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

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**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 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 *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 15-20% (w/v) glucose and 35 ˚C. Reference strain *Saccharomyces cervisiae* MTCC 11815 produced ethanol in relatively low concentrations at similar conditions of substrate concentration, pH and temperature. With 20% (w/v) initial glucose concentration 86.1 and 87.9 g/L ethanol was obtained in shake flasks and laboratory batch fermenter experiments, respectively. This study revealed that *P. kudriavzevii* SK1 could be utilized 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. 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). Some non-*Saccharomyces* yeast species have also been reported to improve the fermentation behaviour of yeast starter cultures and the analytical composition of wine, in terms of more complex aroma ( Esteve-Zarzoso et al 1998; Heard 1999). Non-*Saccharomyces* yeast are known to possess proteases and lipases, which are otherwise lacking in *Saccharomyce* (Esteve-Zarzoso et al 1998). Many non- *Saccharomyces* strains have been commercialized and are available in wine making. In past, non-S*accharomyces* strains have been used for ethanol production e.g. *Torulaspora delbrueckii* strains were used in production of German wheat beers (Tataridis et. al, 2013). Dhaliwal et. al.(2011) employed galactose adapted *Pichia kudriavzeii* cells for ethanol production from sugarcane juice.

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. Previous studies have reported that non-*Saccharomyces* strains, such as those belonging to the genera *Klyuveromyces* and *Pichia* have the potential to ferment sugars at higher temperatures (≥ 40 °C) and tolerate high ethanol concentration. (Oberoi et al. 2011). Chamnipa et. al. (2018) were able to produce 35.51 g/l and 33.81 g/l ethanol from a thermotolerant *Pichia kudriavzeii* strain at 37 °C and 40 °C, respectively. Naunpeng et. al. (2016) reported a *Saccharomyces cerevisiae* strain capable of fermenting sugars at 37-40 °C.

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). Therefore, the present work was

designed to isolate a non-*Saccharomyces* strain with above said characteristics and identify and characterize the isolated strain and assess its potential for ethanol production at an industrial scale in future. *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

## 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

The yeast cultures were isolated from rotten fruits viz. apple, grapes and pears etc.. The fruits were allowed to rot at room temperature, and the extracted juice was collected in sterile containers aseptically. The juice was appropriately diluted and plated on glucose yeast peptone agar. 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 (Dhaliwal et al., 2011). 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 (Dhaliwal et. al. 2011).

## 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 $1\times10^8$ 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.

**2.3 Ethanol production by *P. kudriavzevii***

On the basis of the comparative evaluation results, an experiment was performed with *P. kudriavzevii* SK1 at 20% (w/v) glucose concentration (obtained by saccharification of rice carried out at 20% substrate in water, pH 5.3, temperature 55˚C and enzymes, 30 IU/g α-amylase and 50 IU/g glucoamylase), initial pH and incubation temperature of 5.0 and 35 ˚C, respectively, though the isolate showed potential to grow and ferment sugars, even at 40 and 45 °C and variable pH levels. The setup for fermentation was same as described in previous experiments. Samples were drawn regularly at 6-h interval up to 60 h and analyzed for glucose and ethanol concentrations. All the experiments were conducted in triplicates and the data were analyzed statistically.

**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 as described previously) 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 $1\times 10^8$ 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 and ethanol were determined with HPLC [Ultimate 3000, Dionex Corporation, Sunnyvale, CA, USA] according to the conditions given by Oberoi et al. (2012). Peaks were detected by the RI detector and quantified on the basis of area and retention time of the standards.

## 3. 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 $1\times10^{8}$cells/ml or more 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 g/L 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 rRNA 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 rRNA 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. The species ascribed to genus *Issatchenkia* has been clustered within *Pichia*, and thus all isolates of *Issatchenkia* are replaced with *P. kudriavzevii* for taxonomic entity (Kurtzman 2008, Oberoi *et al* 2010)

### 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, raffinose, sucrose, trehalose, arabitol etc. 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.

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

Comparative evaluation of the two strains revealed that the rate of ethanol production increased with increase in glucose concentration from 10 to 20% (Fig. 1 (a), (b)). However, ethanol production rate decreased at 25 and 30% glucose concentrations during fermentation, though ethanol concentration was found to be higher from higher glucose concentrations at the end of fermentation period. *Pichia kudriavzevii* SK1 produced about 20% higher ethanol as compared to *S. cerevisiae* MTCC11815 after 48 h of fermentation. It is noteworthy to mention here that the time taken to complete fermentation was 60 h with initial glucose concentration of 30%, while fermentation could be completed in 48 h at initial glucose concentration of 20% or less.

