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Studies on Bioethanol Production from Rice Straw by *Saccharomyces cerevisiae*

Brajeshwar Singh[1] and Neeru Choudhry[2]

[1]Department of Microbiology, Faculty of Basic Sciences, SKUAST-Jammu, INDIA.

[2]Department of Microbiology, Faculty of Basic Sciences, SKUAST-Jammu, INDIA.

[1]Corresponding Author: brajeshbhau@yahoo.co.in

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ABSTRACT

The present study aims to determine the bioethanol producing potential of locally isolated *Saccharomyces cerevisiae* strain using Rice straw as a substrate. Fifteen yeast strains were isolated from fruit sample (Apple, Papaya, Sugarcane, Grapes, Orange, Pineapple) and Kaladi samples collected from local markets of Jammu and Udhampur district respectively. Potential yeast isolates were identified on the basis of morphological and microscopic characteristics which were similar to that of MTCC 170 culture procured from IMTECH-Chandigarh. Out of 15 isolates, 14 show a positive change in the color of screening medium indicating the production of ethanol. S-9 culture was further selected based on its highest bioethanol producing ability of 17.88 mg/mL at 24 h of fermentation on YEPD medium and hence chosen for the fermentation of rice straw hydrolysate. Rice straw was subjected to alkaline and acidic pretreatment which was followed by enzymatic hydrolysis. Ethanol Fermentation of the total Rice Straw Hydrolysate obtained after pretreatments using S-9 culture revealed maximum ethanol concentration of 12.26 mg/mL at 24h having fermentation efficiency of 69.5%, 50.7% of sugar conversion occur during the fermentation process and volumetric productivity of 0.1702 g/L/h. Ethanol content per gram of sugar utilized was estimated to be 0.168g/g. Further, S-9 culture show the maximum efficiency for bioethanol production on YEPD medium at temperature of 30°C, pH of 5.6 and under static conditions.

Keywords- Fermentation, *Saccharomyces cerevisiae*, Bioethanol, Rice straw, Fermentation Efficiency.

I. INTRODUCTION

Throughout the world, fossil fuels are the primary source of energy. Due to its non-renewable nature and increased carbon dioxide emission, it is a major cause of global warming. The depriving fossil fuel resources has forced mankind to depend on an alternative fuel which must be renewable, and bioethanol is one of them. Bioethanol is considered as an important renewable fuel to partly replace fossil derived fuels. Fuel or energy sources that are obtained from organic by-products or naturally occurring, living organisms are called as Biofuels. Paper and wood waste, agrowaste, kitchen waste are major sources of biofuels. Ethanol or Ethyl alcohol is an oxygen containing organic compound having the chemical formula -C_2H_5OH. It is a monohydric primary alcohol which boils at 78.5 °C (Rahman, 2013). Ethanol is used as a solvent, a germicide, a beverage, an antifreeze, a fuel and as a chemical intermediate for organic chemicals. It is a colorless, clear, flammable and slightly toxic chemical with acceptable odour. It can be produced from petrochemical feedstock by acid-catalyzed hydration of ethene which account for only 3-4% of the total production while the rest is produced from biomass feedstock through fermentation (Licht, 2006). A blend of 10% dry ethanol and unleaded gasoline was commercially introduced into the US and continues to be marketed mainly in the Midwestern States (Hansen *et al.*, 2005). The octane number and combustion efficiency of bioethanol is greater than gasoline. It has high compression ratio, reduced burning time and high

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heat of vapourisation (Carrillo-Nieves *et al*., 2019, Balat *et al*., 2008). After combustion, ethanol emits less quantity of particulate matters, hydrocarbons and NO_x because of the oxygen content i.e., 35% oxygen therefore it's emission and toxicity are relatively less than other fuels such as diesel, petroleum (Toor *et al*., 2020). Increase in bioethanol from 50 million m^3 in 2007 to over 100 million m^3 in 2012 across the world. Brazil and United States contribute 80% of the world supply, mostly using corn or sugarcane (Kang *et al.*,2014). Fermentation is the process of chemical changes by which breakdown of larger molecules into smaller molecules occur by the microbial intervention. Alcoholic fermentation is a widely used process for production of Bioethanol. Ethanol fermentation is a biochemical process by which breakdown of sugars such as glucose, fructose, and sucrose occur in order to yield energy and gives ethanol and carbon dioxide as biproducts. Yeasts are unicellular eukaryotic microorganisms which belongs to kingdom fungi. About 80% of ethanol is produced by anaerobic fermentation, caused by yeast belongs to *Saccharomyces cerevisiae* (Nofemele *et al.*,2012). It is widely distributed in natural habitat and classified under division Ascomycota. They are heterotrophic, require water for diffusion and can grow aerobically as well as anaerobically.

