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# Media Optimization for Bioethanol Production Using a Native Isolated Strain from Mahua Flowers

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***Abstract:*** *Traditionally mahua flowers are collected by the tribals and used for making liquor is practiced in many states in Northern India. These flowers are rich in sugars and hence harbours native yeast flora capable of carrying out natural fermentation. In the current work previously isolated strain IL2 from Mahua flowers was used for bioethanol production following optimization of physicochemical parameters and nutritional parameters of the media using shake flask fermentation. The bioethanol production for IL2 was 10.65 % (w/v) whereas the reference Saccharomyces cerevisiae MTCC174 was 15.76% (w/v) under optimized conditions. The production of the native isolate can be enhanced by carrying out fermentation in a fermenter and improvising the downstream protocols in future.*

***Keywords:*** *Media optimization, native yeast, Mahua flowers, Bioethanol, fermentation.*

## I. Introduction

*Madhuca longifolia* commonly called as *mahua* is a non- timber tree found in the states of Andhra Pradesh, Bihar, Chhattisgarh, Gujarat, Jharkhand, Karnataka, Madhya Pradesh, Maharashtra, Orissa, Uttar Pradesh and West Bengal. The flowers of this tree are traditionally used in preparation liquor by tribals. The flowering seasons begins from January and lasts until April. The flowers are collected by the locals and are dried to prepare other edible products apart from liquor making. The estimated production of *mahua* flowers is more than one million tons in the country. Various products like alcohol **[1]**,brandy**[2],** acetone**[3],** lactic acid **[4]** and other fermented product have been prepared from the dry *mahua* flowers. *Mahua* flower extract has also being used for fortification of red wine to enhance the overall quality and palatability of red wine **[5].**The current study focuses on bioethanol production from a natural isolate IL2 belonging to genus *Saccharomyces***[6]** in the previous work from *mahua* flowers using *mahua* flowers as substrate by shake –flask fermentation.

## II. Materials and Methods

### 2.1 Fermentation Substrate

*Mahua* flowers were collected and were dried for two days to reduce the moisture content to 15 % followed by sterilizing the flowers by moist heat sterilization in the autoclave at pressure 10 lb/inch2 for a period of 15 minutes **[7].**The total sugar estimated using Anthrone method was found to be 55%

### 2.2 Yeast strains and maintenance

Yeast strain used for study was isolate IL2 belonging to genus *Saccharomyces* isolated in the current research work was maintained on MGYP agar and the reference strain *Saccharomyces cerevisiae* MTCC 174 was maintained as per standard guidelines for the maintenance of the strain.

### 2.3 Preparation of the seed culture

The seed culture was prepared using slight modifications of earlier reports **[7,8].** The fermentation slurry of *Mahua*: Distilled water (1:4) was added instead of glucose, Malt extract 15 grams/ liter, yeast extract 15 grams/liter and peptone 25 gm/liter was autoclaved at 121°C, 15 lbs pressure for 30 minutes. The media was cooled and 2-3 loops (Himedia) of original maintained cultures were added aseptically the flasks for both isolate IL2 and MTCC 174 were incubated at 30°C on rotary shaker for 48 hours at 80 rpm.

### 2.4 Estimation of ethanol [9,10]

The crude fermented sample was qualitatively checked for the presence of ethanol by Jones Reagent test in which Crude fermented broth, 2% Potassium dichromate and Concentrated Sulphuric acid in a ratio of 1:2:1 give bluish green colour indicating presence of ethanol. The fermented broth was distilled using simple distillation apparatus and the recovered distillate was determined by measuring specific gravity as per Pharmacopeia of India (1985**).** The ethanol percentage was calculated using Specific gravity method and percentage in v/v was obtained standard reference table using specific gravity The percentage v/v was converted

to w/v by multiplying with a factor of 0.79 (specific gravity of ethanol). The ethanol can also be estimated by Cole's method, Gas Chromatography and spectrophotometric method.

