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# Condition optimization for ethanol production from waste substrate of different broken rice varieties (IR-36, IR-64, MTU-1010 and Danteshwari)

**[1] OP Suryawanshi, [2] D Khokhar, [3] S Patel**

[1] SRF, Ph.D Research Scholar, Department of Food Process Engineering, NIT Rourkela, Odisha, India

[2] Asst. Proff. & Scientist, Department of Plant Physiology Agricultural Biochemistry Medicinal and Aromatics Plant, AICRP, IGKV Raipur, Chhattisgarh, India

[3] Proff. & HOD, Department of Agricultural Processing and Food Engineering, Faculty of Agricultural Engineering and Technology, IGKV Raipur, Chhattisgarh, India

**Abstract**
Fermentation technology is one of the useful methods in the value addition of rice (paddy) which are generally not fit for human health or less preferred for consumption. Rice (*Oryza sativa* L.) is the major staple food crop in India. In India, different varieties of rice are grown under the diverse agro-climatic conditions likewise irrigated, lowland and upland cultivable fields etc. One of the major rice producing state is "Chhattisgarh" and is known as a bowl of rice. Chhattisgarh states occupy the average of 3.6 million hectares cultivable fields with the productivity between 1.2 to 1.6 t/ha. The rainfall, which is a major factor determining rice productivity, is quite high with an irrigated area of nearly 28%. In Chhattisgarh generally, rice is grown in *kharif* season also in irrigated areas during the summer season with an area of 2.21 lakh hectare with 7.4 lakh tons production during 2012-13. It has been found that abruptly increase in the rice production from Chhattisgarh in summer season (Chhattisgarh state Agriculture Department 2012-13). An experiment was carried out to identify the yeast culture and inoculum size, during value addition of broken rice as ethanol preparation from locally available different rice varieties viz. MTU-1010, IR-64, IR-36, and DANTESHWARI. The acid ($H_2SO_4$) and enzyme (α-amylase) pretreatments were used to obtain maximum reducing sugars, for ethanol preparation using standard yeast strains (Saccharomyces cerevisiae). The enzymatic pretreatment with α-amylase was selected for hydrolysis as it releases the highest reducing surges compared to acid pretreatment. Highest ethanol percentage was obtained by Saccharomyces cerevisiae NCIM 3281@5% inoculum with IR 36 rice variety.

**Keywords:** broken rice, ethanol, *Saccharomyces cerevisiae*, enzymatic pretreatment, anaerobic fermentation

## Introduction
Rice (*Oryza sativa* L.) a native to South-East Asia is one of the leading food crops of the world. Rice is predominantly an Asian crop, 95 percent of it is being produced and consumed in the South-east Asian countries extending from Indo-Pakistan sub-continent to Japan. India has the largest area under paddy in the world and ranks second in the production after China (Annonymous, 2010).

Rice is one of the most important staple food crop in India. The nearly three-fourth population of the country subsists on it. It is grown in India under diverse agro-climatic conditions including irrigated, upland and lowland conditions. In India, rice production was (*kharif + rabi*) 101.8 million tons in the year 2012-13 (Annonymous, 2013).

Chhattisgarh state is known as a bowl of rice. Rice occupies an average of 3.6 million hectares with the productivity of the state ranging between 1.2 to 1.6 t/ha. The rainfall, which is major factor determining rice productivity, is quite high with an irrigated area of nearly 28%. In Chhattisgarh rice is grown in irrigated areas during the summer season with an area of 2.21 lakh hectare with 7.4 lakh tons productions. It has been found that there is an increase in the rice production from Chhattisgarh in summer season (Chhattisgarh state Agriculture Department 2012-13). It is well known that during summer season farming the environments are very hot due to increased temperature and artificial irrigation (almost no rainfall) as only the source of water supplement. Due to hot climatic conditions and insufficient water supply, the rice kernel has some cracks and fissure. During the milling of cracked kernel paddy resulting uneven milling quality. From the research point of view, it has been found that summer season grew (*rabi* season) rice carries a larger number of broken rice than *kharif* season.

The major constituent of rice is starch which fulfills about 90 per cent of rice in dry weight. Except for edible application, the starch finds its application in food, pharmaceutical, textile, paper industries *etc.* (Suresh *et.al* 1999) [101]. It is also processed for the production of maltose, dextrose, glucose syrups *etc.* (Arun Kyalakond 2005). One may consider the utilization of broken rice in biomass formation, saccharification, ethanolic and acetic fermentation. The broken rice of different locally available rice varieties, which are less preferred or almost not preferred commercially for regular use as staple food, can be used for the ethanol production. They are very less preferred since the grains are medium bold to bold type *i.e.*, not of fine-grain or fine quality, the rice will be sticky after cooking or has a high broken percentage.