Ethanol fermentation is usually carried out using sugars, starch and cellulose as substrate. First generation bioethanol includes the production of ethanol from lignocellulose material as feedstock, second generation include sugar based raw material and third generation include algal bioethanol production (Kang *et al.*, 2014). Sugar and starch are currently the main raw material but due to their demand for human feed and an expensive feedstock in the near future, this would guide the attention towards the cellulosic matter as the only potential feedstock for the production of ethanol (Taherzadel *et al*.,2007).

Fuel ethanol derived from biomass appears to be a promising alternative to traditional fossil fuels, having the potential to be used as a sole fuel in specialized engines or in fuel mixes. Agricultural or forestry residues, municipal garbage, and energy crops are all examples of lignocellulosic biomass. It is a plentiful, economical, and renewable feedstock with disposal issues. LB is an intrinsically complex and recalcitrant material in which cellulose, hemicellulose, and lignin are tightly linked, making it difficult for hydrolyzing enzymes to access. (Pin *et al.*, 2019; Trinh *et al.*, 2018).

As a result, a pretreatment method is an absolute requirement for achieving a cost-effective reduction in the downstream operation costs of fermentable sugars recovery from biomass (Tomas-Pejo *et al.*, 2008, Curreli *et al*., 2002). For acid treatments, (a) high temperature and low acid concentration, while (b) low temperature and high acid concentration are being used. H_2SO_4 is often used in diluted form for a wide range of biomasses, including switch grass, maize stover, spruce, poplar, kans grass, coconut fiber, and rice/wheat straw (Zhu *et al.*, 2008). The production of several microbial growth inhibitors such as acetic acid, furfural, and 5-hydroxymethylfurfural, that must be detoxified before fermentation, is the major disadvantage of acid treatment (Palmqvist and Hahn-Hägerdal, 2000).

In general, a higher pretreatment temperature and a shorter residence period result in better xylose recovery and enzymatic digestibility. All existing investigations in the same research field used a diluted H_2SO_4 concentration of (0.2–2%) and a higher temperature (121–372**ºC**) to promote the most critical functions such as hemicellulose hydrolysis, cellulose exposure for digestion, and heavy metal solubilization (Balat, 2011). Rice straw can be utilized to make bio-ethanol, providing additional revenue and a long-term use. It would also provide a clean energy answer to India's ever-growing energy demand. However, it is becoming increasingly crucial to investigate the long-term viability of bio-ethanol production from rice straw, as well as how it fits into the current Indian agricultural landscape (Singh *et al.*, 2002).

II. MATERIAL AND METHOD

2.1 Collection of samples from fruit and dairy sources

Fruit samples (Grapes, Apples, Orange, Pineapple, Papaya and Sugarcane) were collected from the local markets of Jammu District for the isolation of *Saccharomyces cerevisiae*. Kaladi, a fermented milk product was also collected from the local markets of District Udhampur for the studies. The collected samples were stored at 4ºC for further studies.

Lyophilized *Saccharomyces cerevisiae* MTCC-170 procured from IMTECH-Chandigarh was used as a check. This culture was revived on YEPD slants and incubated at 30ºC for 24 h. It was stored at 4ºC for further use.

2.2 Sterilization of glassware

Glassware was washed with distilled water and allowed to dry for all the laboratory experiments. The sterilization of glassware was done before use in hot air oven at 180ºC for 25 minutes.

2.3 Preparation of media

For the isolation and screening of isolates three media were used namely YMA (Yeast Mannitol agar), YEPD (Yeast extract -10g/L, peptone-20g/L, dextrose-20g/L and agar-15 g/L) and Screening media (peptone-10g, dextrose-5g, phenol red, and NaCl-5g in 1 Lt of distilled water).

Sterilization of 500 mL of conical flask was done in hot air oven at 180ºC for 25 minutes. 250 mL of prepared media components was added in a 500 mL of sterile conical flask and autoclaved at 121ºC,15 psi for 15 minutes before the experiment.

