

RESEARCH PAPER

# Comparative study of Simultaneous Saccharification and Fermentation (SSF) and Separate Hydrolysis and Fermentation (SHF) for Rice Wine production by *Pichia kudriavzeii*

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## Abstract

The present study was aimed at preparation of rice wine using *Pichia kudriavzeii* by simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF). Two varieties of rice PUSA 1121 and PR116 were used to produce ethanol by SSF and SHF. Ethanol concentration of 7.64% (w/v) and 6.82% (w/v) (72 hrs) from PUSA 1121 and PR116, respectively was obtained by SHF giving fermentation efficiency of approx. 87% in both the cases. SSF showed decrease in ethanol production 5.88% (w/v) ethanol from parmal rice (PR116) in 72 h with fermentation efficiency of 82.08% and from basmati rice (PUSA 1121), 6.82% (w/v) ethanol was produced with fermentation efficiency of 72% only in 72 h. Hence, SHF proved to be advantageous in rice saccharification and subsequent ethanol production.

**Keywords:** Separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), rice, ethanol, fermentation.

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The two main techniques for ethanol production are: separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). In the process of SHF, the raw material is hydrolysed at optimal conditions and the saccharified wort is subjected to alcoholic fermentation, while in SSF, the hydrolysis and fermentation processes are carried out simultaneously, by co-introduction of enzymes and yeast. A major issue with SHF is the need of different temperatures for hydrolysis and fermentation. While yeast ferments best at around 25°C and pH of 4-5, the hydrolysis process performs

best at temperatures of 47°C (Palmqvist 2000). Also, the yeast and enzymes cannot be separated from the fermented wort for reuse (Olofsson *et al.* 2008). The major advantage obtained from SSF is the economy of time and money. Simultaneous saccharification and fermentation not only saves the cost of energy for carrying out the processes separately but also the time.

With SHF, both enzymes and yeast are able to perform at the optimum temperature and pH. Also, it ensures the complete conversion of starch to sugars and hydrolyzed sugars to ethanol whereas in SSF

starch hydrolysis is slower than that carried out in SHF conditions.

Rice is staple food in India after wheat. In 2018, rice production is estimated at 110 million tons from cultivated area of 41 million hectares (Commodity profile for Rice, 2018). Milled rice has 50-90% starch and can be considered as most suitable raw material for fermentation to produce alcoholic beverages especially after amylolytic conversion of starch into fermentable sugars. Rice variety is known to effect the final ethanol content during fermentation, with finer varieties giving higher ethanol yield and wine from broken rice being the lowest in alcohol content (Kyalakond *et al.* 2006). The field of ethanol and bioethanol production from cereal grains and damaged food grains, respectively is being explored as an alternative source for food and fuel grade ethanol production. The aim of the study was to compare ethanol production by SSF and SHF processes for rice ethanol production and hence optimize the conditions for rice wine fermentation at pilot scale level.

## MATERIALS AND METHODS

### Materials

Rice was procured from Department of Agronomy, Punjab Agricultural University, Ludhiana, India. Indian rice variety PUSA 1121 and PR116 were used for ethanol production in present study.

### Chemicals

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). The analytical chemicals and dehydrating media were procured from Fisher Scientific (Mumbai, India) and Hi-Media Laboratories (Mumbai, India), respectively.

### Inoculum preparation

The yeast inoculum was prepared in GYE broth for which a loopful of 24 h old culture was inoculated taking 100 ml broth in 250 ml Erlenmeyer flasks and incubated at  $30 \pm 2^\circ\text{C}$  on a rotary shaker (200 rpm) for

24 h. The yeast inoculum so prepared was added to the fermentation wort @ of 5% (v/v).