Although from ancient times fermented products of rice are popular in various parts of the world and also in some part of India. The rice ethanol plays one of the important roles as the fermented product of rice. Ethanol has been developed from very primitive Thai rice wines to highly sophisticated Japanese Sake which itself developed from the very primitive beverage. Even the Korean beverages yakju and takju were originally made from rice which is ancient beverages popular among the common people (Park *et. al.,* 1977) [83].

Considering all the above facts and problems, the present investigation was taken up with broken of summer season grown rice varieties namely; IR-36, IR-64, MTU-1010 and Danteshwari for ethanol production.

**Materials and Methods**

An experiment was conducted with the entitled "Condition optimization for ethanol production from smashed of different rice varieties (IR-36, IR-64, MTU-1010 and Danteshwari)" during 2013-2015 at the Department of Agricultural Processing and Food Engineering in collaboration with the Department of Plant Physiology, Agriculture Biochemistry Medicinal and Aromatic Plant, at Indira Gandhi Krishi Vishwavidyalaya, Raipur (C.G.) to study the production of ethanol from different rice varieties having high broken percentage. The broken raw rice was pre-treated with acid and enzyme to convert the rice starch to free sugars i.e. reducing sugars and pretreated starch was inoculated with the three different yeast strains at different concentrations as cultures for ethanol production. The conditions were optimized for maximum production of ethanol.

**Procurement of rice sample**

To conduct the experiment locally available summer season grown different varieties of paddy are selected on the basis of their broken percentage, human consumption rate, utilization rate as animal feed and market value. The four varieties of paddy namely: IR-36, IR-64, MTU-1010 and Danteshwari were collected from the food grain storage section of the Indira Gandhi Krishi Vishwavidyalaya, Raipur Chhattisgarh. Before milling, the collected samples are allowed to absorb the moisture content up to 14-16 % (weight basis) by alternating wetting and drying. Milling of paddy was done separately by using small laboratory rubber roller (rice sheller 62007) without any treatment or parboiling. All the brown rice (dehusked) was collected and their broken percentage were determined by screening i.e. sieve analysis BIS (sieve no. JEL-200).

**Preparation of substrate**

A known quantity of each rice variety was steeped in 30℃ water for 1 hour separately each in different steel sample pots afterward cooked for 5 min. in a pressure cooker with one whistle and mashed separately. Further 25 g of each mashed substrate weighed separately and volume was made to 35 ml with distilled water for hydrolysis to release reducing sugars (Arun Kyalakond 2005).

**Enzymatic pretreatment**

To release the maximum reducing sugars from starch, the prepared substrate was treated with commercial α- amylase (Diastase α -amylase) of 1 per cent solution which was prepared with 10 mM $CaCl_2$ buffer and allowed it for saccharification (hydrolysis) for different incubation periods at different temperature (Vijay Wadhai *et. al* 2011) [66].

**Optimization of Substrate**

The prepared substrate was diluted with distilled water at different concentration i.e. 1:0.5, 1:1 and 1:1.5 concentrations (Substrate: distilled water). This was mixed with prepared 1% of commercial α-amylase enzyme solution and kept it for different periods of incubation at a different temperature. The pretreated substrate was kept for incubation at 60° C to release maximum reducing sugar (Vijay Wadhai *et. al* 2011) [66].

**Optimization of hydrolysis time**

To determine the optimum time to get most of the starch as reducing sugars, the pretreated substrates were kept in an incubator for different time intervals at a constant temperature.

**Standardization and Fermentation**

The TSS of hydrolysate from the pretreatment was maintained @ 24° brix by adding cane sugar and pH was adjusted to 3.5 by adding baking soda or Sodium bicarbonate. The activity of the natural flora of the prepared substrate or hydrolysate was suppressed by adding 200 ppm of Sodium sulfite and kept for 4-5 hours. The substrate was supplemented with diammonium hydrogen phosphate at the rate of different level in terms of g/l as a source of Nitrogen and Phosphorus to achieve proper growth of yeast cultures (Arun Kyalakond 2005).

The ameliorated must was inoculated with *Saccharomyces cerevisiae* (previously prepared in 100 ml of a hydrolysate of each variety) at the rate of 5% of different cultures. The substrates were allowed to ferment anaerobically at 30±1 °C (3 weeks) at different agitation speed (Vijay Wadhai *et. al* 2011) [66].