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2.4 Isolation and Purification of Saccharomyces cerevisiae

2.4.1 From fruit samples

The collected fruit samples were washed under tap water. After cutting it into small pieces it was kept in sterile Petri plate at 30ºC for 96 h. 1 gm of peal was subjected to serial dilution method. 10^{-5} and 10^{-7} dilution was used for spreading over YMA (Yeast Mannitol agar) plate and incubated at 30°C for 24 h in a BOD incubator. Selected colonies were transferred to fresh YMA plate. Pure cultures were obtained through streak plate method. Isolated pure culture was transferred to fresh YMA slants and stored at 4°C for further studies.

2.4.2 From dairy samples

Milk product (Kaladi) was cut in small pieces. It was followed by Serial dilution technique. 10^{-5} and 10^{-7} dilution was used for spreading over YMA (Yeast Mannitol agar) plate and incubating it at 30°C for 24 h in a BOD incubator.

Selected colonies were transferred to fresh YMA plated. Pure cultures were obtained through streak plate method. Isolated pure culture was transferred to fresh YMA slants and stored at 4°C for further studies.

2.5 Characterization of Isolated cultures

2.5.1 Morphological characterization

Individual isolated culture was streaked over YEPD agar plates with the help of sterilized inoculation loop. The plates were incubated at 30°C for 24 h and analysed visually for colony characteristics which include color, texture, margins etc.

2.5.2 Microscopic characterization

Freshly grown cultures on YEPD Medium were examined under microscope in order to study the microscopic characteristics. Isolated yeast cells were stained using Lactophenol Cotton Blue Staining Method. A drop of lactophenol cotton blue statin was placed over clean and grease free slide. With the help of flamed and cooled inoculating loop, a small drop of 24 h old isolated cultures on YEPD agar plate were placed into Lactophenol cotton blue drop. It was finally spread to form a thin layer and a coverslip was placed over. First it was observed under 10x and then at 40x magnification under compound microscope in order to record the microscopic characteristics.

2.6 Screening for Bioethanol production

The screening of isolates was based on the ethanol producing ability of *Saccharomyces cerevisiae* on screening media. The screening media is red due to the presence of phenol red indicator and a change in color from red to yellow after fermentation indicates the production of ethanol from the Isolates. 15 mL of screening media was poured into 30 mL of screw capped tubes which was autoclaved at 121ºC and 15 psi for 20 minutes. Isolates were transferred to fresh YEPD slants and incubated at 30ºC for 48 h. 48 h old isolated culture was used to inoculate autoclaved fermentation medium and kept for fermentation under static conditions at 30ºC for 72 h (Nasir *et al.*, 2017).

2.7 Quantification of Isolates for Ethanol production

In order to select high bioethanol producing isolate from the selected isolates quantification was done spectrophotometrically.

Screened isolates were transferred to fresh autoclaved YEPD slant with the help of sterile inoculating loop. After 24 h. of growth, it was used for the preparation of inoculum. YEPD broth (Yeast extract -0.5%, peptone 1% and Dextrose 2%) was used as inoculation medium. In a 250 mL flask, 50 mL of inoculation medium was prepared and autoclaved at 121°C and 15 psi. For the preparation of inoculum, loop full of 24 h old culture was used to inoculate the inoculum media, which was incubated at 30°C for 24 hours at 120 rpm. YEPD broth (1% yeast extract, 1% peptone, and 12% Dextrose) was used as fermentation media. 20 mL of fermentation medium was poured into 30 mL screw-capped tubes and autoclaved at 121ºC at 15 psi for 20 min. Inoculation of screw capped tubes was done in sterilized conditions @ 10 % with the inoculum prepared and incubated at 30ºC under stationary conditions for 72h. Ethanol fermentation was also executed by MTCC 170 culture in YEPD medium as a control.

2.7.1 Spectrophotometry

Ethanol content produced in the fermentation medium was determined after various time intervals from 24 to 72 h using spectrophotometer. Acidified potassium dichromate was prepared by adding 34 g of potassium dichromate in 500 mL of concentrated sulfuric acid and raising the volume to 1 L with distilled water. This was stored at 4ºC for further use.

A 3.0 mL of acidified potassium dichromate was incubated with 500 µL of standard ethanol solutions at room temperature for 30 min and absorbance was analyzed at 590 nm wavelength using spectrophotometer. A standard curve was prepared by plotting absorbance obtained against the respective ethanol concentrations and an equation was obtained.

