Original Research Article

Isolation and characterization of non-*Saccharomyces* yeasts with improved functional characteristics for ethanol production

Abstract

Out of the twenty yeasts isolated from over-ripened fruits and naturally fermenting sugarcane juice, four isolates showing relatively higher fermentation ability were screened for their fermentation potential. Isolate Y-4 produced relatively higher ethanol than the other isolates from a glucose concentration of 15% (w/v) and was thus selected for future experiments. Microscopic observations revealed that the cells of isolate Y-4 produced ascospores but not arthrospores or ballistoconidia. Scanning electron micrographs (SEM) of the selected yeast showed oval to spherical cells with diameter ranging from 4.5 to 6.2 µm. On the basis of the SEM images and 28s rRNA gene sequencing, isolate Y-4 was identified as a strain of *Issatchenkia orientalis* (*Pichia kudriavzevii*) and designated as *P. kudriavzevii* SK1. *Pichia kudriavzevii* SK1 metabolized glucose, galactose, mannose, maltose and fructose. It showed the potential to grow at a glucose concentration of 30% (w/v) and ferment at elevated temperatures of 45 ˚C, though the best results were observed at glucose concentrations of 15-20% (w/v) and temperatures in the vicinity of 35 ˚C. HPLC determinations revealed ethanol concentrations of 86.1 and 87.9 g/L from an initial glucose concentration of 20% (w/v) in shake flasks and laboratory batch fermenter experiments, respectively. This study revealed that *P. kudriavzevii* SK1 could be exploited for pilot scale fermentation studies at higher temperatures and glucose concentrations than those practiced for industrial fermentation, thereby obviating high refrigeration costs.

Key words: Ethanol productivity; Glucose concentration; *non-Saccharomyces* yeasts; *Pichia kudriavzevii*; Thermotolerant yeasts

1. 2. Introduction

Yeasts, in particular *Saccharomyces cerevisiae* have been used since ancient times in brewing, alcohol production and baking processes (Lee et al. 2003). Non-*Saccharomyces* yeast strains have normally been excluded from fermentation due to production of spoilage metabolites, off odours and low fermentative ability (Chatonnet et al. 1995)

One of the major limitations with the *Saccharomyces* spp. in general has been their inability to grow at high temperatures and ferment under high sugar and high ethanol concentrations. Previous studies have reported that non- *Saccharomyces* strains, such as those belonging to the genera *Klyuveromyces* and *Pichia* have shown the potential to ferment sugars at higher temperatures (≥ 40 °C) and tolerate high ethanol concentration. (Oberoi et al. 2011). From the commercial perspective, a strain capable of tolerating high ethanol and sugar concentrations and possessing invertase activity is desirable (Osho et. al. 2010), especially in high gravity (VHG) fermentations which are common in the ethanol industry wherein the yeast cells are subjected to tolerate high sugar concentrations at the beginning of the fermentation process and high ethanol concentration at the end of the process (Tikka et al. 2013).

A strain that produces a favourable metabolite, thereby enhancing the quality of final product can be selected for industrial application (Ciani et al. 2010). During the isolation and screening of different yeasts, we came across a non-*Saccharomyces* isolate which showed potential to grow and ferment sugars at relatively elevated temperatures. Therefore, the present work was designed to identify and characterize the isolated strain and assess its potential for ethanol production so that the non-*Saccharomyces* strain could be exploited for ethanol production at an industrial scale in future.

Materials and methods

2.1 Materials

Over-ripened grapes, apples, pears and naturally fermenting sugarcane juice were used for isolation of yeasts. Standards for sugars (glucose, fructose, sucrose, xylose, arabinose, galactose and

rhamnose) used during the HPLC determination were procured from Sigma- Aldrich (St. Louis, MO, USA). Chemicals used during analytical work and dehydrating media were procured from Fisher Scientific (Mumbai, India) and Hi-Media Laboratories (Mumbai, India), respectively.

2.1.1 Isolation of yeasts

Yeast peptone dextrose agar (YPD) medium comprising of glucose 2%, peptone 1%, yeast extract 0.5%, agar-agar 1.5% (w/v) was used for isolation of yeasts by pour plate method. Initial pH of the medium was adjusted to 5.0 with 5 mol/L HCl or NaOH. Morphologically, characteristic yeast colonies were picked; cultures were purified by streaking and preserved on YPD agar slants. The potential for ethanol production of selected isolates was tested using 5% glucose, 2% peptone, 2% yeast extract, 2% $MgSO_4$ at pH 5. All the flasks were incubated at 30 °C in an incubator shaker which was maintained at 100 rpm. Samples were periodically drawn at 6 -h interval until 48 h and analyzed for ethanol production and residual glucose concentration. Isolates that showed maximum ethanol production efficiency were finally selected for further studies.

2.1.2 Identification of the screened yeast isolate

The screened and selected yeast isolate was grown for 24 h at 30 °C in 50 ml YPD broth. For DNA extraction method was same as followed by Harju et al. (2004). The D1/D2 region of the large sub-unit (LSU) of the 28S rDNA region was amplified with PCR using forward primer 5'ACCCGCTAACTTAAGC3' and reverse primer 3'GGTCCGTGTTTCAAGACGG5'. The PCR amplified products were then purified using Qiagen Mini elute Gel extraction kit and subjected to automated DNA sequencing on ABI 3730xl Genetic Analyzer (Applied Biosystems, USA) (Sandhu et. al. 2012).