**Estimation of ethanol**

The ethanol was estimated by a colorimetric method as described by Caputi *et al*. (1968) [23]. One ml of representative samples from each treatment was transferred to 250 ml round bottom distillation flask connected to the condenser and was diluted with 30 ml distilled water. The sample was distilled at 74-75°C. The distillate was collected in 25 ml of 0.23 N $K_2Cr_2O_7$ reagents, which was kept at receiving end. The distillate containing ethanol was collected till total volume of 45 ml was obtained. Similarly, standards (05-25 mg ethanol) were mixed with 25 ml of $K_2Cr_2O_7$ separately. The distillate of samples and standards were heated in water bath at 60ºC for 20 minutes and cooled. The volume was made up to 50 ml with distilled water and the optical density was measured at 600 nm using systronics visible spectrophotometer-106. The standard curve was plotted considering the concentration against absorbance. The amount of ethanol in each sample was determined by using the standard curve of ethanol (Arun Kyalakond 2005).

**Result and Discussion**

The experimental results on optimization of various standardized conditions for ethanol production were as follows:

**Broken rice percentage**

Summer grew rice varieties *viz.* MTU-1010, IR-36, IR-64, Danteshwari, Mahamaya and Sona Masuri were procured. It is clear from the Table 1 that maximum broken percentage was in IR-64 (28.21%) rice variety and followed by other varieties IR-36, Danteshwari and MTU-1010.

**Table 1:** Broken percentage of rice varieties

| S. No | Rice varieties | Broken % |
|---|---|---|
| 1 | MTU-1010 | 23.41 |
| 2 | IR-36 | 26.32 |
| 3 | IR-64 | 28.21 |
| 4 | Danteshwari | 24.12 |
| 5 | Mahamaya | 23.20 |
| 6 | Sona Masuri | 22.13 |

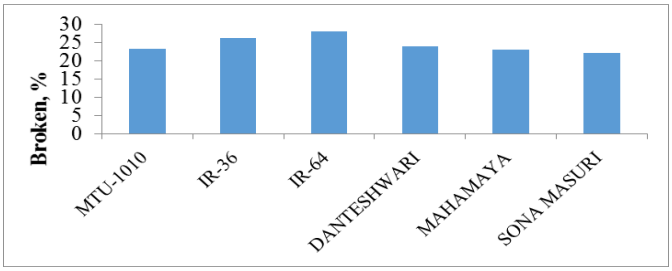


**Fig 1:** Broken percentage of procured varieties

**Selected rice varieties**

From the above table (table 1), the following rice varieties were selected on the basis of higher broken rice percentages (which are higher than normal broken percentage) for further experiments.

**Table 2:** Selected rice varieties

| S.N. | Name of the Rice variety | Source |
|---|---|---|
| 1. | MTU-1010 | I.G.K.V. Raipur |
| 2. | IR-36 | I.G.K.V. Raipur |
| 3. | IR-64 | I.G.K.V. Raipur |
| 4. | Danteshwari | I.G.K.V. Raipur |

**Ethanol Production**

*Saccharomyces cerevisiae* strains are known for ethanol production from a various raw material containing carbohydrates as well as starch. In this experiment, raw materials used for ethanol production were a substrate of broken rice after pre-treatment (different percentage of α-amylase treatment for 6h). Pre-treated rice from all the varieties was further incubated with three different yeast strain of *Saccharomyces cerevisiae* namely: *viz.* NCIM 3570, NCIM 3281 and NCIM 3640 for ethanol production. The ethanol produced after fermentation was analyzed using standard method and ethanol content expressed on a percent basis.

**Effect of different yeast strain on ethanol production from different varieties**

Table 3 and Fig. 2 indicates that maximum ethanol production was found in IR-36 ranging from 4.064 to 4.039% with all three different cultures, with the mean 4.063 which is significantly higher in comparison to other rice varieties, while IR-64 produces the least ethanol (3.862%) on the mean basis. On the other hand, significantly higher ethanol (4.039) percentage on mean basis was produced with yeast strain NCIM 3281.

**Effect of different culture on ethanol production from enzymatic pre-treated different rice varieties**

Rice varieties differ with respect to the production of ethanol content (Table 4 and Fig. 3). The amount of ethanol was found to be significantly higher from IR-36 variety (6.386%). While the maximum production of ethanol from variety IR-64 and Danteshwari was 6.336 % and 6.335% was at par. From the results, it is also revealed that the yeast strain NCIM 3281 produce the highest ethanol in all the varieties.

