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Research Article

BIOTECHNOLOGICAL PRODUCTION OF ETHANOL BY *SACCHAROMYCES CEREVISIAE,* USING DIFFERENT SUBSTRATES

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ABSTRACT
Research efforts are needed to design and improve the process, which would produce sustainable and economically feasible transportation fuel. The present investigation was undertaken to determine the availability of carbohydrates in hydrolysates derived from different substrates *Acacia arabica*, *Delbergia sisso, Peltophorum Pterocarpur* and *Perkia biglobosa* pods, in the production of ethanol.
The enzymatic hydrolysis of the substrates has yielded the significant amount of reducing sugar from the substrates by comparing the effect of enzymes on hydrolysis. The *Acacia arabica* pods has showed the higher production of reducing sugars when treated with 4% a-amylase whereas *Peltophorum pterocarpum* has produced lowest yield of reducing sugar at 4% a-amylase enzyme. The optimum temperature required for the activity of a-amylase enzyme in the production of reducing sugars using different substrates were revealed that at 30°C the *Acacia arabica* has yielded maximum sugars whereas the *Perkia biglobosa* has showed the minimum yield of reducing sugar. The optimum period of enzyme activity in the production of reducing sugars using different substrates was indicted that the *Acacia arabica* has showed the maximum yield of reducing sugars during the incubation period of 24 hours whereas minimum yield was observed in *Perkia biglobosa*. The optimum incubation period of *Saccharomyces cerevisiae* in the production of ethanol has showed that the seven days of incubation has yielded maximum amount of ethanol using the substrate *Acacia arabica.*
Key words: Ethanol**,** *Saccharomyces cerevisiae, Acacia arabica*, *Delbergia sisso, Peltophorum pterocarpur* and *Perkia biglobosa* pods.

INTRODUCTION

It is almost impossible to go anywhere or do anything without being reminded of the fuel situation in the country. Since 20th century, out major energy demand has been supplied by fossil fuels such as oil, coal and natural gas. Fossil fuels originate from deceased organisms that leaved several millions ago and by time have been embedded in the earth's crusts. Insertion of this fossil results in a net increase of today's carbon dioxide levels. Environmental issues such as the threatening increasing temperature caused by the green-house effect and the fact and the fossil fuels are non-renewable resources have increased the interest in producing fuels from renewable resources such as biomass.

In India there are 295 distilleries producing 1058 million liters of alcohol as against annual requirements of 1266 million liters[1]. At present, India is consuming about 7.8 million lit of petrol annually and ethanol requirement for blending is estimated to be 3.9 lakh kiloliters, which provides an immense scope for the agricultural sectors. But the existing level of ethanol production from sugar cane molasses is only 2.0 lakh kiloliters which contributes about 90 percent of total alcohol production[2]. The installed capacities of India ethanol industry during 2003-2004 was 3.2 billion liter per annum which was underutilized (41%) and produced to the tune of 1.3 billion liters therefore this deficit can be met through alternative feed stock such as sorghum, wheat, straw, rice straw, sugar beet and starchy substances like potato, cassava, Sweet potato etc.[3] The current ethanol production process using crops such as sugar cane and corn are well established. However, utilized of cheaper substrates for production of ethanol could make bio-ethanol more competitive with fossil fuel[4]. The present investigation was undertaken to determine the availability of carbohydrates in hydrolysates derived from different substrates *Acacia arabica*, *Delbergia sisso, Peltophorum Pterocarpur* and *Perkia biglobosa* pods, in the production of ethanol.

MATERIALS AND METHODS

Collection of yeast strain and characterization

For the present study, the yeast strain *Saccharomyces cerevisiae* (Baker's yeast) was selected. The strain was obtained from the laboratory, Department of Microbiology and Biotechnology, Karnatak University, Dharwad. The strain of the main culture is sub-cultured and streaked on Petri plate containing yeast extract, peptone and dextrose agar (YEPDA) medium and incubated for 3 days for the development of typical small colonies. Yeast were characterized for assimilation of different carbon source, Growth at 37°C, Alcohol production

Preparation of starter culture

One loop full inoculum of yeast culture was transferred to a test tube containing 5 ml of sterilized yeast extract peptone dextrose broth. Then it was incubated over nigh at 25°C. Then 5ml of yeast culture was added to 100 ml sterilized extracted pod substrates (5% inoculum) in 250 ml flask. It was kept at 25°C for 22 hours in an incubator having shaker facility at 120rpm. Then, it was used at 5 percent (v/v) for fermentation.

Collection of substrate for fermentation studies

In order to select a suitable substrate for the production of ethanol various substrates like *Acacia Arabic* pod, *Dalbergia sisoo* Pod, *Peltophorum pterocarpum* , and *Parkia biglobosa* were collected from Botanical Garden, Karnatak University, Dharwad.

Preparation of substrate

Peeled potato was boiled and ground to paste. The volume made to a known quantity with distilled water for each and then subjected to microbial pretreatment and crude enzymatic.