### Alcoholic fermentation

For simultaneous saccharification and fermentation, yeast culture @ of 5% (v/v) was added to each flask sugars after 6 h of hydrolysis. Along with inoculum, 0.2% (v/v) ammonium sulfate, 0.2% (v/v) potassium dihydrogen phosphate, 0.2% (v/v) magnesium sulphate, 0.1% (v/v) yeast extract were added as nutrients for yeast cells. The fermentation samples were drawn every 6 h and ethanol was estimated. For separate hydrolysis and fermentation, 20% of respective rice variety was saccharified at pH 5.3, temperature  $55^\circ\text{C}$  and enzymes, 30 IU/g  $\alpha$ -amylase and 50 IU/g glucoamylase. The fermentation was carried out using 5% inoculum of *P. kudriavzeii* having  $9.6 \times 10^6$  cells/ml viable cell count.

### Fermenting Microorganism

The yeast strain *Pichia kudriavzeii* (*Issatchenkia orientalis* SK1) (GenBank accession number JX537791.1) was isolated in our lab and was used for fermentation. The yeast was stored at  $4^\circ\text{C}$  on glucose yeast extract agar slants.

### Hydrolysis of rice starch

Saccharification of rice starch was done under standardized conditions of 20% substrate in water, pH 5.3, temperature  $55^\circ\text{C}$  and enzymes, 30 IU/g  $\alpha$ -amylase and 50 IU/g glucoamylase for SHF. For SSF, wort was kept at  $50^\circ\text{C}$  for initial six h. After six h, yeast inoculum was added and temperature was maintained at  $35^\circ\text{C}$ . The conditions for two processes were standardized using RSM model comprising of family of four variables i.e substrate range (10-20%), pH 4-7, temperature  $40$ - $60^\circ\text{C}$  and enzyme units 30-50 IU/g and three levels according to central composite design.

### Analytical techniques

The yeast cell count was measured with a haemocytometer (Hausser Sci., U.S.A) 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]. The column and the RI detector were maintained at 80 and 50 °C, respectively. Deionized degassed water was used as a mobile phase for determination of sugars while 0.05N H<sub>2</sub>SO<sub>4</sub> was used as a mobile phase for ethanol determination (Oberoi *et al.* 2012) Peaks were detected by the RI detector and quantified on the basis of area and retention time of the standards.

## RESULTS AND DISCUSSION

A 20% basmati rice var. PUSA 1121 in water was saccharified using standardized procedure which yielded 172.09 mg/ml glucose and the same amount was used for fermentation. As is seen in (Fig. 1), there was a gradual increase in the concentration of ethanol produced vis-a-vis fall in the concentration of glucose as the fermentation proceeded. At 60 h the alcoholic concentration stood at 68.33 mg/ml, which increased to 76.41 mg/ml at 72 h, hence fermentation was taken as complete and fermentation efficiency was calculated to be 87.29%.

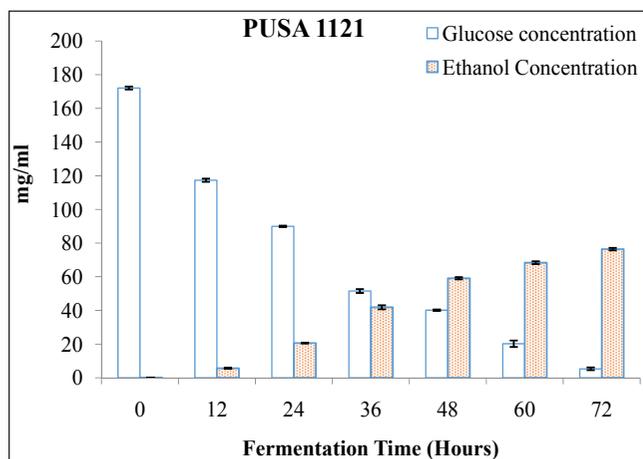


Fig. 1: Ethanol produced using PUSA 1121

From 20% parmal rice var. PR 116, saccharified using standardized procedure yielded 140 mg/ml glucose. The fermentation was complete in 72 h giving final ethanol concentration of 62.18 mg/ml with 87.08% fermentation efficiency (Fig. 2).