**Table 3:** Interaction table of different culture and rice varieties

| Variety | NCIM 3570 | NCIM 3281 | NCIM 3640 | Mean |
|---|---|---|---|---|
| MTU-1010 | 4.013 | 3.998 | 4.005 | 4.005 |
| IR-64 | 2.766 | 4.038 | 3.781 | 3.862 |
| IR-36 | 4.064 | 4.085 | 4.039 | 4.063 |
| Danteshwari | 4.014 | 4.037 | 4.019 | 4.023 |
| Mean | 3.964 | 4.039 | 3.961 | |
| | | | C.D. | SE(m) |
| | | Variety | 0.010 | 0.004 |
| | | Culture | 0.009 | 0.003 |
| | | Interaction | 0.018 | 0.006 |

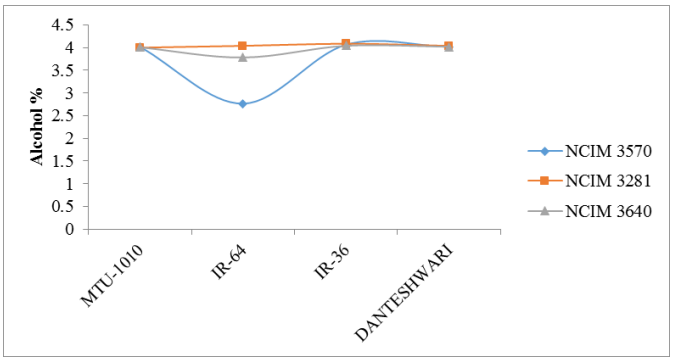


**Fig 2:** Interaction of different culture and rice varieties

**Table 4:** Mean Table for the effect of Enzyme pre-treatment, different cultures and Rice varieties on ethanol production

| | MTU-1010 | | | IR-64 | | | IR-36 | | | DANTESHWARI | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | B1 | B2 | B3 | B1 | B2 | B3 | B1 | B2 | B3 | B1 | B2 | B3 |
| C1 | 0.496 | 0.495 | 0.493 | 0.496 | 0.493 | 0.490 | 0.495 | 0.495 | 0.493 | 0.492 | 0.492 | 0.492 |
| C2 | 2.965 | 2.958 | 3.023 | 2.914 | 2.987 | 2.958 | 3.052 | 3.110 | 3.038 | 2.929 | 3.016 | 3.001 |
| C3 | 6.277 | 6.248 | 6.248 | 6.335 | 6.336 | 6.342 | 6.343 | 6.349 | 6.328 | 6.298 | 6.306 | 6.277 |
| C4 | 6.313 | 6.291 | 6.255 | 5.320 | 6.335 | 5.333 | 6.344 | 6.386 | 6.298 | 6.335 | 6.335 | 6.306 |
| | | | | | | | | | | | C.D. | SE(m) |
| | | | | | | | | | | Variety | 0.010 | 0.004 |
| | | | | | | | | | | Culture (B) | 0.009 | 0.003 |
| | | | | | | | | | | Enzyme treatment (C) | 0.010 | 0.004 |
| | | | | | | | | | | Interaction AxBxC | 0.036 | 0.013 |

