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Studies on Physico-Chemical and Nutritional Parameters for the Production of Ethanol from Mahua Flower (Madhuca indica) Using *Saccharomyces Cerevisiae – 3090 Through Submerged Fermentation (smf)*

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Abstract

Effect of various Physico-Chemical and Nutritional parameter for the production of ethanol from mahua flower using *Saccharomyces cerevisiae -3090* through submerged fermentation have been studied. Substrate mahua flower contains 68% Total sugar. Yeast strain *S.cerevisiae-3090* was obtained from National Collection of Industrial Microorganisms (NCIM), Pune, South India. Maximum production of ethanol obtained at different optimized parameters such as substrate concentration at 28%, p^H at 5.0, Inoculum level at 2%, Inoculum age at 48 hours, Temperature 30^0c, Urea at 0.06 %, Copper sulphate 3 ppm, Sodium Potassium Tartrate 1.0 g/l and fermentation period is 48 hours is 13.450%(w/v). Effect of Sodium Potassium Tartrate and Urea showed the maximum production of ethanol. It is also conformed that the designed media is suitable for ethanol yield for large scale production.

Keywords: Mahua flower; Submerged fermentation; Yeast; Ethanol; GLC

Introducton

Fermentation of fruit juices to alcoholic beverages is known to man since 3000 B.C even in Vedas mentioned about "*SURA*" and "*SAMURA*"are two alcoholic products. Ethanol is proved to be the nearest to the "Universal Solvent", and alternative fuel for the future. Fermentation has contributed significasntly to different industrial activities in the present world today. Extensive exploitation and chemical process have caused ecological imbalances.

Biotechnology has played a major role in the microbial fermentation of cheap raw materials. Microorganisms are used as a tool by fermentation biotechnologists for the conversion of sugar into ethyl alcohol. Due to severe energy crisis in todays world, ethanol is considered to be the most suitable energy source amongst different fossil fuels. It has been producing by using a variety of substrates such as sugar cane juice, Sago starch, corn beat molasses, Jaggery etc. Mahua flowers (Wealth of India, CSIR, 1962) are rich source of sugars, contains 72.9 %, proteins 4.4%, Fat 0.5%, calcium 150 mg, Iron 15mg/100 gm, magnesium, copper and vitamins. In India, various parts of Andhra Pradesh Maharashtra, Chhattisgarh, some tribal communities cultivating and harvesting mahua flowers for alcoholic beverages using traditional methods (Yadav et al., 2009). The extraction of sugars were carried by 1kilogram of mahua flowers autoclaved with 2 liters of water at 1 steam pressure for 15 minutes (Rao et al., 1961; Yadav et al., 2009).

Mahua flowers were used for the production of vinegar (Wealth of India, CSIR, 1964) and alcohol production is likely to continue where they are available at low cost and where alcohol intended for used as potable spirits. Sarkar and Chatterjee (1984) studied structural feature of the polysaccharides (Sarkar and Chatterjee, 1984) of mahua flowers. Preparation of sugars and analysis of flowers from various districts have been studied, the analysis revealed that mahua contains 65-70 % sugars, reducing sugars 48-55%, Invert sugar 14-18,crude protein 4.0-6.5, Ferrous 21-48 % fat 0.9-1.3, ash 2.5-5.2%, calcium 177-266% (Sutaria and Magar, 1955). Biocatalyst *Saccharomyces cerevisiae-3090* was obtained from the (NCIM), Pune. Ethanol tolerance yeast isolated from fermenting cashew juice (Osho, 2005). Mahua flower is a abundant in India and it is having good keeping qualities. If the utilization of mahua flower as a substrate for the production of ethanol though submerged fermentation, it will become a great economic advantage in the Indian context. Based on the above advantages and necessities of the usage of raw material for the production of ethanol, mahua flower was chosen as a substrate in the present work (Yadav et al., 2009).

Materials and Methods

Substrate

Mahua flowers are obtained from Badrachalam, Khammam district, Andhra Pradesh, South India. Mahua scientific name is Madhuca Indica belongs to the family *Sapotaceae*. The extraction of sugars from these flowers is carried with distilled water and mahua flower in 2:1 ratio and autoclaved at pressure 10Ib/inch^2 for a period of 15 minutes.