After 24h, 48h and 72h of fermentation ethanol content was determined by adding 10 µL of fermentation medium in a test tube and raising the volume upto 500µl by adding distilled water. In the test tube, 3 mL of acidified potassium dichromate was added and incubated at room temperature 30ºC for 30 minutes. Measure the Absorbance at 590nm wavelength. The ethanol concentration of the fermentation media was calculated by substituting the absorbance value obtained into the equation of the standard curve.

2.8 Pre- treatment of Agro-waste

2.8.1 Raw Material

The rice straw was collected from Chatha Research Farm and dried at 60ºC. The dried rice straw was cut into small pieces and blended in a blender to obtain uniform size. Rice straw is composed of 38% cellulose, 25% hemicellulose and 12 % lignin (Hung *et al.*, 2020). Pretreatment helps to break down the rigidity of lignocellulosic biomass so that the lignin,

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hemicellulose, and cellulose molecules may be exposed, allowing the yeast to access fermentable sugars (Toor *et al.*, 2020).

2.8.2 Delignification of the rice straw by alkaline pre-treatment

Blended rice straw was subjected to alkaline treatment for the removal of lignin. It would help to expose the cellulosic content. 5gm of blended rice straw was added into 50 mL of 0.2 M KOH solution and finally incubated at 35ºC for 4 h. After that, it was filtered with muslin cloth and the biomass was rinsed several times under tap water to achieve a neutral pH (Yadav et al., 2011).

2.8.3 Acid Pretreatment of the De-lignified rice straw

The acid pre-treatment of the delignified rice straw was done in a biphasic manner using sulfuric acid. The first phase acid hydrolysis was done by incubating delignified rice straw in 100 mL of 1% Sulphuric acid at 121°C for 15 minutes. It was filtered and the solid residual were used for second phase acid hydrolysis. In Second phase hydrolysis the solid residues were incubated with 100 mL of 2% Sulphuric acid at 121°C for 15 minutes. Solid and liquid portions were further separated through filtration. Acid pretreated biomass was dried at 40ºC and further subjected to enzymatic pretreatment (Yadav et al., 2011).

2.8.4 Enzymatic pre-treatment

Biphasic acid pretreatment (1% SA, 2% SA) along with enzymatic saccharification was more efficient for saccharification of rice straw (Gupta *et al.*,2015). Enzymatic saccharification was done by using cellulase enzyme. The dried acid pretreated biomass was weighed and pre inoculated with acetate buffer at 10% substrate consistency having concentration of 50mM and pH, 5.6 for 15 min at 37°C. After that cellulase loading of 15 CMcaseIU/G was added and further incubated at 50 °C and 150 rpm for 48 h in a water bath. Finally, the liquid and solid fractions were separated using filtration.

Prehydrolysate were mixed for ethanol fermentation as total hydrolysate and analysed for reducing sugar content by DNSA (3, 5- Dinitrosalicylic Acid) method (Miller Gl, 1985).

2.9 DNSA Test

Table 1: DNSA reagent Components

S. No.	Chemicals	Percentage (w/v)
1	3,5-Dinitrosalicylic acid	1
2	Phenol	0.2
3	Na_2SO_3	0.05
4	NaOH	5

DNSA reagent was prepared by mixing 3,5-Dinitrosalicylic acid (1%w/v), Phenol (0.2%w/v), Na_2SO_3 (0.05%w/v) and NaOH (5%w/v) and raised the volume up to 100 mL by adding distilled water. DNSA reagents was finally stored in dark colored bottles for further use.

100µl of the sugar hydrolysate were mixed with 900µl of distilled water in test tube and incubated with 1 mL of DNSA reagent at 95°C for 10 min in a water bath. 1mL of NaK Tartrate (40% w/v) was added after that content was cooled under tap water and absorbance was analyzed at 575nm.

A standard curve for glucose was formed and the concentration of glucose was determined by comparing OD with this curve. Initial sugars and final residual sugars were estimated.

2.10 Ethanol fermentation of the Rice Straw Hydrolysate

Ethanol Fermentation of the total Rice Straw Hydrolysate were performed using best isolate of yeast culture (*Saccharomyces cerevisiae)*, MTCC-170 culture.