2.1.3 Biochemical characterization of selected yeast isolate

The selected yeast isolate after microscopic examination and molecular characterization was found to be a strain of *Pichia kudriavzevii* and designated as *P. kudriavzevii* SK1, described elsewhere in

this paper. Carbohydrate assimilation capacity of cells to metabolize different sugars and urease enzyme activity were analyzed using KB009 Hi-Carbohydrate and KB006 Hi-Candida kits, respectively (Hi-Media Laboratories Pvt. Ltd, Mumbai, India). Resistance to 1% acetic acid, 0.01% and 0.1% cycloheximide was assessed by incorporation of acetic acid and cycloheximide at concentrations mentioned above in the sterilized YPD broth flasks that were inoculated with *P. kudriavzevii* cells. Flasks were incubated at 30 ºC for 24 h in an incubator shaker. All the experiments were performed in triplicates.

2.2 Comparative evaluation of ethanol production

Fermentative ability of *P. kudriavzevii* SK1 cells was compared with that of *S. cerevisiae* MTCC 11815 cells in ethanol production using synthetic medium. Inoculum preparation and incubation conditions remained same as described previously. Flasks containing 150 ml fermentation medium composed of 15% glucose, 0.2% (w/v) yeast extract, 0.2% (w/v) peptone and 0.2% (w/v) $MgSO_4$ were used for conducting fermentation trials. Flasks were inoculated with 10 ml inoculum having a cell concentration of 1×10^8 cells/ml. Experiments were performed using one factor at a time approach with glucose concentration varying between 100-300 g/L, pH varying between 3 to 6 and temperature ranging from 25-45 °C. Experiments were planned with varying levels of one parameter with the other two parameters kept at their optimum values. Since, the objective of the study was to evaluate the strains under limited stress, optimal values selected for glucose concentration, temperature and pH were 200 g/L, 35 °C and 5.0, respectively. All the experiments were performed in the flasks fitted with a three piece air lock system which were incubated in an incubator shaker at 100 rpm. A set of three flasks was removed from the incubator shaker at regular interval of 6-h until 60 h and analyzed for glucose and ethanol concentrations.

2.4 Ethanol production in laboratory batch fermenter

On the basis of the preliminary results, ethanol production was carried out in 2.5 L batch reactor (Minifors, Infors HT, Switzerland). About 1600 ml broth containing 200 g/L glucose (obtained by sachharification of rice) was supplemented with 0.2 % yeast extract, 0.2% peptone and 0.2 %

$MgSO_4.H_2O$. After sterilization and cooling, pH of the medium was adjusted to 5.0 with the sterilized 5 mol/L HCl solution and the medium after cooling was inoculated with 10% (v/v) yeast cells at a cell concentration of 1×10^8 cells/ml. Agitation, pH and temperature were maintained at 100 rpm, 5.0 and 35˚C, respectively throughout the fermentation process. Samples were drawn regularly at 6-h intervals up to 60 h and analyzed for glucose and ethanol concentrations. The experiment was conducted three times in the same fermenter and results were statistically analyzed.

2.5 Analytical methods

Yeast cell count was determined with a haemocytometer (Hausser Sci., USA) and the cell viability was assessed by staining the cells with 0.1% methylene blue solution (Borzani and Vario, 1958).Reducing sugars were determined by the dinitrosalicylic acid (DNS) method (Miller 1959).Glucose was determined with HPLC [Ultimate 3000, Dionex Corporation, Sunnyvale, CA, USA] using a Shodex SP-0810 column (300 × 7.8 mm) fitted with a SP-G guard column (Waters Inc., Milford, MA, USA). Ethanol was determined using IC-Pak ion exclusion column (300 × 8.0 mm) [Waters Inc., Milford, MA, USA] (Oberoi et al, 2012). Peaks were detected by the RI detector and quantified on the basis of area and retention time of the standards.

2.6 Statistical analysis

All the experiments were conducted in triplicate and the mean and standard deviation values for the data were calculated using MS excel software. Wherever necessary for finding significance between the treatment means, t-test was used for test of significance using CPCS software developed by the Department of Maths, Statistics and Physics of Punjab Agricultural University, Ludhiana, India.

2. Results & Discussions

Twenty yeast isolates were selected on the basis of microscopic examination and their pure cultures were maintained on YPD slants. Selected isolates were tested for different characteristics, such as growth in presence of 5% ethanol and ethanol production potential. Seventeen isolates were able to grow at 30 °C in presence of 5% ethanol but only 13 could ferment glucose to ethanol. Out of the 13 isolates,

four isolates, Y-4, Y-6, Y-10 and Y-15 showed characteristic diversity in terms of colony and cell morphology and also high cell count in the range of 1×10^{8}cells/ml [illegible]ore in 48 h as compared to the remaining isolates. Y-4, Y-6, Y-10 and Y-15 produced 23.08, 21.96, 22.66 and 21.03 [illegible] ethanol, respectively from an initial 5% (w/v) glucose concentration, which was relatively higher (>80% fermentation efficiency) than the other isolates that showed capability to ferment glucose to ethanol in presence of ethanol. Considering their ability to produce ethanol efficiently, the above four isolates were selected for further studies.