**2.5 Effect of various physico-chemical and nutritional parameters on bioethanol production**

**2.5.1 Effect of substrate concentration**

The substrate concentration was varied from 5 % to 35 % the other parameters such as pH was maintained at 5, inoculum age 72 hours, agitation 80 rpm, temperature RT, concentration of urea 0.04 % was kept constant.

**2.5.2 Effect of pH**

The effect of pH was studied by varying the pH from 2 to 10 of the fermentation media using 0.1 M Sodium hydroxide and 0.1 M Hydrochloric acid.

**2.5.3 Effect of temperature**

The effect of temperature was studied as temperature affects the ethanol production due to volatility of ethanol. The temperature was varied from 25°C to 50°C.

**2.5.4 Effect of inoculum age**

The process of fermentation was carried out for a period of 7 days and after every 24 hours the ethanol yield was calculated.

**2.5.5 Effect of inoculum level**

Inoculum level was studied to determine the percentage of inoculum in the media for optimum fermentation. The range selected was from 0.5 % to 3 %.

**2.5.6 Effect of agitation**

Agitation helps the organisms to utilize maximum sugars and thereby increasing the yield of alcohol production than static conditions. The flasks were agitated at 50, 80, 100, 120 and 150 rpm on a rotary shaker.

**2.5.7 Effect of magnesium ions**

The effect of magnesium ions on the yeast activity was studied for varying concentrations of magnesium ions ranging from 1 ppm to 10 ppm.

**2.5.8 Effect of Urea**

Microbes use nitrogen source for growth and metabolic activities hence urea concentration was altered in the medium ranging from 0.01 gm/liter to 0.1gm/liter.

All the above parameters were studied and the production was carried out using the optimized parameters the percentage yield was carried for isolate IL2 and compared with the standard reference strain *Saccharomyces cerevisiae* MTCC 174.

## III. Results

**3.1 Effect of substrate concentration**

The substrate concentration was varied between 5 % to 35 % and the results were obtained by running three sets in different time span. It was found that 20% substrate concentration was best suited for IL2 and 30% substrate concentration was best suited for *S. cerevisiae* MTCC 174. The minimum fermentation time was 72 hours for both the strains after which even though the substrate concentration increased the yield decreased. Thus the optimum yield was **8.03 %** (w/v) at **20 %** utilization of substrate for IL2 and 8.34 % (w/v) at 20 % utilization of 20 % substrate for *S. cerevisiae* MTCC 174 however the yield was **9.54** (w/v) for **30 %** substrate utilization for the standard strain.

To compare the effect of changes in the physico-chemical and nutritional parameters on production of bioethanol the substrate concentration 20% was kept constant and other parameters were studied. The results obtained are reported in **table 4.1 and figure 4.1.**

**3.2 Effect of pH**

The effect of pH was studied by varying the pH from 2 to 10. The ethanol yield was lower in acidic pH as well at highly alkaline pH. The optimum pH was **5** which showed **8.48 %** (w/v) for isolate IL2 and **9.10 %** (w/v) for *S. cerevisiae* MTCC 174. The results obtained are reported in **table 4.2.1, table 4.2.2 and figure 4.2.**

**3.3 Effect of temperature**

The effect of temperature was studied on the ethanol production. The temperature was varied from 25°C to 50°C. The optimum temperature was **30°C** as yielded **8.78 %** (w/v) for isolate IL2 and **9.11 %** (w/v) for *S. cerevisiae* MTCC 174. The isolate IL2 could not tolerate 50°C hence no fermentation was obtained in the flask. The results obtained are reported in **table 4.3 and figure 4.3.**

**3.4 Effect of inoculum age**

The process of fermentation was carried out for a period of 7 days and after every 24 hours the ethanol yield was calculated. It was found that the optimum yield of **8.34%** (w/v) for isolate IL2 and **8.71%** (w/v) for *S. cerevisiae* MTCC 174 was found after **72 hours.** The fermentation continued for all seven days but dropped down drastically to 2.53% (w/v) and 2.79 % (w/v) for IL2 and *S. cerevisiae* MTCC 174 respectively. The results obtained are reported in **table 4.4 and figure 4.4.**