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Moisture context

Moisture content is Estimated by using Association of Official Agricultural Chemistry (AOAC, 1960). The moisture content of the mahua flower used for the present study is found to be 15 %.

Estimation of total sugars

Total sugar of mahua flower is estimated by using Anthrone method, and found to 68% of sugars.

Estimation of phenolics

During fermentation, total phenolics in fermented media were estimated using (Basha et al., 2004; Amerine et al., 1965).

Microorganism

Yeast strain *Saccharomyces cerevisiae-3090* was obtained from "National Collection of Industrial Microorganism" (NCIM) National Chemical laboratory, Pune, Maharashtra, South India.

Medium for seed culture

A 10L bioengineering AG (Switzerland) fermentor was used for large scale production of cell. The fermentor was equipped with temperature, pH, and RPM controls. The volume kept was 5Litres. The p^H was maintained. The fermentor was operated for 24 to 98 hours continuously to get maximum inoculum. Yeast strain *S.cerevisiae*-3090 culture is maintained on the yeast extract, glucose, malt retract, peptone (*YGMP*) medium at 30°C temperature and maximum growth obtained at P^H5 this nutrient agar medium containing Malt extract 15gm/liter, Glucose 50 gm/liter, Yeast extract 15gms/liter, Peptone 25gm/liter and the seed culture for fermentation was prepared as 5000 ml of *YGMP* medium and medium is autoclaved at 15 lbs pressure, 2-3 loops of original culture were transferred aseptically and incubated at 30°C for a period of 48 hours on orbital shaker at 50 rpm. The total cells estimated by heamocytometer, thus one ml of broth contains $6.2x10^7$ cells. S.cerevisiae is maintained in YGMP medium and for every 30.

Estimation of Ethanol

Gas chromatographic method by Anthony (1984) was used in the present work. The fermented samples were centrifuged for 10 min. The supernatant was used for ethanol analysis by Gas chromatography (Nucon 5765) using a flame ionization detector (FID) and porapak Q column (100 to 120 mesh). The temperature (°C) of 180, 240, 240 for oven, injector and detector respectively were maintained. In this n-propanol was used as an internal standard. Standard curve was prepared with different ethanol concentrations and the percentage of ethanol % (v/v) was estimated as follows

$$\text{Percentage ethanol} = \frac{\text{peak hight of ethanol}}{\text{peak hight of n-propanol}} \text{ x F}$$

where F is the slope from standard curve (Anthony, 1984). The percentage of ethanol v/v was converted to percentage w/v by multiplying with a factor of 0.79 (specific gravity of ethanol at 20°C). Ethanol is also estimated by Potassium dichromate oxidation method (Horwitz, 1980) and spectrophotometric method (Caputi et al., 1968).

Experiment

Effect of substrate concentration

Effect of substrate concentration for production of ethanol from mahua flower using *S.cerevisiae-3090* was carried out by varying substrate concentration from 4w/v to 36w/v other parameters such as Inoculum age 72 hours, Inoculum level 1 gm/100ml, Agitation 50 rpm, concentration of Urea about 0.04%, pH 6, at room temperature 37°C.

It can be seen in Figure1, that the maximum yield of ethanol is 7.551% (w/v) obtained at 28w/v concentration of substrate mahua flower and fermentation time was observed is 2 days and it can be seen from Figure 2, that the production of ethanol was decreased with increase in substrate concentration. It was conformed that substrate mahua flower extract of 28 w/v concentration is an optimum substrate concentration. This in view, substrate concentration at 28w/v was taken as an optimum, and effect of other physico-chemical and nutritional parameters such as P^H, Temperature, Inoculum level, Inoculum age, agitation, effect of nitrogen source i.e. Urea, Ammonium sulphite, effect of Metal ions Mg^{+2} and Cu^{+2}, EDTA and Sodium Potassium Tartrate were studied.

Effect of pH

In order to study the effect of P^H the experiment was setup by varying the P^H from 1 to 10 p^H of the fermentation medium, it was adjusted with 0.1 *M* Sodium Hydroxide (NaOH) and 0.1 *M* Hydrochloric acid (*HCL*).

