylose reductase oxidizes D-xylose to D- xylitol which is then converted to D-xylulose by the enzyme xylitol dehydrogenase. D-xylulose thus produced is phosphorylated to D-xylulose-5 phosphate by the enzyme xylulose kinase. Conversion of D- xylulose-5 phosphate into ethanol occurs via pentose phosphate and Embden Meyerhoff Parnas pathways. Within the PP cycle, xylulose-5-phosphate is metabolized to glyceraldehyde-3 phosphate and fructose-6-phosphate, thenthese compounds are converted to pyruvate via EMP pathway (Eric et al. 2010). The pyruvate is finally converted to ethanol by alcohol dehydrogenase enzyme. Many of the genetically engineered yeast cells have been developed that can utilize pentose as well as hexose sugars under aerobic conditions also (Lee et al. 2017).

Ethanol production from pear fruits during batch fermentation

Pear fruits (*Pyrus communis*) are one of the major fruit crops of hilly regions of Uttarakhand with maximum production share of around 38% (India 2012). It is a highly nutritive, minerals and vitamin rich perishable

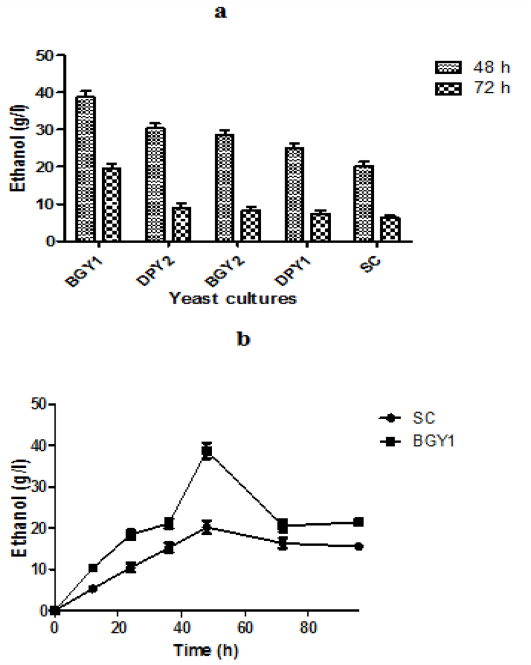


Fig. 5. Ethanol yield from diluted whole pear fruit pulp fermented with different yeast cultures for 72 h under liquid state batch fermentation conditions.

fruit. However, a major portion of total fruit produce (around 20-30%) is spoiled and goes waste due to its highly perishable nature, lack of proper transportation and post-harvest processing technologies in the remote hilly areas. However, this inferior quality or rotten fruits can be utilized for bioethanol production upon microbial fermentation. Considering these facts, attempts were made during present investigation for utilization and bioconversion of these underutilized fruits into bioethanol by newly isolated yeast strains which were having better stress tolerance as compared to the commonly used *S.cerevisiae.* Four selected yeast isolates and *S. cerevisiae* cultures were compared for their efficacies for bioethanol production from diluted fruit pulp samples under liquid state batch fermentation conditions. The fermenting yeasts varied in their ethanol producing capabilities utilizing fruit pulp as the substrate. Ethanol production was monitored upto 72 h incubation time at 28 ± 2 ^{0}C. Though, the selected isolates and *S. cerevisiae* varied in their ethanol producing capabilities from fruit pulp, but all the test cultures showed better ethanol yield after 48 h of fermentation that varied from 20.2 g/l (*S. cerevisiae*) to 38.7 g/l (BGY1) (Fig. 5a). The newly isolated strain BGY 1 showing maximum ethanol yield from the fruit pulp during liquid state batch fermentation was selected as high efficacy superior strain among all the cultures and *S. cerevisiae*. Variability in ethanol production by different strains of *S.cerevisiae* and *Zymomonas mobilis* has been well documented (Li et al. 2011). Further, to optimize the fermentation period for ethanol production by the selected isolate BGY1, the fermented samples were analyzed for ethanol content during batch fermentation of pear fruit upto 96 h at an interval of 12 h and compared with that of *S. cerevisiae* (Fig. 5b). Maximum ethanol yield obtained at 48 h further conformed the optimum fermentation period of 48 h for alcoholic fermentation of diluted pear fruits by alcohol fermenting BGY1 and *S. cerevisiae* and higher alcohol producing efficacy of BGY1 as compared to *S. cerevisiae*.