Ethanol production medium (EPM) having pH 5.6 was prepared. EPM medium was prepared by adding peptone-1 g/L, yeast extract-1.5g/L, dipotassium phosphate-1g/L, ammonium sulphate-1 g/L and magnesium sulphate-1g/L into rice straw hydrolysate. 20 mL of the EPM medium prepared was added into 30 mL of screw capped tubes and autoclaved at 121ºC, 15psi for 15 minutes (Vaid *et al.*, 2017). The media was inoculated with 24 h old yeast cultures at the rate of 1.5% (v/v), and incubated at 30ºC. Ethanol content was determined after various time intervals from 24 to 72 h as discussed earlier.

Experimental ethanol yield and Fermentation efficiency was calculated using the formula in equation no. 1 and 2 (Vogel et al., 2011).

All the experiments were conducted in triplicates, and data presents the mean values.

$$\text{Ethanol from hexoses and pentoses (mL/g)} = \frac{1.1\times 0.51 \text{ ethanol per glucose}}{\text{Specific volume of ethanol (0.789 g/mL)}} \quad \ldots(1)$$

$$\text{Fermentation efficiency (\%)} = \frac{\text{Experimental yield} \times 100}{\text{Theoretical yield}} \quad \ldots(2)$$

2.11 Optimization of culture conditions for bioethanol production

S. cerevisiae is capable of very rapid rates of glycolysis and ethanol production occur under optimal condition. The culture conditions of high ethanol producing isolate was optimized which include pH, Temperature and Agitation. Classical one factor at a time was used for the optimization parameters. To examine the effects of temperature, initial pH and agitation on ethanol production, isolates were cultivated at a range of temperatures 25, 30, 35ºC; various pH 4.6, 5.6 and 6.6 and various agitation rate 0, 120, 150 rpm.

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2.11.1 pH optimization

50 mL of inoculation medium (Yeast extract - 0.5%, peptone 1% and Dextrose 2%) was inoculated with loop full of 24 h old culture, and further incubated at 30°C for 24 hours at 120 rpm in an incubator shaker. YEPD broth (1% yeast extract, 1% peptone, and 12% Dextrose) was used as fermentation media having initial pH of 4.6, 5.6 and 6.6. Inoculation of fermentation medium was done in sterilized conditions @ 10 % with the inoculum prepared and incubated at 30ºC under stationary conditions for 72 h. Concentration of ethanol was measured spectrophotometrically at 24, 48 and 72 h.

2.11.2 Temperature optimization

Inoculation medium (Yeast extract -0.5%, peptone 1% and Dextrose 2%) was inoculated 24 h old culture and incubated at 30°C for 24 hours at 120 rpm in an incubator shaker. 20 mL of fermentation medium broth (1% yeast extract, 1% peptone, and 12% Dextrose) having initial pH of 5.6 was inoculated in sterilized conditions @ 10 % with the inoculum prepared and incubated at 25ºC, 30ºC and 35ºC under stationary conditions for 72 h. Concentration of ethanol was measured spectrophotometrically at 24, 48 and 72 h.

2.11.2 Agitation

Inoculation medium (Yeast extract -0.5%, peptone 1% and Dextrose 2%) was inoculated by 24 h old culture and incubated at 30°C for 24 hours under shaking conditions. 100 mL of fermentation medium broth (1% yeast extract, 1% peptone, and 12% Dextrose) was inoculated with the inoculum prepared @ 10 % and incubated at 30ºC under static conditions, 120 rpm and 180 rpm for 72 h. Concentration of ethanol was measured spectrophotometrically at 24, 48 and 72 h.

2.12 Quality parameters

Quality parameters include Initial glucose concentration in the total hydrolysate before fermentation, final glucose concentration after 72 h of fermentation, Percentage of Sugar consumption, Fermentation efficiency, Ethanol yield per gram of sugar consumed and Volumetric productivity.

Initial and final glucose concentration of the EPM was determined by DNSA method as discussed earlier. Percentage of sugar consumption was calculated by taking the difference of final and initial sugar concentration then diving it with initial sugar content and finally multiplied by 100 (Rezania *et al.*, 2018). Ethanol yield per gram of sugar consumed is the amount of ethanol produced(mg/mL) divided by the sugar consumed. Ethanol volumetric productivity was calculated by dividing the ethanol concentration(g/L) of the fermentation medium with the fermentation time in hours. It was expressed as volumetric productivity (g/L/h) (Rezania *et al.*, 2018).

III. RESULT

In the present study *Saccharomyces cerevisiae* was isolated from fruit and kaladi samples. The potential isolates were identified on the basis of morphological and microscopic characters. They were further screened on the basis of ethanol producing potential of *Saccharomyces cerevisiae*. Quantification on YEPD media was done spectrophotometrically. Selected isolate was used for the fermentation of rice straw after several pretreatments and quantification was done. Optimization of culture condition of best isolate was also done.