It was found to be very important since P^H profiles gives a

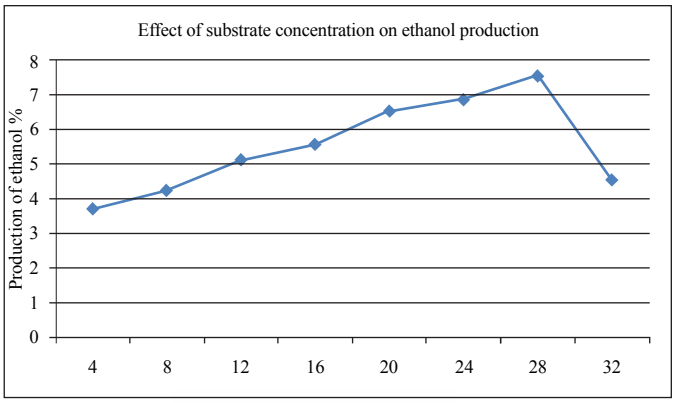

Figure 1: Substration concentration g/1.

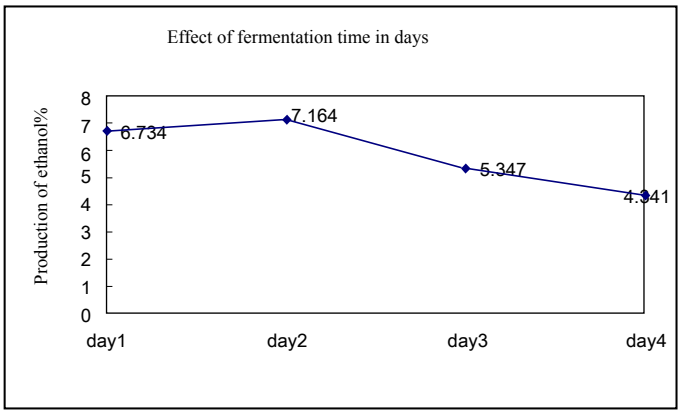

Figure 2: Fermentation time in days.

sharp peak. The ethanol yield is low at P^H 2 and further increase in P^H decreases ethanol production. It can be observed in Figure 3, that the maximum ethanol 8.256% (w/v) was obtained at P^H 5.

Effect of temperature

Temperature has a profound effect on ethanol production, since ethanol is volatile. The effect of temperature on Ethyl alcohol (C_2H_5OH) yield was studied by varying the temperature from 10°C to 50°C, it can be seen in the Figure 4, the yield of ethanol is very low at 10°C, but gradually increases up to 30°C, at this temperature, maximum ethanol yield obtained is 9.431%(w/v).

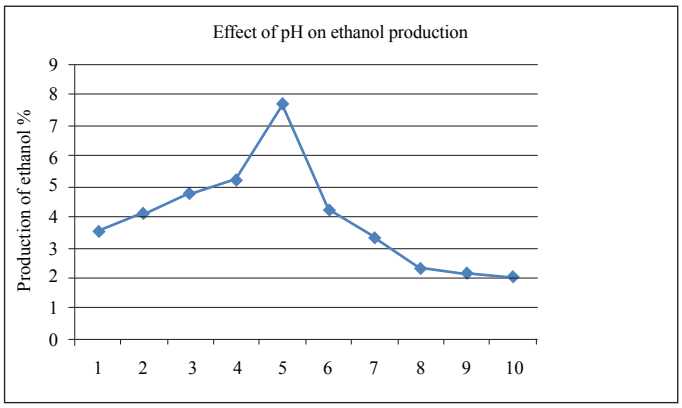

Figure 3: pH

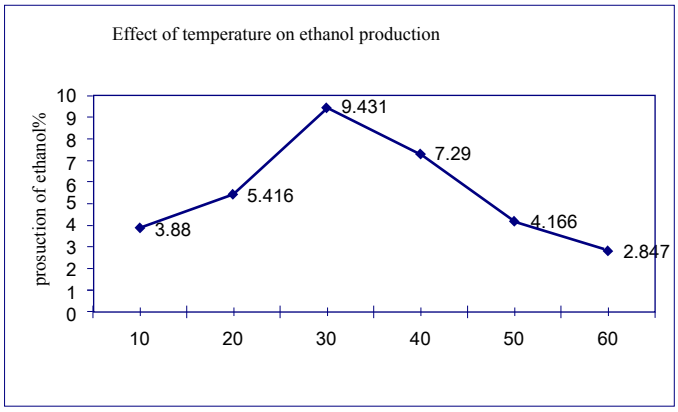

Figure 4: Temperature °C.