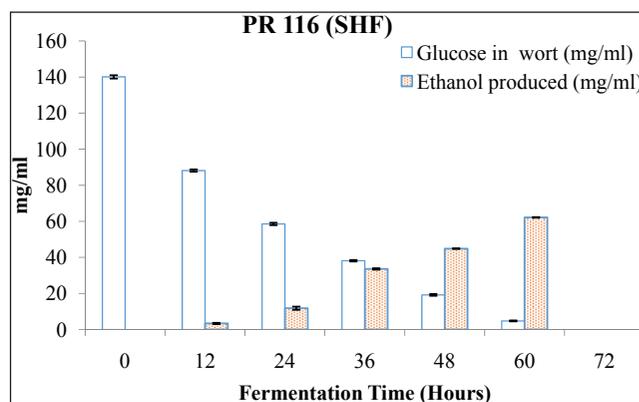


Fig. 2: Ethanol produced by PR 116

\*The experiment was conducted in triplicates.

Organism: *P. kudriavzeii*, Volume: 500ml

Initial sugars obtained after saccharification of 20% substrate:

PUSA 1121: 17%, PR 116: 14%, Temperature: 30°C

During SSF studies, 20% substrate concentration was taken and for 6 h, the saccharification temperature was maintained at 54°C. After 6 h, yeast inoculum was added and temperature was adjusted to 35°C. For ethanol production from rice var. PUSA 1121, 8.23 mg/ml ethanol was produced after 12 h, which increased to 36.27 mg/ml in 24 h and 45.08 mg/ml after 45 h. The fermentation was complete in 63 h producing 60.14 mg/ml ethanol. There was an increase of 2 mg/ml only in next 12 h as after 72 h fermentation time, the ethanol concentration was 62.8 mg/ml, with 8.23 mg/ml as residual sugar (Fig. 1 & 2).

During SHF, ethanol production during initial 12 h was very less but increased with time (Fig. 1). After 24 h was 20.59 mg/ml ethanol was produced which doubled in next 12 h i.e. 41.86 mg/ml after 36 h. The trend continued till 48 h with 59.10 mg/ml ethanol concentration in wort. Similarly, during alcoholic fermentation of rice var. PR 116, only 11.82 mg/ml ethanol was produced during initial 24 h (Fig. 2), but there was 3 fold increase in ethanol concentration

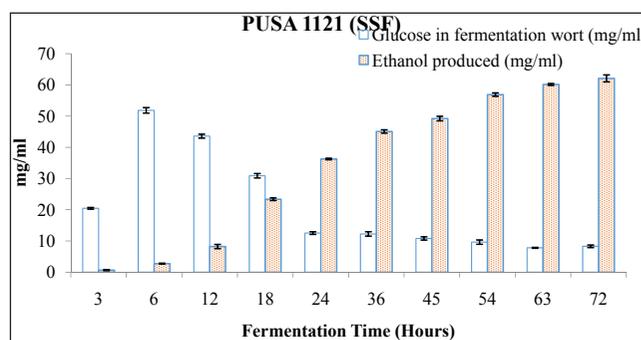
after 36 h (33.61 mg/ml). This trend of increase in ethanol concentration after an initial lag was observed during alcoholic fermentation with both the rice varieties i.e. PUSA 1121 and PR 116. *P. kudriavzeii* is slow fermenting yeast, which shows a lag period at the beginning of fermentation (Deenanath, 2009). The reason for this lag can be due to presence of aerobic conditions at the beginning of fermentation. But as the fermentation proceeds, the anaerobic conditions begin to dominate and the rate of ethanol production increases. Also the increased level of alcohol in later stages may affect the cell membrane structure, which leads to fall in further alcohol fermentation (Briggs et al. 2004).

There was a constant decrease in glucose concentration since the beginning of the fermentation (Fig. 1 & 2), though the ethanol productivity was low during the initial hours. The decrease can be due to metabolic activity of yeast and major part of glucose consumed was used for increase in biomass only than increase in ethanol concentration. A supply of oxygen is necessary during brewery propagation and early fermentation to generate yeast biomass and ensure that yeast is in optimum physiological conditions for effective fermentation (Hammond, 2000; Hulse, 2003).

In the process of simultaneous saccharification and fermentation (SSF), the glucose produced is consumed simultaneously by yeast cells for ethanol production. The process has the advantage of reduction in time and energy input but faces some major problem with difference in saccharification and fermentation temperature and changing pH, which affects enzymatic activity. In our experiment on ethanol production by SSF, we observed that the final ethanol concentration was less than that obtained by the process of SHF.