3.1 Ethanol production by the selected yeast isolates

In order to find the most efficient isolate among the four isolates, the four yeast isolates were further compared for their ethanol producing abilities at relatively higher glucose concentration of 15% (w/v) and temperature of 35 °C, than usually practiced for industrial fermentations. Isolate Y-4 produced 68.03 g/L ethanol showing highest fermentation efficiency of 92.1% as compared to the other three isolates. Ethanol production levelled off after 36 h for all the four isolates corresponding to ethanol productivity of 1.89, 1.65, 1.74, 1.44 g/L/h for isolates Y-4, Y-6, Y-10 and Y-15, respectively. Isolate Y-4 produced 68.03 g/L ethanol showing highest fermentation efficiency of 92.1% as compared to the other three isolates. High glucose consumption and ethanol yield are known to be indicators of osmotolerance by yeasts (Favaro et al. 2013). On the basis of high ethanol producing ability isolate Y-4 was selected for further fermentation studies.

3.2 Identification of isolate Y-4

Sequencing and analysis of the 28s rDNA region of the yeast strain revealed that this region had the highest identity with *I. orientalis* F701. Phylogenetic relationships were drawn using the alignment and cladistic analysis of homologous nucleotide sequences of known microorganisms. The isolated yeast strain belonged to the same branch as *I. orientalis* F701 with 100% homology in the 28s rDNA region. On the basis of the morphology and the comparison of 28s rRNA gene sequence, the isolated yeast strain was confirmed as a strain of *P. kudriavzevii* and was designated as *P. kudriavzevii* SK1. The 28s rRNA gene sequences for *P. kudriavzevii* were submitted to GenBank with accession number JX537791.1.

3.3 Biochemical and microscopic characteristics of the selected isolate

Pichia kudriavzevii SK1 cells were able to utilize maltose, fructose, dextrose, galactose and mannose, but were unable to use several sugars, such as xylose as carbon source. Microscopic observations revealed that the yeast cell produced ascospores but not arthrospores or ballistoconidia. The isolate could not grow in the presence of cycloheximidine and lacked lipolytic activity and was unable to metabolize starch. The isolate showed some desired characteristics like high osmotolerance and thermostability. An ideal microorganism used for ethanol production should have rapid fermentation potential, appreciable thermotolerance, ethanol tolerance and high osmotolerance (Limtong et al. 2002) and on the basis of results obtained the isolate was explored for its fermentative abilities. The isolate was identified as *Pichia kudriavzeii.* Similar characteristics for *P. kudriavzevii* have been reported previously in literature (Oberoi et al. 2011). Lee et al. (2003) reported an isolate *I. orientalis* DY252 that could utilize glucose, fructose and ethanol but not sucrose and maltose.

3.4 Comparison of ethanol production by *P. kudriavzevii* SK1and *S.cerevisiae* MTCC11815

Comparative evaluation of the two strains revealed similar ethanol concentration at temperatures of 25 and 30 ˚C but as the temperature increased, efficiency of *S.cerevisiae* MTCC11815 decreased and it could produce only 53 g/L ethanol at 35 °C with ethanol concentration further declining to 20.9 g/L and 10 g/L at 40 and 45 °C respectively (Figure S1). *Pichia kudriavzevii* SK1 however produced ethanol in concentrations ranging between 80-92 g/L at temperatures ranging from 25-35 °C. These results confirmed that *P. kudriavzevii* SK1 showed higher fermentation efficiency than *S. cerevisiae* MTCC11815 at 35 °C. *Pichia kudriavzevii* SK1 produced 60.4 g/L and 48 g/L ethanol at 40 and 45 ˚C, respectively [Figure S1(a)] in 48 h, which were nearly three times higher than the ethanol concentration at 40 °C and about five times higher at 45 °C produced by the standard reference isolate. Even at 35 °C, ethanol concentration after 48 h was twice for *P. kudriavzevii* SK1 as compared to *S. cerevisiae* MTCC11815 (Figure S1). However, in most of the cases, ethanol concentration leveled off after 48 h which could be because of the depletion of nutrients, stress due to prolonged growth and production of certain toxic metabolites in the medium.

189 The two strains were tested for ethanol fermentation ability with changes in pH (3-6) (Figure S2).
190 Significant difference in ethanol production was not seen at pH of 3 or 3.5 for either of the isolates with
191 *P. kudriavzevii* SK1 producing a slightly higher ethanol than *S. cerevisiae*. Both the stains showed
192 maximum ethanol production at pH 5 with *P. kudriavzevii* SK1 producing about 22% more ethanol than
193 *S. cerevisiae* MTCC11815.