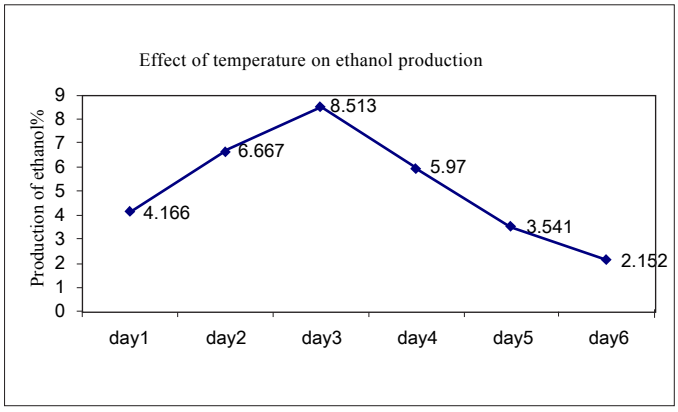

Figure 5: Innoculum age in days.

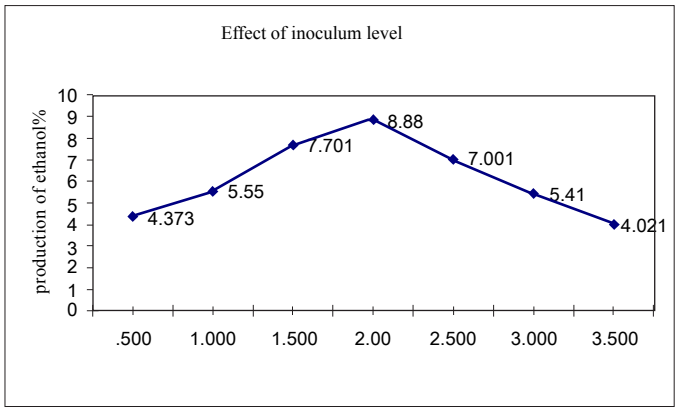

Figure 6: Inoculum level in mg/ml.

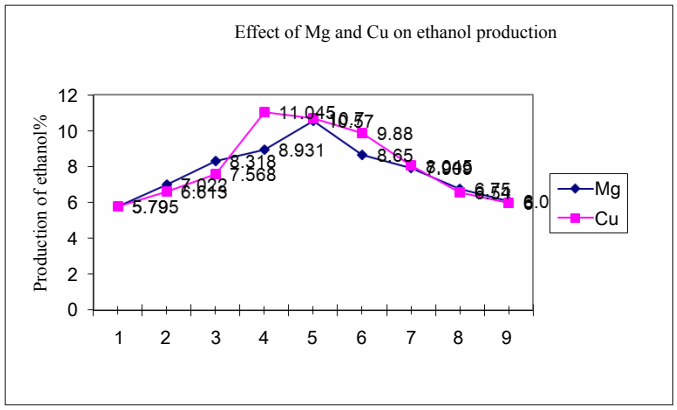

Figure 7: Concentration of Metal ions in ppm level.

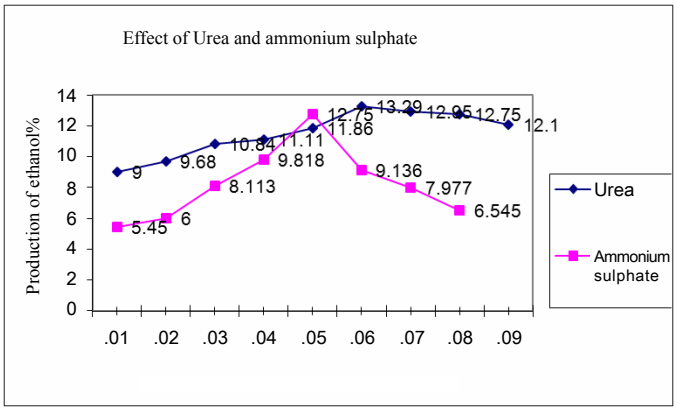

Figure 8: Effect of Nitrogen source in mg/ml.