3.1 Isolation and purification of Saccharomyces cerevisiae:

Isolation of *Saccharomyces cerevisiae* was done from fruit and dairy samples collected from local market of Jammu and Udhampur district respectively. Fruit samples include Apple, Orange, Pineapple, Papaya, Grapes and Sugarcane while Kaladi was taken as dairy sample. Total fifteen isolates (three from sugarcane, two from pineapple, two from grapes, one from orange, one from papaya, one from apple and five from kaladi) were obtained from the collected samples.

3.2 Microscopic and colony characteristic

In the present study microscopic and colony characteristics were examined based on the growth of isolates on YEPD media. Isolated cultures show smooth texture, entire margins and whitish or off-white color. Microscopic characteristics show ovoid and elongated cells. The morphological and colony characteristics were compared with that of MTCC 170 strain of *Saccharomyces cerevisiae*.

3.3 Screening of potential isolates for bioethanol production

The potential isolates were screened for the production of bioethanol. They were allowed to grow in the screening medium for about 72h and analyzed for the color change. The change in the color of screening medium after 72h of fermentation from red to yellow indicate the production of bioethanol. The results presented in table 3 show that 15 out of 14 (S-1, S-2, S-3, S-4, S-5, S-7, S-8, S-9, S-9, S-10, K-1, K-2, K-3, K-3, K-4 and K-5) show a positive color change and indicate the production of ethanol.

3.4 Quantification of Isolates for bioethanol production

Quantification of screened isolates was done spectrophotometrically. Ethanol was used as a standard. Isolates were grown on YEPD medium and ethanol content was estimated after various time intervals from 24 to 72 h of fermentation using acidified potassium. S-9 isolate show maximum ethanol concentration of 17.88 mg/mL and fermentation efficiency of 69.78% on YEPD media.

3.5 Estimation of initial reducing sugar content by DNSA method.

Total sugar hydrolysate obtained after various pretreatments were analyzed for the content of reducing sugar by DNSA method. It helped us to know the efficiency of pretreatment and the quantity of fermentable sugars exposed for yeast to act. Initial glucose concentration of total hydrolysate was 7.05±0.4922 mg/mL.

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3.6 Estimation of ethanol content after fermentation with the rice straw hydrolysate

Ethanol content was determined spectrophotometrically after the fermentation with S-9 culture using Rice straw hydrolysate. MTCC 170 culture was used as a control. Results in table 6 show that S-9 culture maximum ethanol concentration of 12.26 mg/mL and fermentation efficiency of 69.5% at 24h. The concentration of ethanol produced by S-9 isolate reduces to 7.37 mg/mL and 2.01 mg/mL after 48h and 72h of fermentation, respectively. MTCC-170 culture produced maximum ethanol concentration of 14.98 mg/mL and fermentation efficiency of 84.9% at 24h the concentration of ethanol produced by MTCC 170 also decreases to 6.38mg/mL and 1.29 mg/mL after 48h and 72h of fermentation respectively.

3.7 Estimation of Final reducing sugar content by DNSA method

The final reducing sugar content of the fermentation media was analyzed by DNSA method. S-9 and MTCC 170 culture show the final glucose concentration of 3.41±0.046 mg/mL and 2.32±0.032 mg/mL respectively after 72h of fermentation.

3.8 Optimization of culture conditions

Optimization of culture conditions for the growth of S-9 isolate includes temperature, pH, and agitation using classical factor one at a time. Three factors were analyzed for each character. Ethanol concentration was determined spectrophotometrically by recording absorbance at 590 nm after 24h, 48h and 72 h of fermentation. The fermentation condition for growth of S-9 isolate were temperature – 25ºC, 30ºC and 35ºC, pH - 4.6, 5.6 and 6.6 and agitation -static, 120rpm and 180rpm.

3.8.1 Effect of Temperature on Ethanol production

During the studies, S-9 culture show maximum ethanol production of 16.9 mg/mL at 24h of fermentation at 30ºC after that the ethanol concentration reduces to 15.35 mg/mL and 11.12 mg/mL at 48h and 72 h respectively (Table 1).