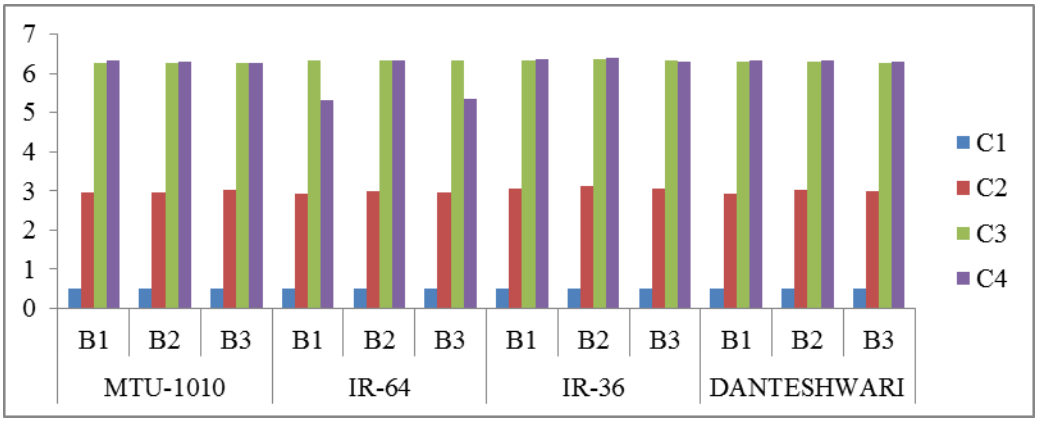


**Fig 3:** Effect of Enzyme pre-treatment, different cultures and Rice varieties on ethanol production

**Standardization of Optimum Condition for Ethanol Production**

**Condition optimization for ethanol production from variety IR-36 with NCIM 3281 strain**

Various factors *viz.* temperature, pH, substrate concentration, agitation and supplementation with micronutrient and nitrogen source affect the microbial growth directly. Hence, the experiments were carried out to optimize the conditions for maximum ethanol production.

**Temperature Optimization**

Temperature directly affects the yeast growth as well as ethanol production. For temperature optimization entire fermentation process was carried out with NCIM 3281 strain and substrate from IR-36 variety treated with 1% α-amylase enzyme for 6h. From the Table 5, it was found that maximum ethanol (6.35%) produced at 30ºC which is significantly higher than other temperatures (Fig. 4). At low-temperature microbes grow slowly and at high temperature, the enzyme and protein denature results in less ethanol production. Similar work was carried out by Arun kyalakond (2005) and results were found that maximum amount of residual reducing sugar was recorded in wine prepared from *Saccharomyces cerevisiae* NCIM 3090 (3.294 mg/g), followed by *Saccharomyces cerevisiae* NCIM 3576 (3.232 mg/g) and lowest in *Saccharomyces cerevisiae* var. *ellipsoideus* CFTRI 101 (3.137 mg/g). With the increase in the inoculum level, the residual sugar content decreased significantly in all the three strains. The highest percent of ethanol was recorded in wine prepared with *Saccharomyces cerevisiae* var. *ellipsoideus* CFTRI 101(5.23%) and least in *Saccharomyces cerevisiae* NCIM 3090 (4.929%). At 5 per cent inoculum level 6.439 per cent of ethanol was observed and 3.908 percent at 1 per cent inoculum level.

**Table 5:** Optimization of temperature for ethanol production

| Temperature ºC | Ethanol % |
|---|---|
| T1 (25) | 5.213 |
| T2 (30) | 6.350 |
| T3 (35) | 5.372 |
| C.D. | 0.006 |
| SE(m)± | 0.002 |

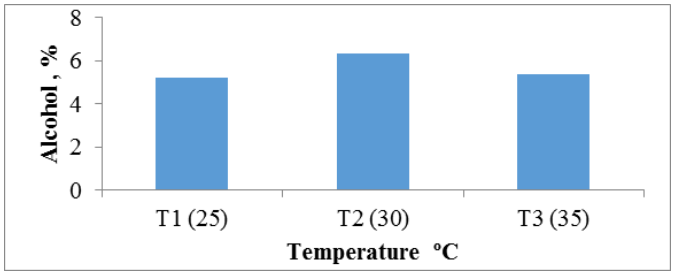


**Fig 4:** Optimization of temperature for ethanol production

**Optimization of Agitation speed (rpm) for ethanol production**

The experiment was carried out at different agitation speed to optimize the optimum agitation speed following other standardized conditions for IR-36. From the experiment, it was found that ethanol production was significantly higher at 100 rpm Table 6 (Fig. 5). At low agitation speed the substrate, culture, and products could not get proper mixing leads to less

growth as well as ethanol production. However, at higher agitation speed microbial cells get destroyed due to high mechanical force.

**Table 6:** Optimization of Agitation speed (rpm) for ethanol production

| Agitation (rpm) | Ethanol % |
|---|---|
| N1 (80) | 5.461 |
| N2 (100) | 6.350 |
| N3 (120) | 5.233 |
| C.D.<br>SE(m)± | 0.004<br>0.001 |

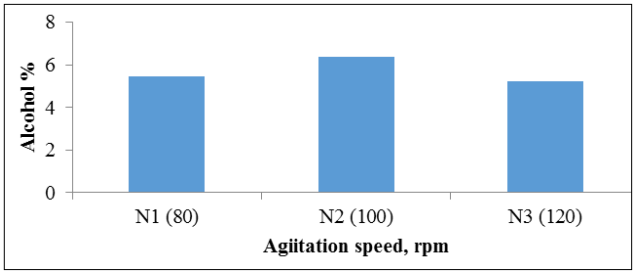


**Fig 5:** Optimization of Agitation speed (rpm) for alcohol

**Optimization of substrate concentration for ethanol production**

An experiment was carried out with selected rice variety and culture with other standardized conditions to optimize substrate concentration for maximum ethanol production. From the experiment it was found that pre-treated substrate diluted at 1:1 (V/V) ratio with distilled water gives significantly higher ethanol production than other (Table 7 and Fig. 6). Optimum substrate concentration is required for microbial growth and enzymes activity; at low concentration of substrate, enzymes do not show their maximum activity while at higher concentration their activity may be inhibited.