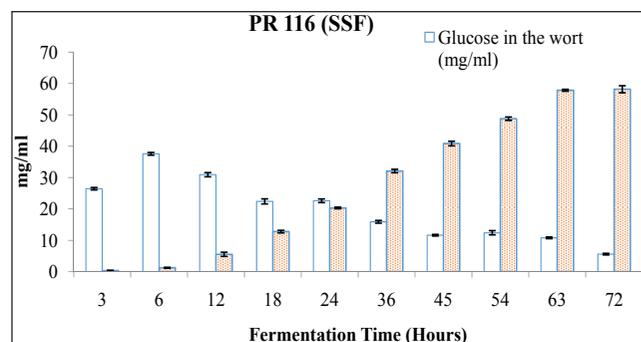
During SSF studies, considering that initial substrate concentration of 20%, releases approximately 17% sugars from rice var. PUSA 1121, the corresponding fermentation efficiency was calculated to be 72% (Fig. 3), which was less as compared to ethanol produced by process of separate hydrolysis and fermentation.

The decrease in fermentation efficiency may be attributed to the decline in hydrolysis temperature from 54°C to 32°C, which led to decreased saccharification efficiency. Also as mentioned, the change in pH due to ethanol produced during fermentation affects enzyme activity. Also, both enzyme and yeast undergo plasma degradation as ethanol concentration increases (D'amore,1991).



**Fig. 3:** Ethanol produced using PUSA 1121

Sugar concentration at the time of addition of inoculum was 26.45 mg/ml (2.64%) for rice var. PR 116 (Fig. 4). It increased to 37.58 mg/ml (3.75%) after 6 h and corresponding ethanol concentration was 1.24 mg/ml (0.52%). After 12 h fermentation, the ethanol produced was 5.55 mg/ml, which increased to 20.32 mg/ml after 24 h. the fermentation ceased after 63 h, producing 57.84 mg/ml ethanol with fermentation efficiency of 82.08%. The residual sugar concentration of 5.6 mg/ml (0.56%) (Table 2).



**Fig. 4:** Ethanol produced by PR 116

\*The experiment was conducted in triplicates.

Substrate conc.: 20%; Temperature: 50°C for hydrolysis for initial 6 h., 32°C after inoculation; Enz units: 50 IU/g.

Similar results were given by Saha *et al.* (2011), who achieved  $21.6 \pm 0.5$  g/l ethanol in 4 h from  $44.1 \pm 0.4$  g/l total sugars at pH 6 by separate hydrolysis and fermentation, whereas they got  $24.9 \pm 0.3$  g/l ethanol in 96 h and  $26.7 \pm 0.0$  g ethanol in 72 h from bioabated wheat straw hydrolyzate by batch simultaneous saccharification and fermentation and fed-batch simultaneous saccharification and fermentation, respectively using a recombinant *E. coli* strain. Ohgren *et al.* (2007) achieved an ethanol yield of 72.4% by SSF as compared to 59.3% by SHF from 8% initial sugars produced from hydrolysis of steam pre-treated corn stover. Chrisnasari *et al.* (2013) found SHF process giving better results as compared to SSF during fuel grade ethanol production from *Sorghum bicolor* grain.

Lertpinyochaithaworn (2007) achieved ethanol concentration of 13.53, 15.24 and 17.20% in polished, paddy and malted rice respectively through separate hydrolysis and fermentation process, giving product yield (g/g) of 0.482 (paddy rice), 0.427 (polished rice) and 0.544 (malted rice). Ethanol concentration of  $149 \pm 7.0$  g/kg malted rice was achieved from waste paddy after 48 h incubation by SmF, which was higher than the yield of  $130 \pm 7.8$  g/kg obtained during SSF after 90 h incubation (Chaijamrus and Mouthung, 2011).