Characterization of BGY1 for osmo-thermo- tolerance

Higher sugar concentration and temperature is known to inhibit the microbial growth during batch fermentations. Since, alcoholic fermentation is an exothermic reaction, therefore, tolerance of fermenting organism to higher temperature is of significant importance as it provides several advantages such as reduced cooling cost, reduced contamination risk by mesophilic microorganisms and increased rates of sugar to ethanol conversion, resulting in higher ethanol productivity. Higher sugar concentration limits the growth by causing osmotic lysis of the cells known as plasmolysis. Moreover, the enzyme alcohol dehydrogenase is regulated by feedback inhibition and the product ethanol represses the enzyme activity. Therefore, the ethanogenic microbial strains with better tolerance to sugar (osmotolerance), temperature (thermotolerance) and ethanol are preferred over others for alcoholic fermentation. The selected strain BGY1 was evaluated for its glucose tolerance in YPD broth containing varying concentrations of glucose (2-30%). The cell growth of yeast cultures was affected by increasing glucose concentration in fermenting broth from 2% to 30%. The optimum growth was recorded at 2% sugar concentration in all the cases while a gradual decline in cell density

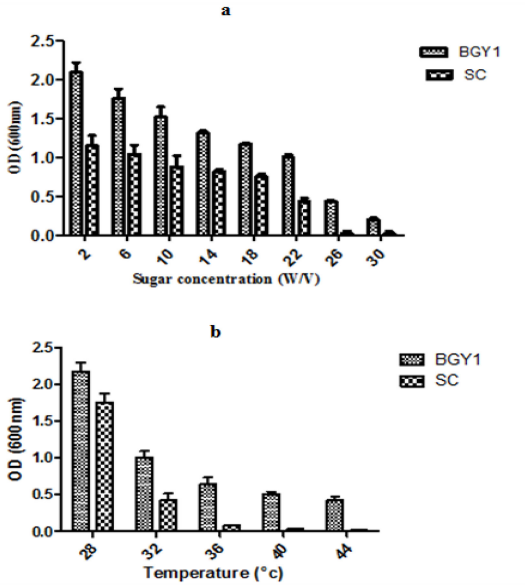
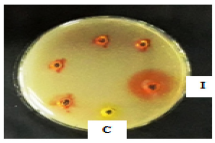


Fig. 6. Osmo- thermo-tolerance of BGY1 and *S. cerevisiae* as determined by *in vitro* growth capabilities at different temperatures and sugar concentrations.

was noted with increasing sugar concentrations. The isolate BGY1 was capable of growing upto 30% sugar concentration while *S. cerevisiae* could grow only upto 22% sugar concentration as indicated by optical densities measured at 600 nm (Fig. 6a). Survival of the cells of BGY1 at higher sugar concentration indicated better osmo-tolerance as compared to *S. cerevisiae*. The results were consistent with the other studies (Ajit et al. 2017). Several yeasts have been reported as osmophilic organisms capable of growing at 50 to 60% sugar concentration (Bubnova et al. 2014). High temperature causes cells to become dehydrated because of breaking of covalent bonds and loosening of ionic interactions; but survival at high temperature (thermotolerance) is one of the major desirable characteristics of the fermenting yeast because of the exothermic nature of the reaction. Therefore, the isolate BGY1 was evaluated for its tolerance to temperature stress upto 44 ^{0}C and compared with *S. cerevisiae*. It is apparent from the data that both the cultures grew optimally at 28 ^{0}C and with increasing temperature a decline in cell growth was recorded (Fig. 6b). The isolate BGY1 could grow upto 44 ^{0}C while *S. cerevisiae* could grow only upto 36 ^{0}C indicating better thermotolerance of the newly isolated strain BGY1 as compared to *S. cerevisiae*. Tolerance of *P. kudriavezeii* to higher temperature (45 ^{o}C) has also been reported by previous workers (Chamnipa et al. 2018)