The two strains were tested for ethanol fermentation ability with changes in pH (3-6) Fig. 2(a), (b). Significant difference in ethanol production was not seen at pH of 3 or 3.5 for either of the isolates with *P. kudriavzevii* SK1 producing a slightly higher ethanol than *S. cerevisiae*. Both the stains showed maximum ethanol production at pH 5 with *P. kudriavzevii* SK1 producing about 22% more ethanol than *S. cerevisiae* MTCC11815.

It was observed that 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 (fig. 3 (a), (b)). *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 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. 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.

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

During fermentation in shake flasks, ethanol concentration of 86.1 g/L (Fig..4) was obtained in 48 h, while an ethanol concentration of 87.9 g/L (Fig. 5) was achieved in a laboratory fermenter during the same corresponding period . Ethanol productivity in shake flasks and batch fermentor were 1.79 and 1.83 g/L/h, respectively during 48 h of fermentation. Although a significant difference in ethanol concentration was not observed in shake flask and laboratory fermenter, higher ethanol concentration and productivity was observed in case of laboratory fermentation experiment. Ethanol production rate decreased after 36 h of fermentation and leveled off after 48 h. The isolate showed ability to work at higher temperature and pH. Kaewkrajay et al (2014) reported ethanol concentration of 42.4 g/L after 48 h at 45 ˚C using a thermotolerant strain of *P. kudriavzeii* in a 7 l jar fermenter. It could be safely concluded from this study that *P. kudriavzevii* SK1 holds promise for conducting fermentation studies 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.

### Conclusion

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* designated as *P. kudriavzeii* SK1 showed the potential of fermenting sugars at substrate concentration (10-25%), pH (4.5-6.5) and temperature ( 25-45 °C) giving best results at 20% substrate concentration, pH 5 and temperature 35°C. From 20% glucose at 35 °C, *Pichia kudriavzevii* SK1 produced 87.9 g/L ethanol 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.

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**Conflict of interest: NONE**

**Ethical Statement**

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

**Author's Contribution**

Authors Rajpal Phutela and Harinder Singh Oberoi designed the study being the major and minor guides of Doctorate program of author Sandeep Kaur. Author Sandeep Kaur conducted the study and wrote the manuscript. Author Harinder Singh Oberoi edited and contributed to the writing of the manuscript. All authors have read and approve the manuscript.

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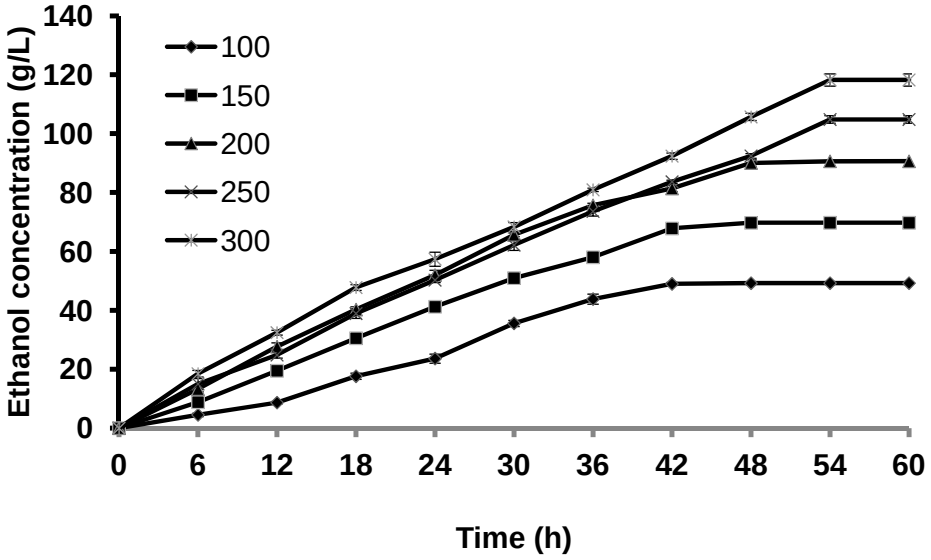
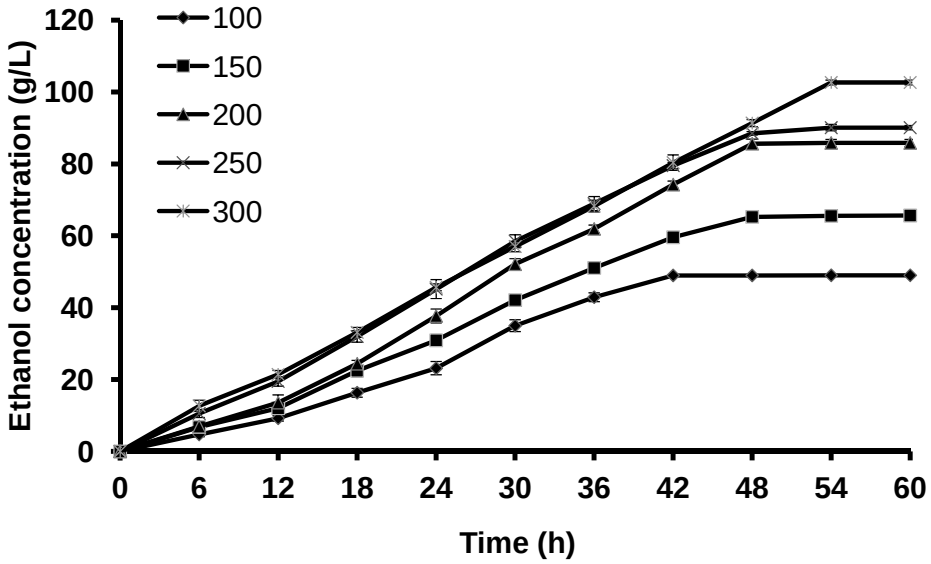
Figure 1:



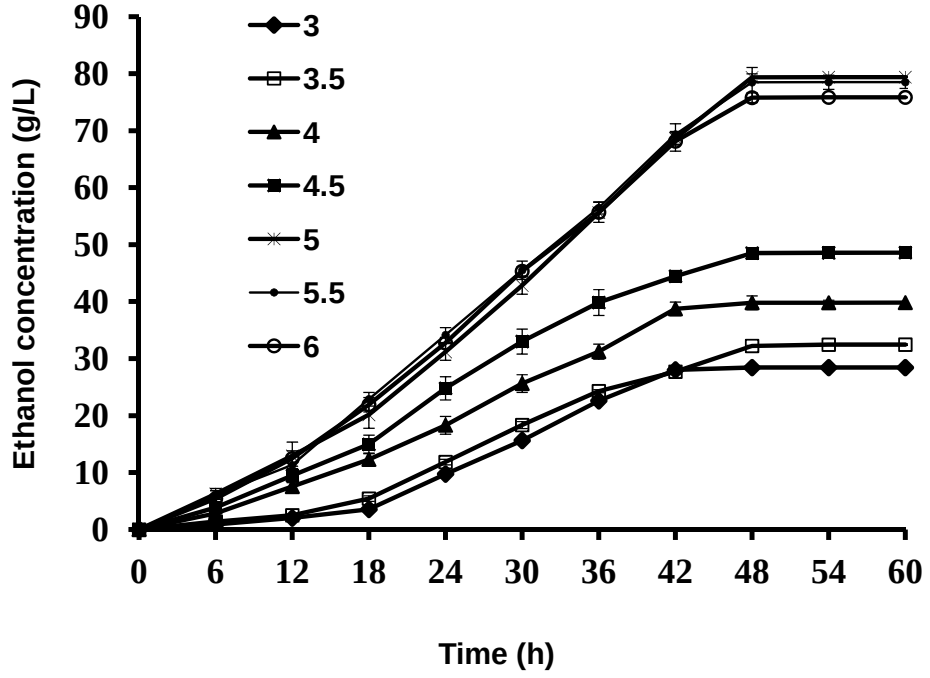

**(a) Effect of glucose concentration on ethanol production by *Pichia kudriavzevii* SK1**



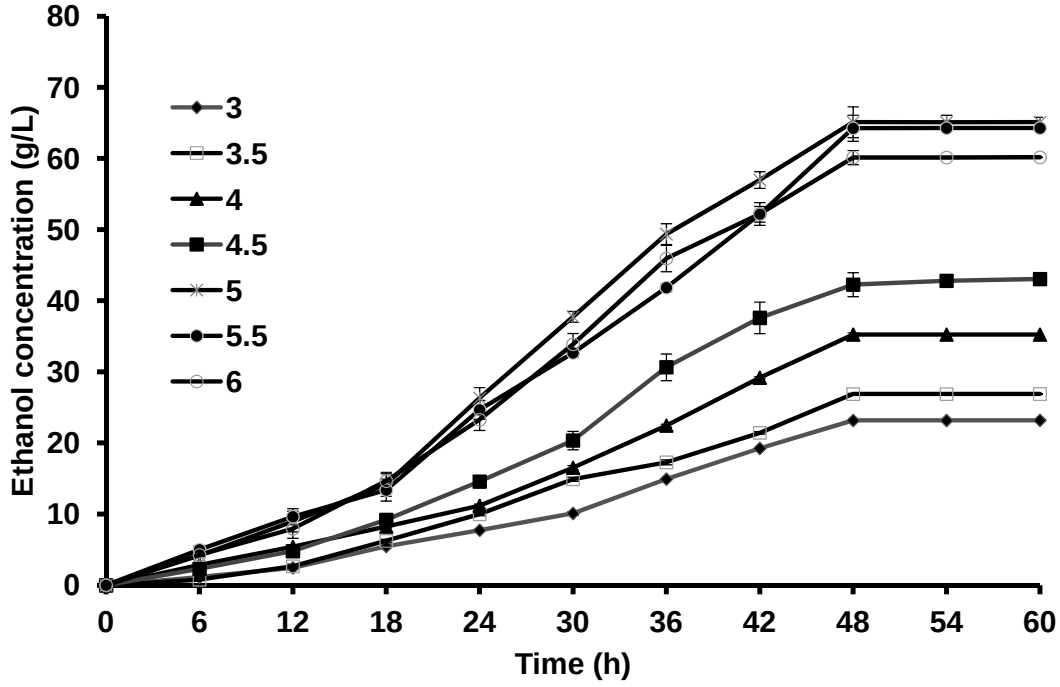

**(b) Effect of glucose concentration on ethanol production by *Saccharomyces cerevisiae* MTCC11815**

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 2



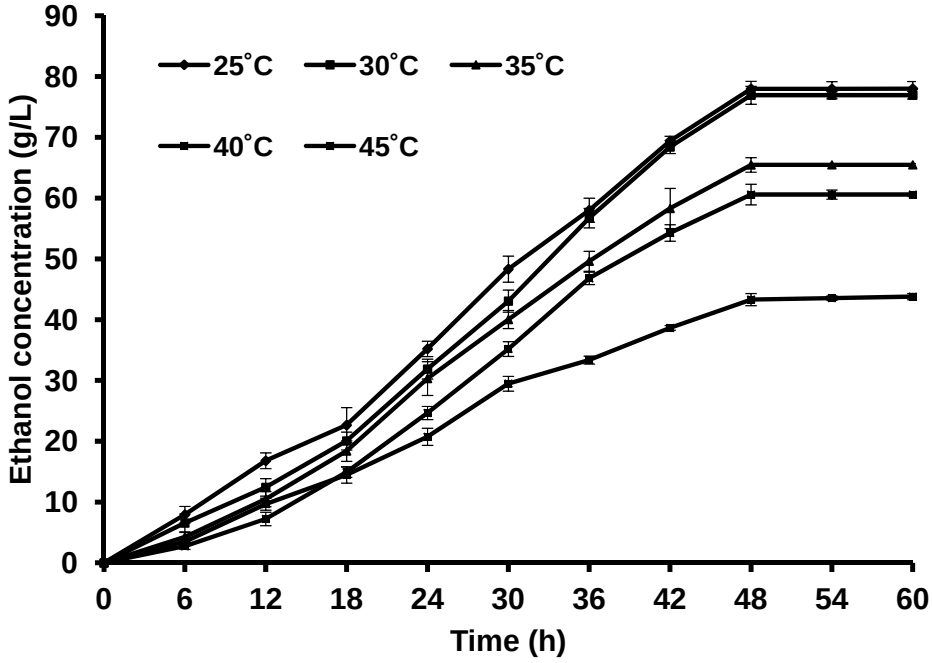