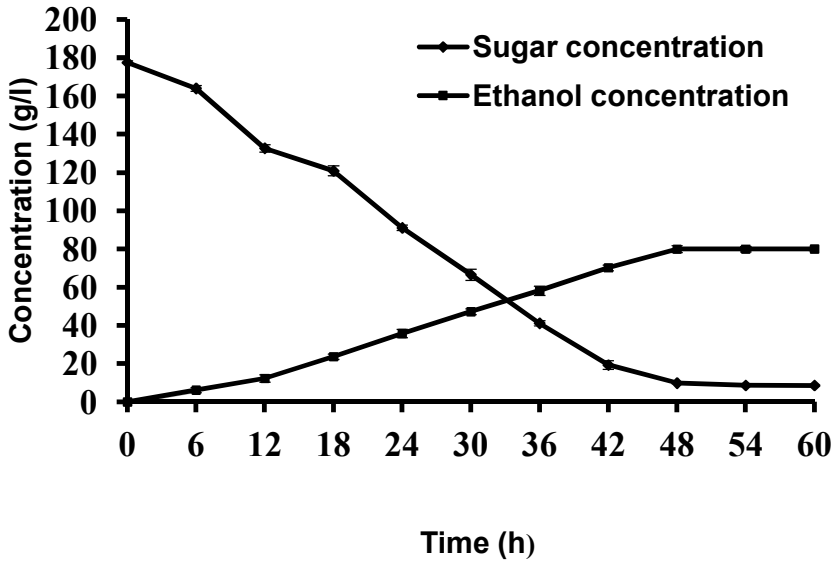
194 It was observed that the rate of ethanol production increased with increase in glucose concentration
195 from 10 to 20%. However, ethanol production rate decreased at 25 and 30% glucose concentrations
196 during fermentation, though ethanol concentration was found to be higher from higher glucose
197 concentrations at the end of fermentation period (Fig. 3S). *Pichia kudriavzevii* SK1 produced about 20%
198 higher ethanol as compared to *S. cerevisiae* MTCC11815 after 48 h of fermentation. (Figure S3). It is
199 noteworthy to mention here that the time taken to complete fermentation was 60 h with initial glucose
200 concentration of 30%, while fermentation could be completed in 48 h at initial glucose concentration of
201 20% or less.

202

203 **3.5 Ethanol production by *P. kudriavzevii* SK1 in a laboratory fermenter**

204 During fermentation in shake flasks, ethanol concentration of 86.1 g/L was obtained in 48 h (Fig. 1),
205 while an ethanol concentration of 87.9 g/L was achieved in a laboratory fermenter during the same
206 corresponding period . Ethanol productivity in shake flasks and batch fermentor were 1.79 and 1.83
207 g/L/h, respectively during 48 h of fermentation. Although a significant difference in ethanol
208 concentration was not observed in shake flask and laboratory fermenter, higher ethanol concentration
209 and productivity was observed in case of laboratory fermentation experiment. Ethanol production rate
210 decreased after 36 h of fermentation and leveled off after 48 h (Fig 1). The isolate showed ability to
211 work at higher temperature and pH. At controlled pH, volatile fatty acids (VFAs) in final product are
212 reduced and specific ethanol production rate and ethanol fermentation efficiency are significantly
213 improved (Lin et al. 2012). Kaewkrajay et al (2014) reported ethanol concentration of 42.4 g/L after
214 48 h at 45 ˚C using a thermotolerant strain of *P. kudriavzeii* in a 7 l jar fermenter. It could be safely
215 concluded from this study that *P. kudriavzevii* SK1 holds promise for conducting fermentation studies
216 using the process parameters optimized in this study at a higher scale of operation. We are now

attempting to conduct experiments with *P. kudriavzevii* SK1 in ethanol production from starchy and lignocellulosic biomass.



Legends to fig. 1: initial glucose conc.= 20% (200 g/l), pH=5, temperature= 35°C
Organism: *Pichia kudriavzeii* SK1.

Conclusion

Species belonging to *Saccharomyces* are being used for alcoholic fermentation since time immemorial. However, because of certain limitations with the *Sachharomyces* spp. such as, low sugar and ethanol tolerance and compromised fermentative ability at elevated temperatures, research focus is gradually shifting to isolation of non-*Saccharomyces* spp. having desired functional characteristics. *Pichia kudriavzevii* SK1 isolated in this study showed potential to tolerate high glucose and ethanol concentrations and also the ability to grow and ferment at elevated temperatures, generally not practiced during industrial fermentations. Ability to ferment sugars at higher temperatures by yeasts is being perceived as a major advantage for industrial fermentations, especially in tropical countries as it obviates the high energy and infrastructural costs associated with refrigeration. *Pichia kudriavzevii* SK1was able to ferment 20% glucose at 35 °C with a fermentation efficiency of about 91% in a laboratory fermenter resulting in a volumetric productivity of 1.83

g/L/h, thereby showing potential for commercial exploitation. Therefore, this study has set a platform for evaluating *P. kudriavzevii* SK1 at a higher scale of operation for its fermentative ability.

Ethical Statement

'This article does not contain any studies with human participants or animals performed by any of the authors."