3.8.2 Effect of pH on Ethanol production

The final ethanol concentration in the medium is affected by the pH of the medium. More acidic and basic conditions result in a decrease in the yield of ethanol due to retarded cell growth and metabolic pathways in yeast (Taherzadeh *et al.*, 2007). Optimum pH for the ethanol production by S-9 isolate was 5.6 with the ethanol concentration of 16.96mg/mL after 24 h of fermentation and having the fermentation efficiency of 66.37% (Table 2).

Table 1: Effect of temperature on production of Ethanol from S-9 Culture on YEPD media

S. No.	Temperature	Ethanol concentration (mg/mL)			Maximum Ethanol concentration (mg/mL)	Fermentation efficiency %
		24hr	48hr	72hr		
1	**25°C**	14.67±0.235	11.19±0.322	6.7±0.654	14.67±0.235	57.41
2	**30°C**	16.9±0.562	15.35±0.385	11.12±0.235	16.6±0.562	66.17
3	**35°C**	15.03±0.225	11.2±0.449	4.89±0.433	15.03±0.225	58.85

Data in mean of three triplicates± S.D

Table 2: Effect of pH on production of Ethanol from S-9 culture on YEPD media

S. No.	pH	Ethanol concentration (mg/mL)			Maximum ethanol concentration (mg/mL)	Fermentation efficiency %
		24hr	48hr	72hr		
1	**4.6**	13.9±0.455	10.41±0.680	4.48±0.708	13.9±0.455	54.5
2	**5.6**	16.96±0.449	11.56±0.290	7.41±0.635	16.96±0.449	66.37
3	**6.6**	13.41±0.449	9.62±0.680	4.78±0.482	13.41±0.449	52.48

Data in mean of three triplicates± S.D

Table 3: Effect of agitation on production of ethanol from S-9 culture on YEPD medium

S. No.	Agitation	Ethanol concentration (mg/mL)			Maximum ethanol concentration (mg/mL)	Fermentation efficiency %
		24hr	48hr	72hr		
1	**Static conditions**	15.77±0.634	11.56±0.612	6.95±0.323	15.77±0.634	61.7
2	**120 rpm**	15.69±0.806	8.75±0.980	4.46±0.484	15.69±0.806	61.43
3	**180 rpm**	14.75±0.449	10.19±0.455	5.12±0.819	14.75±0.449	57.72

Data in mean of three triplicates± S.D

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4.8.3 Effect of Agitation on Ethanol production

Maximum ethanol production of 15.77mg/mL was estimated during fermentation in static conditions. Agitation causes a reduction in the production of ethanol which is due to the increase in cell density. Maximum ethanol production was estimated to be 15.69 mg/mL and 14.75 mg/mL when fermentation occur in shaking conditions at 120 rpm and 180 rpm respectively after 24 h (Table 3).

Table 4: Effect of Temperature, pH and Agitation on Ethanol production by S-9 isolate on YEPD media

S. No.	Optimized conditions		Maximum ethanol concentration (mg/mL)	Fermentation efficiency %
1	Temperature	25°C	14.67±0.235	57.41
		30°C	16.9±0.562	66.17
		35°C	15.03±0.225	58.85
2	pH	4.6	13.90±0.455	54.5
		5.6	16.96±0.449	66.37
		6.6	13.41±0.449	52.48
3	Agitation	Static	15.77±0.634	61.74
		120 rpm	15.69±0.806	61.43
		180 rpm	14.75±0.449	57.72

Data in mean of three triplicates± S.D

Table 5: Ethanol production during the fermentation of pretreated hydrolysate

Culture	Ethanol concentration (mg/mL)	Fermentation efficiency %	Q_p (g/l/h)	$Y_{g/s}$ (g/g)	S_c%
S-9	12.26±0.521	69.5	0.1702	0.168	50.7
MTCC-170	14.98±0.230	84.9	0.208	0.158	66.36

Q_p volumetric productivity, $Y_{g/s}$ Ethanol content per gram of sugar utilized, S_C Sugar consumption %
Data in mean of three triplicates± S.D

3.9 Quality parameters

S-9 isolate show the Ethanol concentration of 12.26 mg/mL, Fermentation efficiency of 69.5%. 50.7% of sugar conversion occur during the fermentation process and volumetric productivity of 0.1702 g/L/h. Ethanol content per gram of sugar utilized was estimated to be 0.168g/g. MTCC 170 culture show a higher ethanol concentration of 14.98 mg/mL. Fermentation of hydrolysate of rice straw occur with fermentation efficiency of 84.9% and 67.09% of sugar consumption was estimated (Table 5).