Effect of Inoculum age

Inoculum age is investigated to determine the potentiality of the *S.cerevisiae* 3090. From the Figure 5, it is seen that the experiment reveals that the maximum growth can be obtained at 48 hours age of yeast and the ethanol yield was obtained is 8.513% (w/v).

Effect of Inoculum level

Inoculum level was studied to determine the wet weight of *S.cerevisiae-3090(16)* cells. In this experiment, wet weight of cells analyzed.

From the Figure 6, It was seen that the maximum ethanol yield was obtained is 8.880% (w/v) at 2 % of Inoculum level. The increase in inoculum level decreases production of ethanol.

Effect of agitation

Agitation is one of the important parameter because at optimum rpm, *S.cerevisiae*-3090 can utilize maximum sugars for their growth and for ethanol production through submerged fermentation. By keeping optimized parameters, agitation was studied. In this experiment the rpm was varied from 50 to 250 rpm with an increment of 50 rpm.

It is seen from the Figure 7, that the ethanol production (7.686%) (w/v) gradually was increased with increase in agitation up to 100 rpm, but further increase in rpm, decrease in ethanol yield was observed. So, optimum agitation at 100 rpm was used for further investigation.

Effect of metal ions magnesium and calcium

Mahua is a suitable substrate for the production of ethanol in large scale process, Because it consists of Phosphorous about 140 mg, Calcium 150mg, and also Magnesium and Copper are present. Since, metal ions has been found as a profound effect on yeast activity. In this experiment the effect of Magnesium and copper were carried out with different concentrations ranging from 1 ppm to 9 ppm level.

From the Figure 8, it was showed that ethanol yields was 10.570% and11.045% at 5ppm of Mg^{+2} and 4ppm of Cu^{+2} respectively.

Effect of nitrogen source

Most microbes utilize nitrogen to metabolize nitrogenous substances for the growth and their activity, effect of nitrogen source (Beltran et al., 2007) of Urea and Ammonium sulfate were studied with different concentrations ranging from 0.0.1gm/lit to 0.09 gm/liter.

From the Figure 9, it can be conclude that maximum production was obtained at 0.06gm/liter concentration of urea, and it can be seen that the ethanol is increased from 0.01gm/liter to 0.06gm/liter, further increase in urea decreases the ethanol production, this may be due to the inhibitory effect of Urea and Ammonium sulphate. This figure showed that the curve of Urea at 0.06gm/liter and Ammonium sulphate at 0.05gm/liter overlap with one other, that indicates urea has in significant effect on the rate of production of ethanol and it may be concluded that it is desirable to use urea of 0.06gms/liter for the maximum production.

Effect of chelating agents

Chelating agents EDTA and sodium potassium tartrate were studied with different concentrations ranging from an increment of 0.2gms/liter respectively.

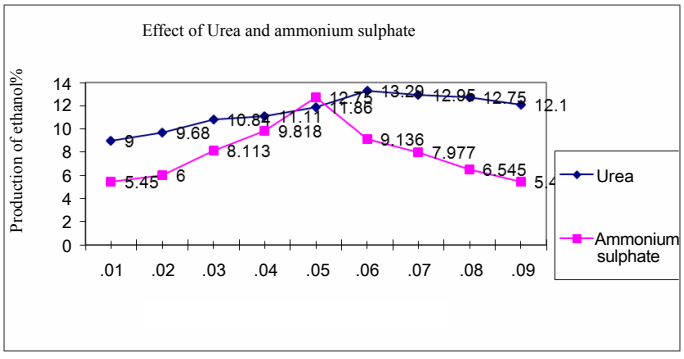

Figure 9: Effect of Nitrogen source in mg/ml.