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300

Legends to Figures

Figure S1: Effect of temperature on ethanol production by

(a) *Pichia kudriavzevii* SK1

(b) *Saccharomyces cerevisiae* MTCC11815

Glucose concentration: 200 g/L, pH: 5.0

Values represented are mean of three observations, *n-3*. Error bars provide information on the variability in data

Figure S2: Effect of pH on ethanol production by

(a) *Pichia kudriavzevii* SK1

(b) *Saccharomyces cerevisiae* MTCC11815

Glucose concentration: 200 g/L, Tempertaure: 35 °C

Values represented are mean of three observations, *n-3*. Error bars provide information on the variability in data

Figure S3: Effect of glucose concentration on ethanol production by

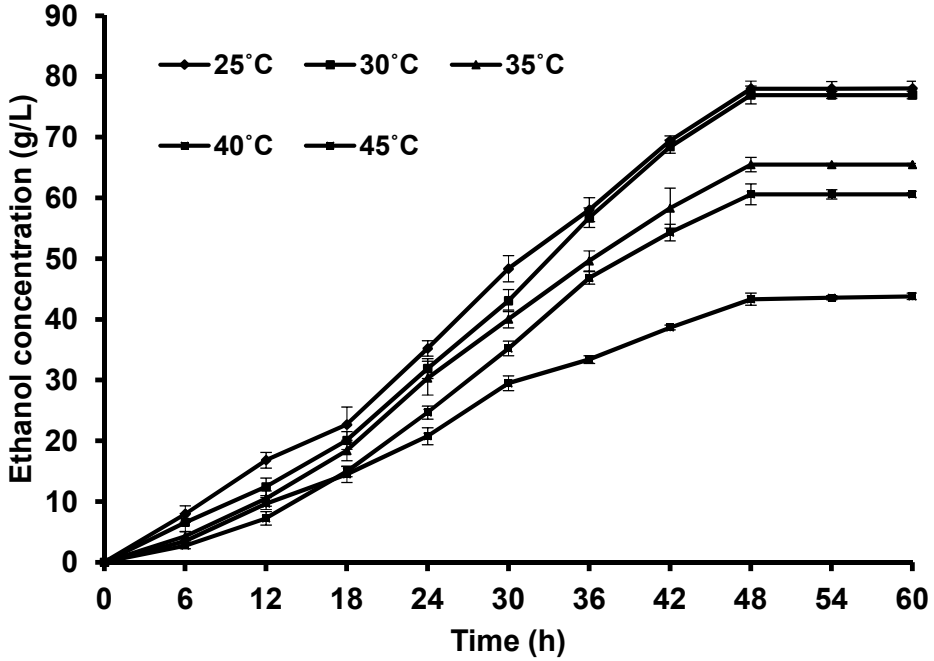
(a) *Pichia kudriavzevii* SK1

(b) *Saccharomyces cerevisiae* MTCC11815

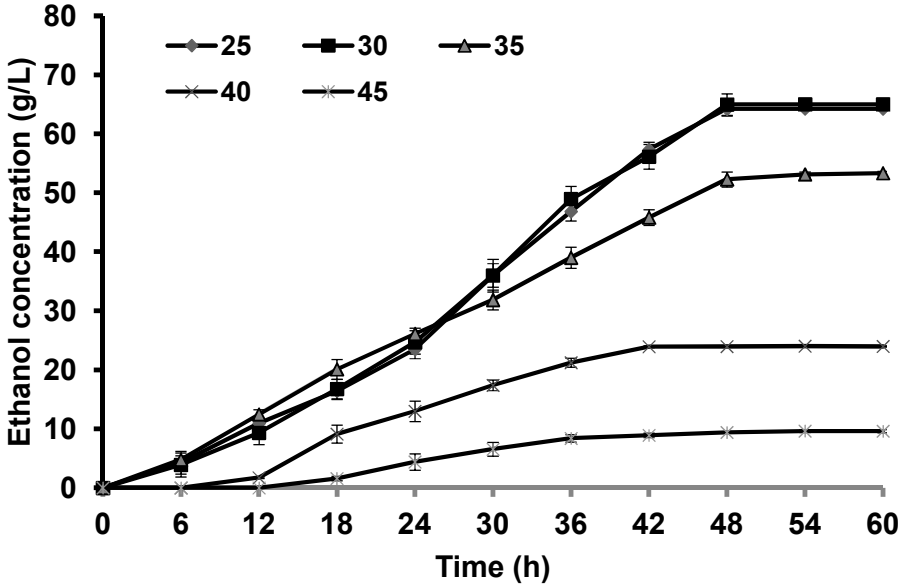
Temperature: 35 °C, pH: 5.0

Values represented are mean of three observations, *n-3*. Error bars provide information on the variability in data

Figure S1:

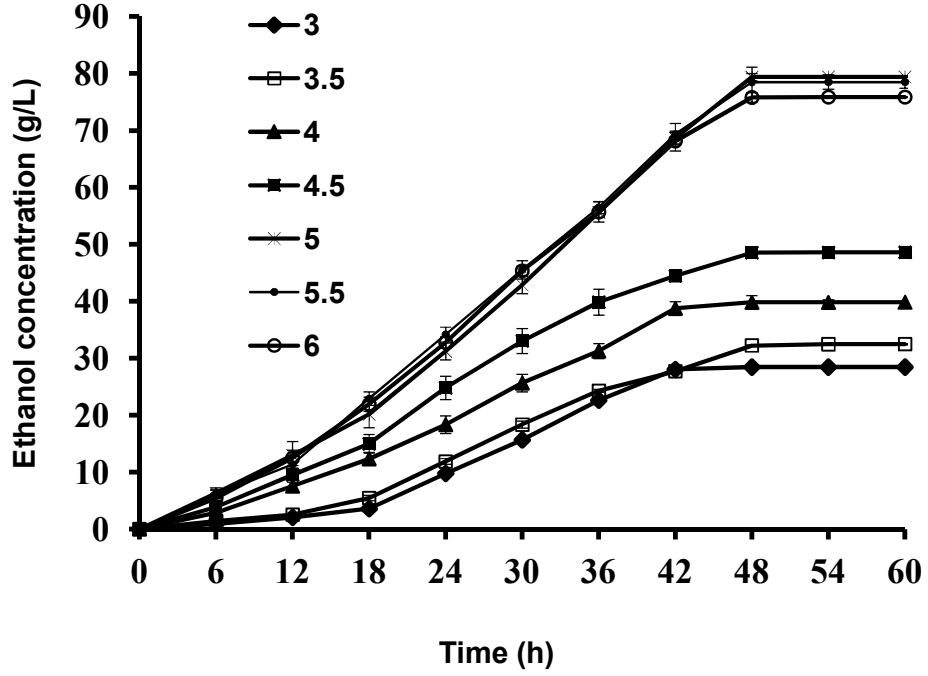


(a): Effect of temperature on ethanol production by *Pichia kudriavzevii* SK1



(b) Effect of temperature on ethanol production by *Saccharomyces cerevisiae* MTCC11815

340 Figure S2



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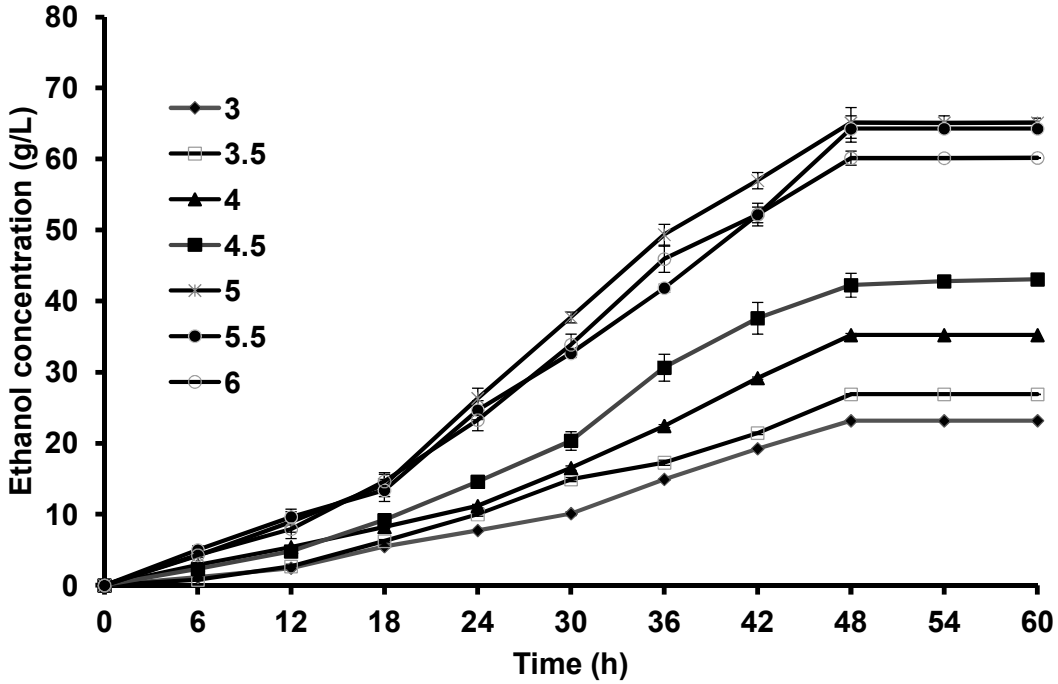
342 (a) Effect of pH on ethanol production by *Pichia kudriavzevii* SK1



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344 (b) Effect of pH on ethanol production by *Saccharomyces cerevisiae* MTCC11815

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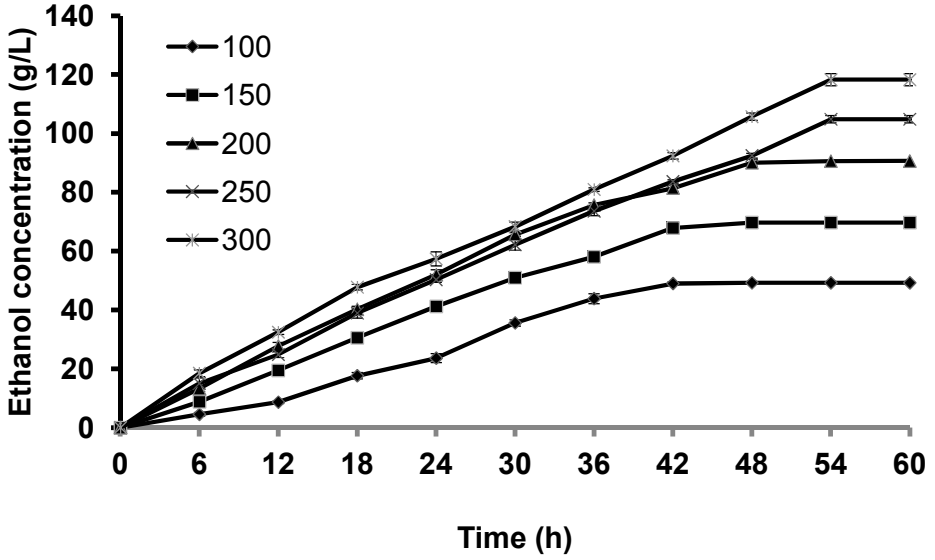
346 Figure S3