**3.5 Effect of inoculum level**

Inoculum level was studied to determine the percentage of inoculum in the media for optimum fermentation. The range selected was from 0.5 % to 3%. It was found that **2.5%** of inoculum was effective to yield **8.56 %** (w/v) for isolate IL2 and **9.0 %** (w/v) for *S. cerevisiae* MTCC 174. The results obtained are reported in **table 4.5 and figure 4.5.**

**3.6 Effect of agitation**

The flasks were agitated at 50, 80, 100, 120 and 150 rpm on a rotary shaker. The optimum yield was at **100 rpm** after which the decrease of yield was observed. The yield for isolate IL 2 was **8.63%** (w/v) and **9.12%** (w/v) for *S. cerevisiae* MTCC 174. The results obtained are reported in **table 4.6 and figure 4.6.**

**3.7 Effect of magnesium ions**

Yeast activity was studied for varying concentrations of magnesium ions ranging from 1 ppm to 10 ppm. The optimum concentration for magnesium ions was found to be **7 ppm** wherein the yield for isolate IL2 was **9.16%** (w/v) and **9.20 %** (w/v) for *S. cerevisiae* MTCC 174 indicating that magnesium ions can activate the yeast for enhanced production. The results obtained are reported in **table 4.7.1 & table 4.7.2 and figure 4.7.**

**3.8 Effect of Urea**

Urea concentration was altered in the medium ranging from 0.01gm/liter to 0.1gm/liter. The optimum concentration was **0.05gm/liter** of urea concentration which gave yield of **9.14%** (w/v) for isolate IL2 and **9.65 %** (w/v) for *S. cerevisiae* MTCC 174. The results obtained are reported in **table4.8.1 & table 4.8.2 and figure 4.8.**

**3.9 Optimization of fermentation process**

From the results obtained of the above parameters the optimum conditions for the fermentation process were set for isolate IL2 and standard reference strain *S. cerevisiae MTCC 174* which is summarized in **table 4.9.** The fermentation was carried out for period of 5 days and the yield of bioethanol produced was estimated and the results obtained are summarized in **table 4.10** and **figure 4.9.**

## IV. Figures and Tables

**Table 4.1** Effect of substrate concentration on bioethanol production

| **Strain** | **5%** | **10%** | **15%** | **20%** | **25%** | **30%** | **35%** |
|---|---|---|---|---|---|---|---|
| **Isolate IL2** | 2.35 | 4.56 | 6.78 | **8.03** | 7.54 | 5.35 | 4.54 |
| ***S. cerevisiae* MTCC 174** | 3.55 | 5.68 | 7.12 | 8.34 | 8.75 | **9.54** | 7.45 |

**Figure 4.1** Effect of substrate concentration on bioethanol production

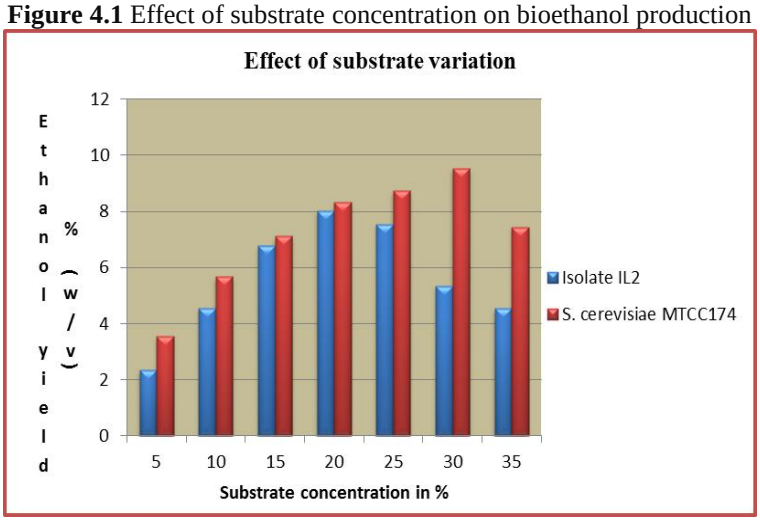


**Table 4.2.1** Effect of pH variation on bioethanol production

| Strain | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|
| **Isolate IL2** | 2.45 | 4.56 | 5.78 | **8.48** | 7.02 |
| ***S. cerevisiae* MTCC 174** | 2.58 | 5.54 | 6.78 | **9.10** | 7.94 |