## CONCLUSION

Separate hydrolysis and fermentation proved to be a better method than simultaneous saccharification and fermentation during the present study. Two rice varieties namely PUSA 1121 and PR 116 were used during the study. Final ethanol content of 76.41 mg/ml with 87.29% fermentation efficiency and 62.18 mg/ml with 87.08% efficiency was obtained with PUSA 1121 and PR116 respectively during SHF from initial sugar concentration of 18% and 14% respectively for two varieties. The corresponding efficiencies during SSF were 72% and 82.08% respectively.

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## CONFLICT OF INTEREST

Authors declare that they have no conflict of interest.

## REFERENCES

- Briggs, D.E., Boulton, C.A., Brookes, P.A. and Stevens, R. 2004. Brewing: Science and Practice. Woodhead, Cambridge, UK.
- Borzani, W. and Vario, R. 1958. Quantitative adsorption of methylene blue by dead yeast cells. *J. Bacteriol.*, **76**: 251-255.
- Chaijamrus, S. and Mouthung, B. 2011. Selection of Thai starter component for ethanol production utilizing malted rice from waste paddy. *Songklanakarin J. Sci. Technol.*, **33**: 163-70.
- Chrisnasari, R., Susetyo, D.H., Sugianto, A.P. and Pantjajani, T. 2013. Optimization Modelling for ethanol production from *shorgum bicolor* grain: Comparison between Separate Hydrolysis and Fermentation and Simultaneous Saccharification and Fermentation. *Microbiology*, **7**(1): 9-16.
- Commodity profile for rice-March 2018. <http://agricoop.gov.in/sites/default/files/Rice%20profile%20March%2C%2C%202018.pdf>
- D'amore, T. 1991. Improving Yeast Fermentation Performance. *J. Institute Brewing*, 375-82.
- Deenanath, E.D. 2009. *Enzymatic hydrolysis of bitter sorghum for bioethanol production*. M.Sc Thesis. University of Witwatersrand.
- Hammond, J.R.M. 2000. Yeast growth and nutrition: Brewing Yeast Fermentation Performance. 1<sup>st</sup> edn (Smart K.A. ed), pp. 77-85. Blackwell Science, Oxford, UK.
- Hulse, G.A. 2003. Yeast propagation. Brewing Yeast Fermentation Performance. 1<sup>st</sup> edn. (Smart K.A. ed), pp. 249-56. Blackwell Science, Oxford, UK.
- Kyalakond, A.V., Savalgi, V.V. and Alagawadi, A.R. 2006. Screening of rice varieties for alcoholic beverage production. *Karnataka J. Agric. Sci.*, **19**(4): 837-40.
- Miller, G.L. 1959. Use of dinitrosalicylic acid for determining reducing sugars. *Anal. Chem.*, **31**: 426-28.
- Oberoi, H.S., Babbar, N., Sandhu, S.K., Dhaliwal, S.S., Kaur, U., Chadha, B.S. and Bhargav, V.K. 2012. Ethanol production from alkali-treated rice straw via simultaneous saccharification and fermentation using newly isolated

- thermotolerant, *Pichia kudriavezii* HOP-1. *J. Ind. Microbiol. Biotechnol.*, **39**: 557-566.
- Ohgren, K., Bura, R., Lesnicki, G., Saddler, J. and Zacchi, G. 2007. A comparison between simultaneous saccharification and fermentation and separate hydrolysis and fermentation using steam pre-treated corn stover. *Proc. Biochem.*, **42**: 834-39.
- Olofsson, K., Magnus Bertilsson and Gunnar Lidén 2008. A short review on SSF – an interesting process option for ethanol production from lignocellulosic feedstocks. *Biotechnology for Biofuels*, **1**: 7.
- Palmqvist, E. 2000. Fermentation of Lignocellulosic Hydrolysates. *Bioresource Tech.*, 25-33.
- Saha, B.C., Nichols, N.N., Qureshi, N. and Cotta, M.A. 2011. Comparison of separate hydrolysis and fermentation and simultaneous saccharification and fermentation processes for ethanol production from wheat straw by recombinant *Escherichia coli* strain FBR5. *Appl. Microbiol. Biotechnol.*, **92**: 865-74.