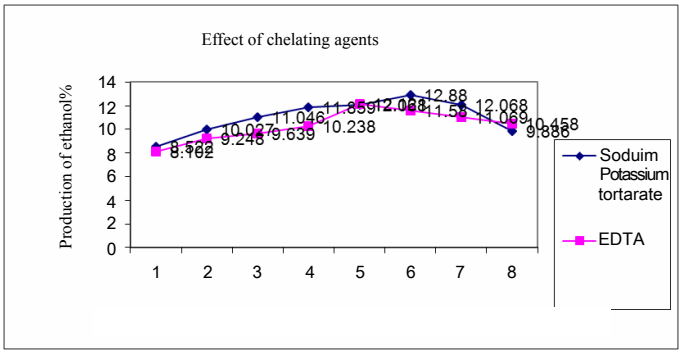

Figure 10: Concentration of EDTA and sodiun potassium tartarate mg/ml.

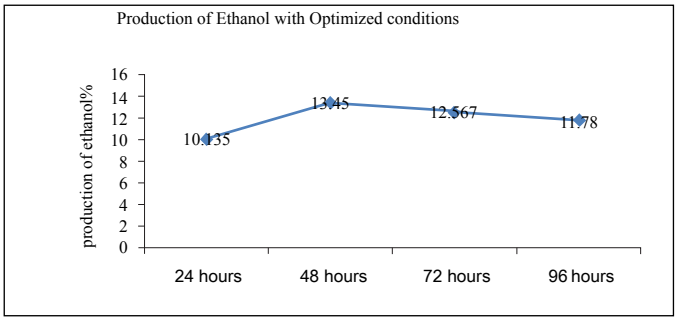

Figure 11: Fermentation time in hours.

Substrate concentration			28 %
pH			5.0
Temperature			30 °C
Inoculum age			48 hours
Inoculum level			2 %
Agitation			100 rpm
Calcium			4 ppm
Urea			1.06 mg/ml
Sodium Potassium Tartrate			1.0 mg/ml
Production of ethanol %			
1st day	2nd day	3rd day	4th day
10.135%	13.450%	12.567%	11.780%

Table 1: Production of Ethanol with optimal conditions evaluated. the experiment showed that the maximum ethanol obtained is13.450 %(w/v).

It can be seen in Figure 10 the curves obtained for EDTA at 1.1gms/liter and sodium potassium tartrate at 1.6gms/liter has profound effect and maximum yield obtained were 12.880% (w/v) and 12.121%(w/v) at 1.1gms/liter of EDTA and 1.0gms/liter of sodium potassium tartrate. It can be concluded that EDTA can be used as suitable chelating agent for the production of ethyl alcohol.

The optimum conditions designed for enhancing the yield of ethanol by *S.cerevisiae-3090* using submerged fermentation process (smf) is mentioned in Table 1.

Based on the result obtained after the study of various physico chemical and nutritional parameters, an experiment was conducted using all the optimized conditions such as Substrate concentration of 28%, P^H-5, Inoculum level 2%, Inoculum age of 48 hours, Sodium potassium tartrate at 1.1%, Agitation is at 100 rpm, It can be seen in Figure 11, that the maximum ethanol obtained is 13.450%(w/v) for 48 hours of fermentation time.

Results and Discussion

Yeast Strain *Saccharomyces cerevisiae*-3090 (Ratnam et al., 2003) is proved as a suitable strain, since it resist high concentration of sugars at 28%. Effect of various optimized parameters such as substrate concentration 28%, Inoculum level 2%, Inoculum age 48 hours, Agitation 100 rpm, Ph 5, Temperature 30°C, Urea at 0.06 %, metal ions such as Mg^{+} at 4 ppm and Cu^{+} at 5ppm and effect of chelating agents such as sodium potassium tartrate 1.0gm/liter showed that maximum ethanol yield is 13.450 % (w/v). It was also found that the urea has a profound effect on ethanol production at 0.06 mg/ml and the ethanol yield obtained is 13.290% (w/v).

Conclusion

Studies on physic chemical and nutritional parameters for the production of ethanol showed that the media designed is cheap and can be easily reproducible. Mahua flower is proved as a suitable substrate, since it consists high sugars 68 % and metal ions such as Mg+, Cu+, phosphorous and protein. It is a cheapest raw material and it can be obtained from any part of India. With all positive features of Mahua flower, it can be used as suitable raw material for large scale production of ethanol. The temperature and pH for maximum were found to be 30°C, and pH 5 which is also optimum temperature and pH for the growth of the organism.

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