**Table 4.2.2** Effect of pH variation on bioethanol production

| Strain | 7 | 8 | 9 | 10 |
|---|---|---|---|---|
| **Isolate IL2** | 6.45 | 4.45 | 3.34 | 2.30 |
| ***S. cerevisiae* MTCC 174** | 7.05 | 5.43 | 4.15 | 2.54 |

**Figure 4.2** Effect of pH variations on bioethanol production

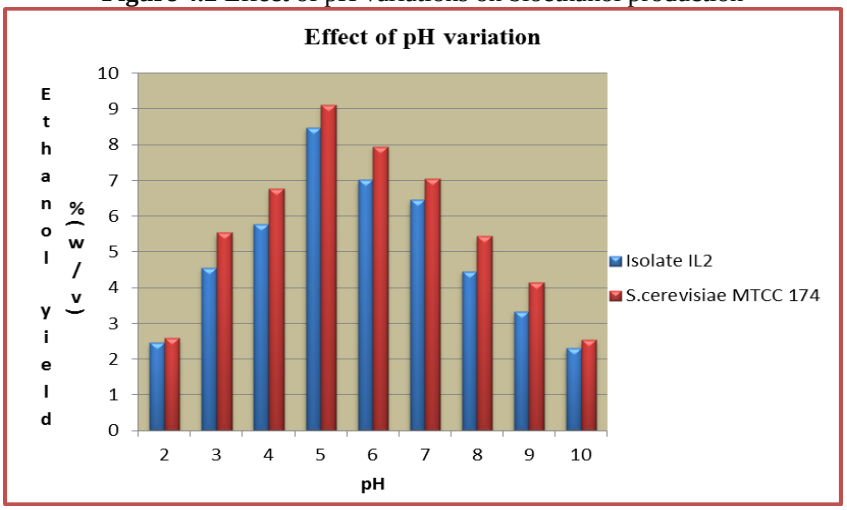



**Table 4.3** Effect of temperature variation on bioethanol production

| Strain | 25 | 30 | 37 | 44 | 50 |
|---|---|---|---|---|---|
| **Isolate IL2** | 7.45 | **8.78** | 8.36 | 3.48 | - |
| ***S. cerevisiae* MTCC 174** | 7.58 | **9.11** | 8.78 | 5.10 | 3.45 |