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348 (a) Effect of glucose concentration on ethanol production by *Pichia kudriavzevii* SK1

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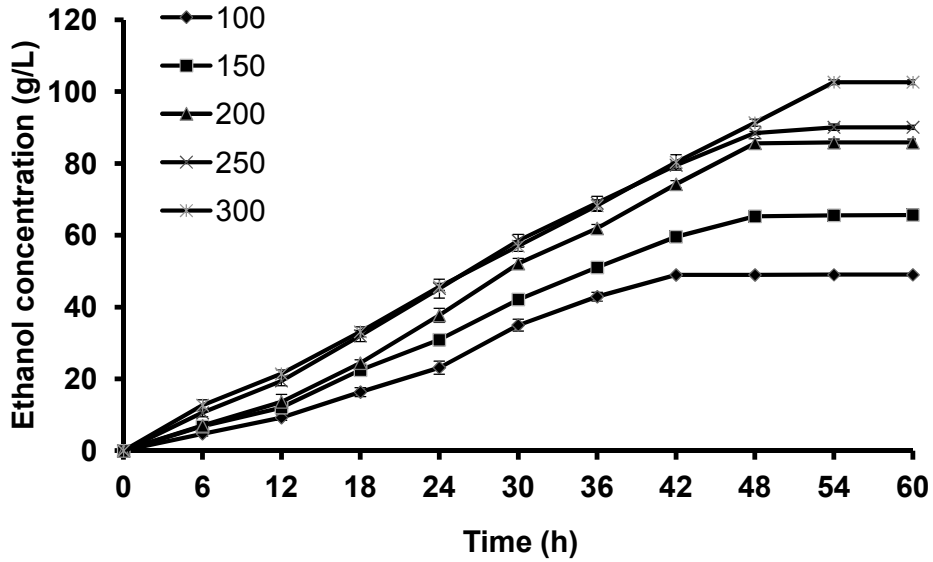

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351 (b) Effect of glucose concentration on ethanol production by *Saccharomyces cerevisiae*
352 MTCC11815

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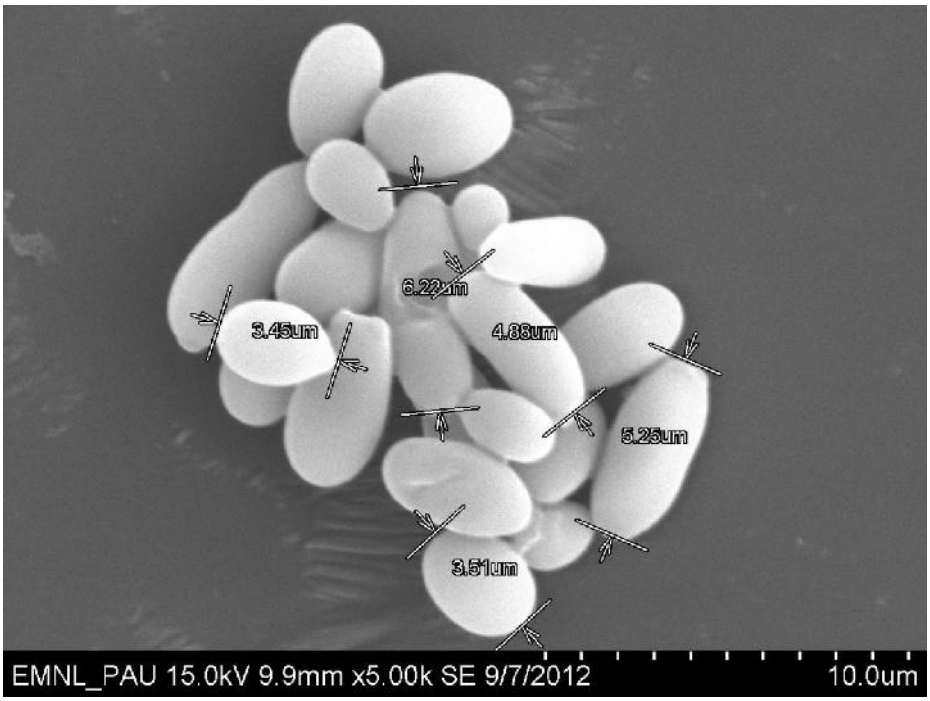

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355 **SEM image yeast isolate (*Pichia Kudriavzeii*)**