Identification and characterization of ethanogenic strain BGY1

During the present study, a number of native newly isolated yeast cultures capable of producing ethanol were screened for their osmo-thermo and ethanol-tolerance. Among all the cultures, the isolate of black grape BGY1 showed higher ethanol production. Initial characterization of the yeast isolate BGY1 was done on the basis of cultural, morphological and biochemical characteristics. Culturally BGY1 formed cream colored, slimy pin pointed raised colonies on YPD agar. Phase contrast and scanning electron microscopic studies revealed the budding nature of the yeast with elongated cells. The isolate was able to assimilate and ferment both hexose (glucose) and pentose (xylose) sugar producing ethanol, showed high intracellular alcohol dehydrogenase activity, higher stress tolerance for sugar, temperature and ethanol as compared to others and was identified as superior strain. The culture was finally characterized using 5.8 S ITS rRna gene sequence as the molecular



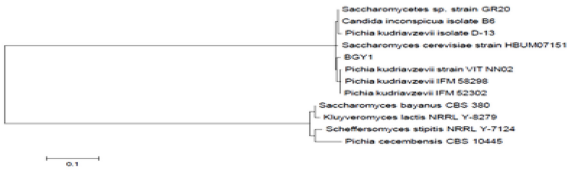


Fig. 7. Qualitative detection for *in vitro* ethanol production during plate assay. C = Control, I = BGY1. **Fig. 8.** Phylogenetic tree showing genetic relativeness of selected yeast isolate BGY1 with other alcohol producing yeast strains based on 5.8 S ITS gene sequence.

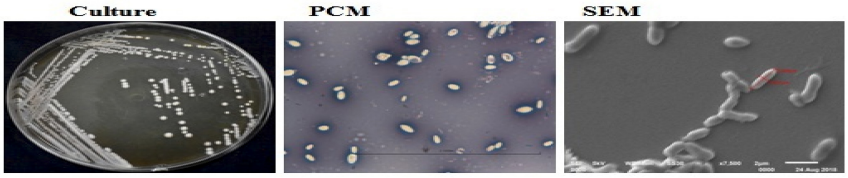


Fig. 9. Culture plate, phase contrast and scanning electron microscopic view of isolate BGY1.

marker. The phylogenetic analysis based on BLAST search using 5.8 S ITS rRNA gene sequence exhibited its maximum homology (98%) with *Pichia kudriavezeii* IFM 5688, *Pichia kudriavezeii* IFM 52302. *Pichia kudriavezeii* IFM 5753 and *Pichia kudriavezeii* IFM 5749 and was thus deginated as *Pichia kudriavezeii* strain BGY1 (Figs. 7–9). Although *Saccharomyces cerevisiae* is restricted to produce ethanol only from hexose sugars but *Pichia* species has been identified as potential glucose and xylose fermenting yeast. Several species of *Candida* and *Pichia* have emerged as good fermentors of D-xylose (Kim et al. 2010) *P. stipitis* and *P. orientalis* have been well identified for ethanol production utilizing D- xylose (Isono et al. 2011, Silva et al. 2011). Higher thermo tolerance of the yeast *Pichia kudriavezeii* BGY1 as observed by us during present investigation has also been documented for high temperature ethanol production by *Pichia kudriavezeii* RZ8-1 by previous workers (Chamnipa et al. 2018).

CONCLUSION

Pichia kudriavezeii BGY1 a novel yeast strain isolated from black grapes was identified as a high efficacy ethanol producing yeast with high alcohol dehydrogenase activity which can effectively ferment hexose (glucose) as well as pentose (xylose) sugars into ethanol. Its higher tolerance to ethanol, heat and sugar streas together with higher alcohol producing and xylose fermenting abilities indicate it as a better alcohol producing strain as compared to *S. cerevisiae.* The efficacy for ethanol production by the yeast can be further enhanced after proper optimization and bioreactor studies. In future, the strain appears to have potential for industrial ethanol production from underutilized/spoiled fruits after proper pretreatment and bioprocessing of the substrate.

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