**Figure 4.3** Effect of temperature variation on bioethanol production

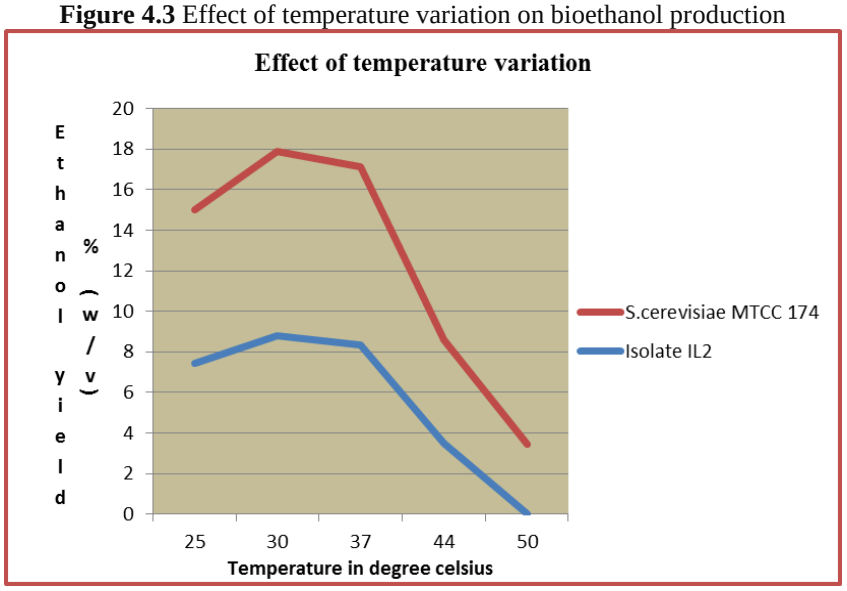



**Table 4.**4Effect of on inoculum age bioethanol production

| Strain | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
|---|---|---|---|---|---|---|---|
| **Isolate IL2** | 3.56 | 5.45 | **8.34** | 7.05 | 5.67 | 3.45 | 2.53 |
| ***S. cerevisiae* MTCC 174** | 4.56 | 7.05 | **8.71** | 7.56 | 6.54 | 4.35 | 2.79 |

**Figure 4.**4Effect of inoculum age on bioethanol production

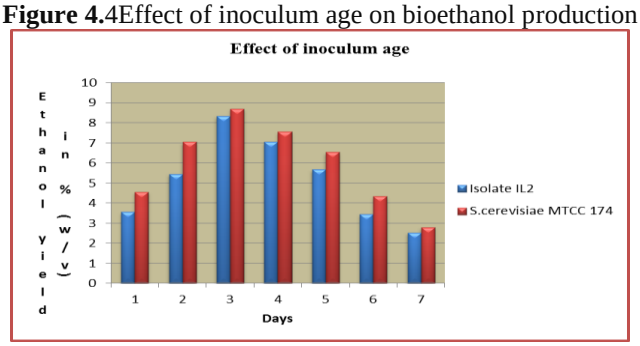



**Table 4.5** Effect of inoculum level on bioethanol production

| **Strain** | **0.5%** | **1%** | **1.5%** | **2%** | **2.5%** | **3%** |
|---|---|---|---|---|---|---|
| **Isolate IL2** | 2.50 | 4.56 | 6.89 | 7.05 | **8.56** | 6.56 |
| ***S. cerevisiae* MTCC 174** | 3.57 | 5.23 | 7.45 | 8.10 | **9.00** | 7.56 |

**Figure 4.5** Effect of inoculum concentration level on bioethanol production

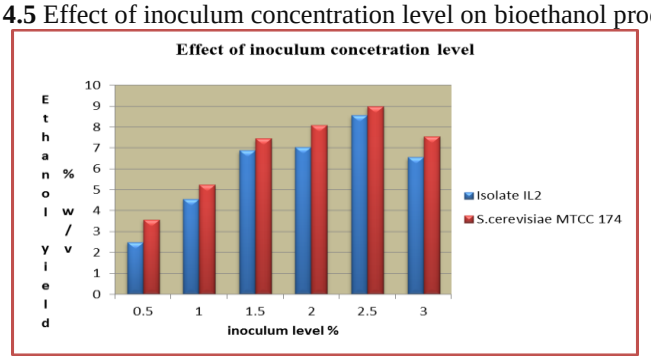



**Table 4.6** Effect of agitation on bioethanol production

| **Strain** | **50 rpm** | **80 rpm** | **100 rpm** | **120 rpm** | **150 rpm** |
|---|---|---|---|---|---|
| **Isolate IL2** | 6.54 | 7.15 | **8.63** | 7.45 | 4.34 |
| ***S. cerevisiae* MTCC 174** | 6.55 | 7.58 | **9.12** | 7.05 | 5.67 |