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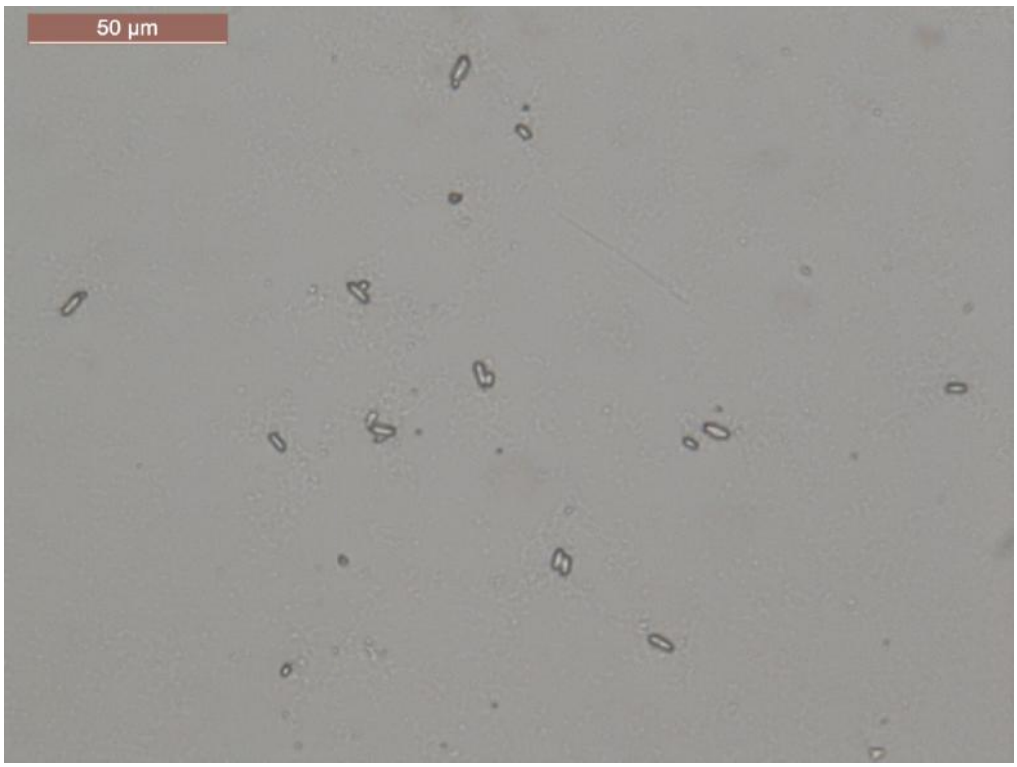

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358 **Phase contrast image of yeast isolate (*Pichia Kudriavzeii*)**

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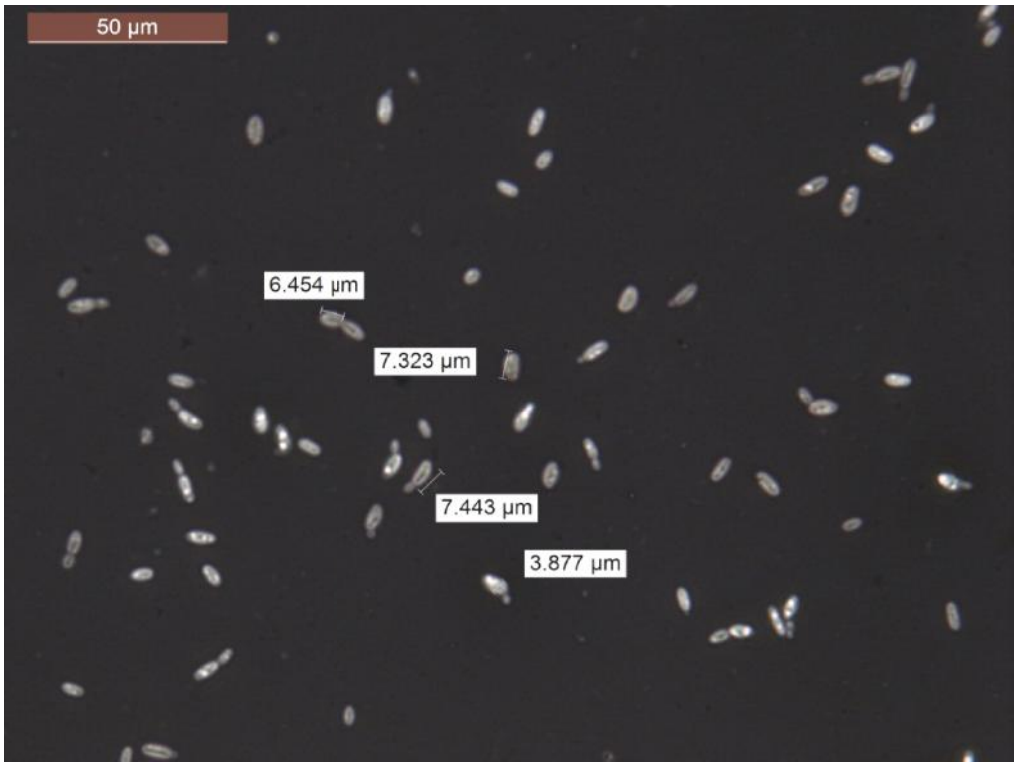

Negative staining of isolate (*Pichia Kudriavzeii*)