IV. DISCUSSION

In the present study, fruit samples and kaladi samples were collected from local market of Jammu and Udhampur district respectively. 15 yeast strains were isolated from Apple, Papaya, Sugarcane, Grapes, Orange, Pineapple and Kaladi samples. Nasir *et al.*, 2017 have isolated *Saccharomyces cerevisiae* from orange and pineapple for bioethanol production. The isolation of *Saccharomyces cerevisiae* from fruit samples has been reported by various workers (Balakumar *et al.*, 2001; Boudjema *et al.*, 2016; Sabate *et al.*, 2002; lee, 2013; Cha *et al.*, 2008, Zin Yu *et al.*, 2018).

Potential yeast isolates were identified on the basis of morphological and microscopic characteristics which were similar to that of MTCC 170 culture. Morphological characters of our culture show smooth whitish, creamish and entire colony on YEPD medium. The cell morphology of our isolated cultures shows ovoid and elongated cells. Our efforts are backed up by the work of Nasir *et al.*, 2017.

Isolates were screened for the bioethanol production on the basis of color change of the screening medium. Nasir *et al.*, (2017) also identified the isolates on the basis of color change of the screening medium. They suggested that the color change of the medium is due to the acid produced and gas evolved during fermentation. The quantification of selected isolates was done spectrophotometrically which revealed that S-9 isolate show maximum ethanol concentration of 17.88 mg/mL at 24 h of fermentation on YEPD medium. Potential yeast strains studies revealed that isolated *Saccharomyces cerevisiae* strain produce maximum ethanol concentration of 16.96mg/mL at 5.6 pH on YEPD medium.

Rice straw is a potential lignocellulosic material for the production of bioethanol. It is composed of high concentration of cellulose and hemicellulose due to

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which it can be converted into fermentable sugars. Pretreatment aims to expose the cell wall materials for enzymatic degradation, enhance the substrate's surface area and porosity, diminish cellulose crystallinity, and alter the heterogeneous structure of cellulosic materials (Gadde *et al*., 2009). Rice straw was pretreated by alkaline, acidic and enzymatic hydrolysis. Fermentation of sugar hydrolysate of rice straw by S-9 isolate produces 12.26 mg/mL of ethanol at 24h of fermentation, Fermentation efficiency of 69.5%. 50.7% of sugar conversion occur during the fermentation process and volumetric productivity of 0.1702 g/L/h. Ethanol content per gram of sugar utilized was estimated to be 0.168g/g.

In our present study, optimization of fermentation condition for growth of S-9 isolate were temperature – 25ºC, 30ºC and 35ºC, pH - 4.6,5.6 and 6.6 and agitation -static, 120rpm and 180rpm.

Optimum pH for the ethanol production by S-9 isolate was 5.6 with the ethanol concentration of 16.96 mg/mL after 24 h of fermentation and having the fermentation efficiency of 66.37%. Our results are in accordance with that of Narendranath and Power, (2005). They reported that, *Saccharomyces cerevisiae* in general is an acidophilic organism and thus grows better under acidic conditions. There are many enzymes functioning within the yeast cell during growth and metabolism. Each enzyme works best at its optimal pH, which is acidic because of the acidophilic nature of the yeast itself. When the extracellular pH deviates from the optimal level, the yeast cell needs to invest energy to either pump in or pump out hydrogen ions in order to maintain the optimal intracellular pH (Narendranath *et al.*, 2001). If the extracellular pH deviates too much from the optimal range, it may become too difficult for the cell to maintain constant intracellular pH, and the enzymes may not function normally. If the enzymes are deactivated, the yeast cell will not be able to grow and make ethanol efficiently. The optimum temperature for the ethanol production for S-9 isolate appears to be 30ºC with maximum ethanol value of 16.90 mg/mL. Maurice, (2011) suggested that the yeasts at 30ºC are under minimal stress and are not inhibited by the produced ethanol. In addition, at 35°C, the ethanol yield decreases with an increase of fermentation temperature. Maximum ethanol production of 15.77 mg/mL was observed during fermentation in static conditions. Agitation causes a reduction in the production of ethanol which is due to the increase in cell density. Maximum ethanol production was estimated to be 15.69 mg/mL and 14.75 mg/mL when fermentation occur in shaking conditions at 120 rpm and 180 rpm respectively after 24 h.

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