**Figure 4.6** Effect of agitation on bioethanol production

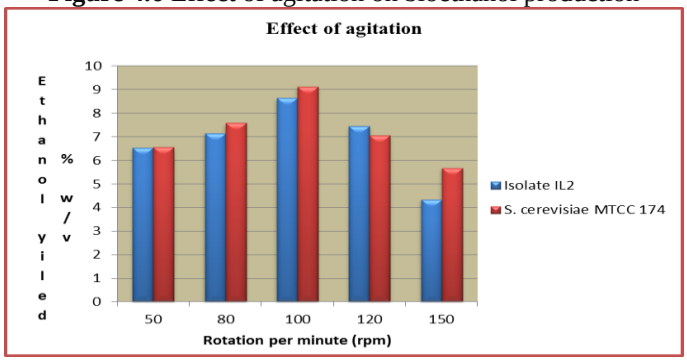



**Table 4.7.1** Effect of magnesium ions on bioethanol production

| **Strain** | **1 ppm** | **2 ppm** | **3 ppm** | **4 ppm** | **5 ppm** |
|---|---|---|---|---|---|
| **Isolate IL2** | 5.67 | 6.55 | 7.58 | 8.65 | 8.78 |
| ***S. cerevisiae* MTCC 174** | 5.87 | 6.78 | 8.00 | 8.54 | 8.96 |

**Table 4.7.2** Effect of magnesium ions on bioethanol production

| **Strain** | **6 ppm** | **7 ppm** | **8 ppm** | **9 ppm** | **10 ppm** |
|---|---|---|---|---|---|
| **Isolate IL2** | 8.96 | **9.16** | 8.56 | 7.65 | 7.45 |
| ***S. cerevisiae* MTCC 174** | 9.06 | **9.20** | 8.88 | 8.54 | 7.30 |

**Figure 4.**7 Effect of Magnesium ions concentration on bioethanol production

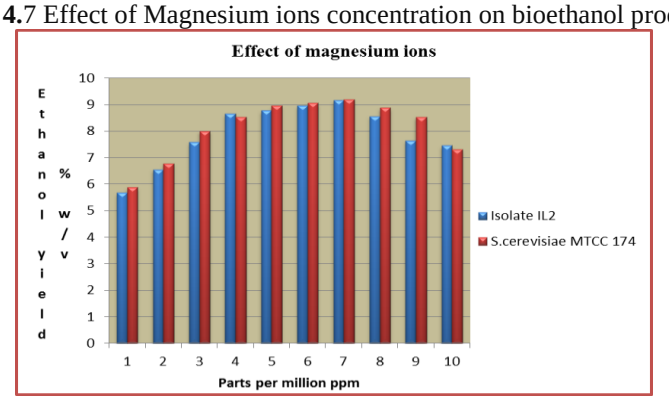



**Table 4.8.1** Effect of urea concentrations on bioethanol production

| Strain | 0.01 gm/l | 0.02 gm/l | 0.03 gm/l | 0.04 gm/l | 0.05 gm/l |
|---|---|---|---|---|---|
| **Isolate IL2** | 5.68 | 6.78 | 6.98 | 8.78 | 9.14 |
| ***S. cerevisiae* MTCC 174** | 5.77 | 7.78 | 8.00 | 9.54 | 9.65 |

**Table 4.8.2** Effect of urea concentrations on bioethanol production

| Strain | 0.06 gm/l | 0.07 gm/l | 0.08 gm/l | 0.09 gm/l | 0.10 gm/l |
|---|---|---|---|---|---|
| **Isolate IL2** | 8.88 | 7.76 | 7.56 | 7.15 | 6.45 |
| ***S. cerevisiae* MTCC 174** | 9.25 | 9.14 | 8.88 | 8.54 | 7.30 |