**(a) Effect of pH on ethanol production by *Pichia kudriavzevii* SK1**



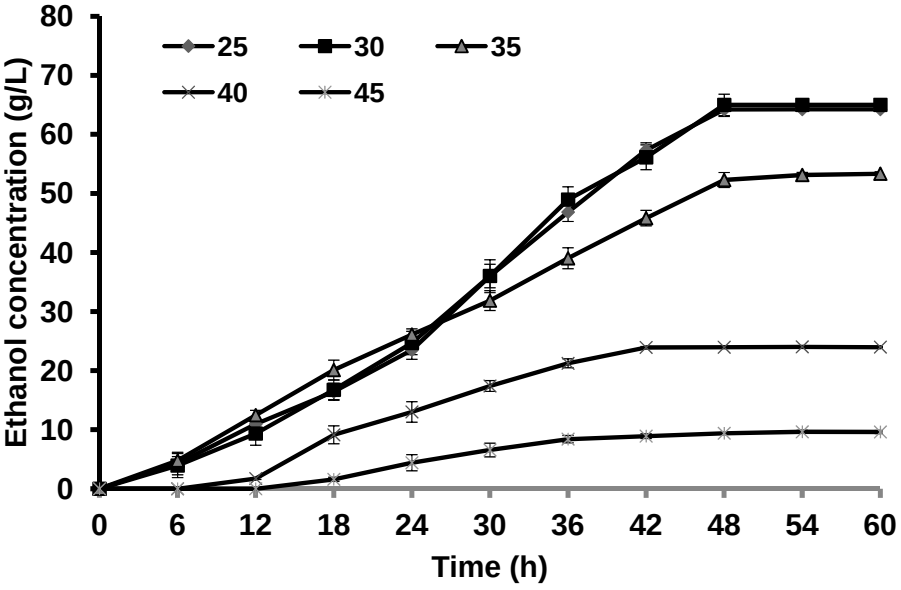

**(b) Effect of pH on ethanol production by *Saccharomyces cerevisiae* MTCC11815**

Glucose concentration: 200 g/L, Tempertaure: 35 Values represented are mean of three observations, *n-3*. Error bars provide information on the variability in data

Figure 3




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