**Table 7:** Optimization of substrate concentration for ethanol production

| Rice variety | Substrate concentration | | |
|---|---|---|---|
| | 1:0 | 1:1 | 1:2 |
| IR-36 | 5.326 | 6.350 | 5.375 |
| C.D.<br>SE(m)± | 0.006<br>0.002 | | |

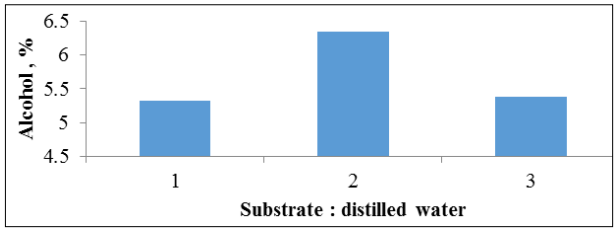


**Fig 6:** Optimization of substrate concentration for ethanol production

**Optimization of another supplement (diammonium hydrogen phosphate) for fermentation**

An experiment was carried out with selected rice variety and culture under other standardized condition to optimize the nitrogen supplement for maximum ethanol production. From the experiment, it was found that diammonium hydrogen phosphate supplied at the rate of 0.5 g/l was significantly better for maximum ethanol production (Table 8 and Fig. 7).

**Table 8:** Optimization of another supplement (diammonium hydrogen phosphate) for fermentation

| Supplement | Ethanol % |
|---|---|
| T(control) | 3.242 |
| T1(0.2gm/l) | 5.244 |
| T2(0.5gm/l) | 6.350 |
| T3(0.7gm/l) | 5.311 |
| C.D.<br>SE(m)± | 0.003<br>0.001 |

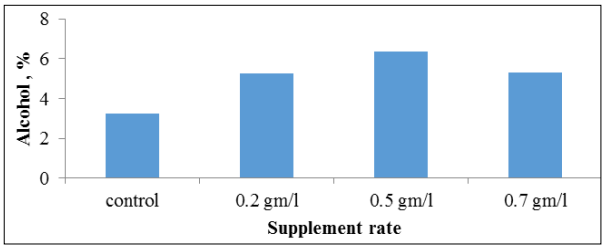


**Fig. 7** Optimization of another supplement (diammonium hydrogen phosphate) for fermentation

**Ethanol production at optimized conditions**

Ethanol was produced by following all the optimized conditions from IR-36 with *S.cerevisiae* NCIM 3281 and it was recorded 6.858%.

**Table 9:** Ethanol production at optimized condition

| Rice | Substrate concentration | Culture | Temperature | Agitation | Ethanol % |
|---|---|---|---|---|---|
| IR-36 | 1:1 | NCIM 3281 | 30±1 ºC | 100 rpm | 6.858 |

**Conclusion**

From the present study, it can be concluded that

1. The present investigation clearly brought out that maximum hydrolysis of rice substrate can be achieved by commercial α-amylase pre-treatment with 1% concentration at 6h.
2. The commercial α-amylase pre-treatment giving maximum reducing sugars was suitable for ethanol preparation using the *Saccharomyces cerevisiae.*
3. *Saccharomyces cerevisiae* NCIM 3281 culture produced highest percent (6.349) of ethanol with IR-36 rice variety followed by NCIM 3570.
4. The optimum temperature for ethanol (6.858%) production is $30\pm1^0$C is with *Saccharomyces cerevisiae* NCIM 3281 using rice from IR-36 at agitation speed 100 rpm and pretreated substrate diluted at 1:1 ratio.

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Reddy2 and Young-Jung Wee3* 1. Department of Microbiology, Yogi Vemana University, Kadapa (A.P.) 516003, India. 2. Department of Biochemistry, Sri Venkateswara University, Tirupati 517502, India. 3. Department of Food Science and Technology, Yeungnam University, Gyeongsan, Gyeongbuk 712-749, Korea. Accepted 4 April, 2011

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