**Figure 4.8 Effect** of urea concentrations on bioethanol production

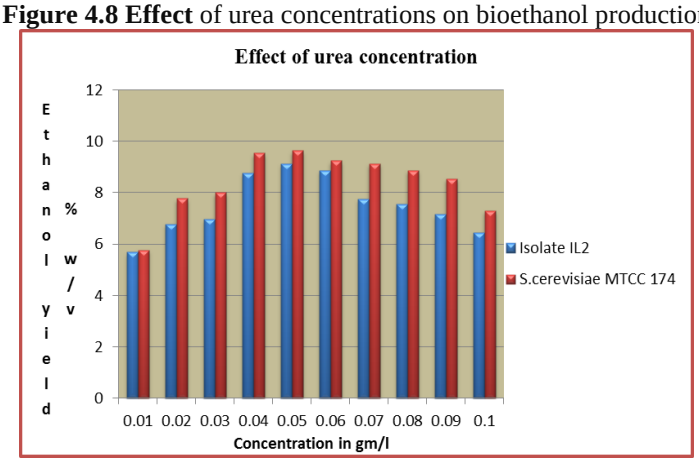


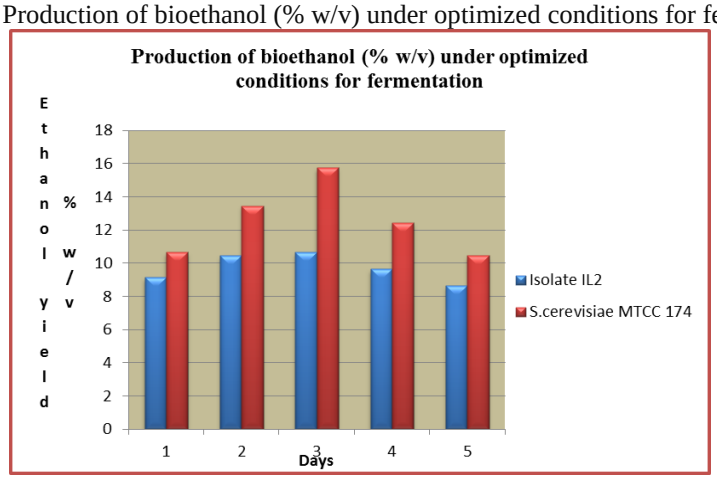

**Table 4.9 Optimized conditions for fermentation process using *mahua* substrate and strains Isolate IL2 and *S. cerevisiae* MTCC 174**

| Parameter | Value |
|---|---|
| Substrate concentration | **20%** |
| pH | **5** |
| Temperature | **30$^0$C** |
| Inoculum age | **72 hours** |
| Inoculum concentration level | **2.5%** |
| Agitation speed | **100 rpm** |
| Magnesium ions | **7 ppm** |
| Urea concentrations | **0.05 gm/l** |

**Table 4.10** Production of bioethanol (% w/v) under optimized conditions for fermentation.

| Strain | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 |
|---|---|---|---|---|---|
| **Isolate IL2** | 9.15 | 10.46 | **10.65** | 9.65 | 8.65 |
| ***S.cerevisiae* MTCC 174** | 10.65 | 13.45 | **15.76** | 12.45 | 10.46 |

**Figure 4.9** Production of bioethanol (% w/v) under optimized conditions for fermentation




## V. Conclusion

In our previous study natural yeast flora was isolated from *mahua* flowers. Out of the isolated strains, IL2 strain was better ethanol tolerant, thermo- tolerant, osmo-tolerant strain. The IL2 strain was selected to ferment *mahua* flowers as a substrate and was compared with standard reference *Saccharomyces cerevisiae* MTCC 174. The fermentation process was optimized considering various factors influencing fermentation process and at optimized process the isolated strain IL2 generated on an average of 10.65% (w/v) and maximum upto14.56% (w/v) of bioethanol after 72 hours of fermentation which was almost similar to the standard reference strain *Saccharomyces cerevisiae* MTCC 174 which produced average of 15.76 and maximum upto 18.71% (w/v) bioethanol. There were certain factors such as substrate concentration, concentration of magnesium ions and temperature which had maximum effect on ethanol yield. **Benerji D.S.N. et. al., 2010** reported 13.45 % for *S. cerevisiae* 3090 under submerged fermentation. **Mandal P and Kathale N. 2012.** reported 302 ml of bioethanol production using *S. cerevisiae* 3044. The isolated strain can be engineered to utilize various substrates and production of bioethanol can be increased in a fermenter which can solve energy crisis and serve as a substitute to fossil fuels.

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