**(b)Effect of temperature on ethanol production by *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

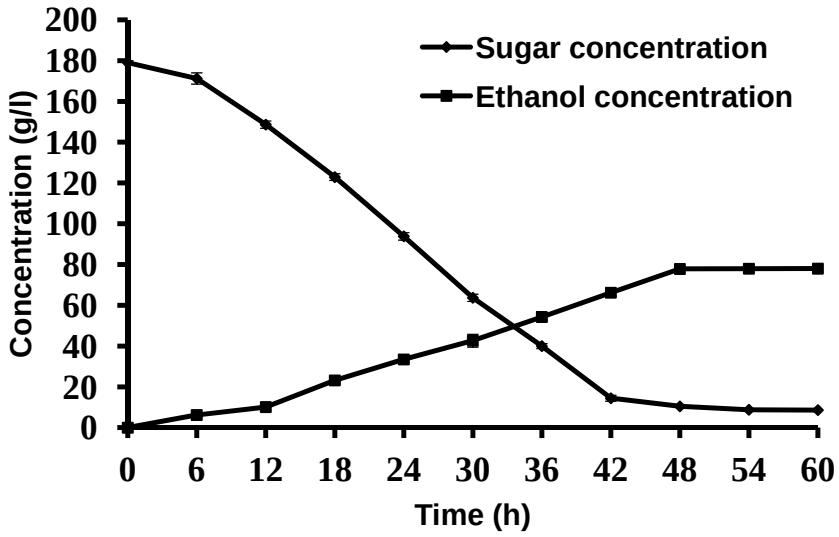



**Fig 4: Ethanol production and glucose consumption by *Pichia kudriavzevii* SK1 in shake flasks**

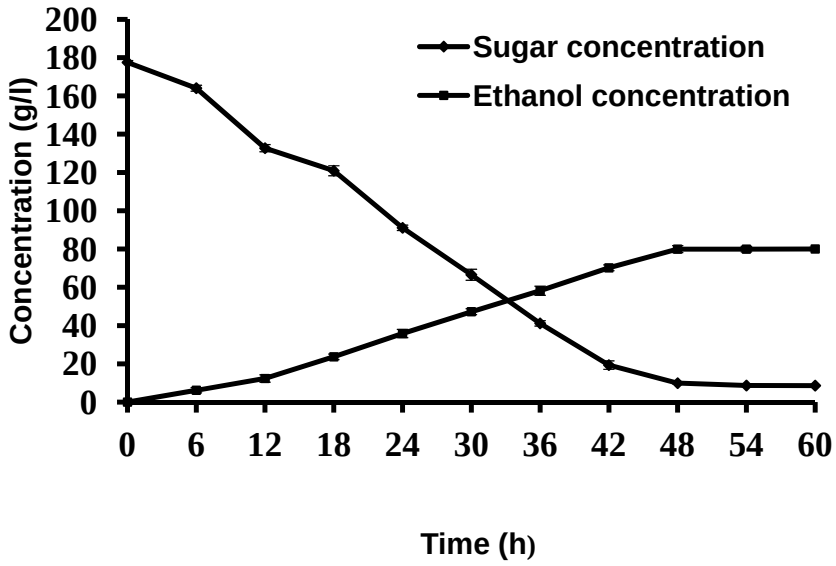



**Fig 5: Ethanol production and glucose consumption by *Pichia kudriavzevii* SK1 in laboratory fermenter**