Microbial Pretreatment

For the prepared substrate, nutrient supplementation of peptone (0.2%), Yeast extract (0.2%), $MgSO_4$ (0.1%) $(NH4)_3$ PO4 (0.02%), was done and Steam sterilized at 121°C for 15

min. Then agar grown yeast cultures were inoculated and incubated for 5 days on rotary shaker (150 rpm) for saccharification. After incubation the amount of reducing sugar released was estimated by DNS method[5].

Extraction of crude amylase from rage seeds

The seeds of rage were soaked in water for overnight and kept for germination for 2-3 days. The germinated seeds were oven dried at 45oC. The dried seeds were crushed at 4°C and stored under refrigeration. One g of sample material was extracted with 5-10 volume of ice-cold 10mM calcium chloride solution for overnight at 4°C. The extraction of enzyme was done by centrifugation (Cooling Centrifuge C – 24) at 10,000rpm at 4°C for 20 min. The supernatant was used as source of crude enzyme.

Optimization of hydrolysis parameters

Optimization of enzyme

To identify a suitable concentration of enzyme for pretreatment, enzyme concentration was taken as 1%, 2%, 3%, 4%, and 5%. These concentrations were mixed with substrate and incubated for saccharification. Amount of saccharification was estimated in terms of release of reducing sugar by DNS method.

Optimization of temperature

The above optimized parameter was tested against the temperature at 20°, 30° and 40°C.

Optimization of incubation period for hydrolysis

The incubation periods for hydrolysis of substrates by enzyme were examined at different intervals of 8, 16, 24 and 48h.

Fermentation

The hydrolysates from all the pre-treatment was adjusted to PH of 4.5 using 1N KOH and inoculated with different yeast culture and incubated under aerobic condition for 24h. Anaerobic condition was then created by plugging with cork, making a provision for trapping CO2 and incubated at room temperature for 5 days. The amount of ethanol produced and residual sugar left unfermented were estimated.

Screening of substrates for submerged fermentation

25gm of the all the four Pod substrates powder was taken and add the 100 ml distilled water in 250 ml conical flasks. And made the cotton plugged. These cotton plugged flasks were autoclaved as 121°C under 151b pressure for 15 min and allowed to cool at room temperature. This suspension was measured for amount of total sugar using Brix method. And pH was adjusted to 3.2 or 5.2. Then the contents of the flasks were inoculated with 5 percent starter cultures. The flasks were incubated at room temperature on a rotary shaker for 5 days. After incubation the substrates were analyzed for ethanol production.

RESULTS

Since plant sources are rich in starch cellulose and free sugars, they are suitable for fermentation of ethanol. Therefore, the powder *Acacia arabica*, *Dalbergia sisso*, *Peltophorum pterocarpum* and *Porkia biglobosa* pods were evaluated of enzymatic hydrolysis, subsequent ethanol production, optimization of various parameters such as selection of yeast, a-amylase activity, temperature and incubation period were also standardized. The results are being presented as follows. The strain of *Saccharomyces cerevisiae* (baker's yeast) found a characteristic white, smooth, wrinkled, raised colonies on yeast extract peptone dextrose agar (YEPDA) medium and also shown characteristic budding and rapid growth.

Assimilation of different carbon sources (Table 1)

Assimilation of different carbon sources, such as glucose, dextrose, sucrose, fructose and lactose were studied. The *Saccharomyces cerevisiae* has shown the good growth on glucose, dextrose, fructose and sucrose, whereas there was no growth on lactose (Table 1). The isolated colonies were taken from two day old culture plate and stained with lacto-phenol cotton blue. The cells were observed under microscope, with 10X and 40X objectives. The budding of cells of yeast was observed and they were maintained on YPEDA slants.

Table 1. Assimilation of different carbon sources in the growth of *Saccharomyces cerevisiae*

Strain	Glucose	Dextrose	Sucrose	Fructose	Lactose
Saccharomyces cerevisiae	++	++	++	++	-

Enzymatic hydrolysis (Graph – I)

The reducing sugars yield indicates an estimation of how much of bound sugars can be liberated during hydrolysis. These sugars yield were based on the treatment of substrate *Acasica arabica*, *Delbergia sisso*, *Peltophorum pterocarpum* and *Perkia biglobosa* using crude a-amylase enzyme (Table 2). The enzyme concentration (1%) on four different substrates in the production of reducing sugars was 27.06, 23.26, 26.09 and 28.14 mg/gm respectively. The 2% enzyme concentration of four different substrates yields the sugars were 28.12, 26.27, 22.92 and 31.97 mg/g respectively. The 3% enzyme concentration on substrates yields the sugars were 33.15, 31.07, 28.15 and 28.37 mg/gm respectively. The 4% enzyme concentration on substrates gives the sugars of 36.27, 34.26, 31.27 and 29.14 mg/gm respectively. Further, the 5% enzyme concentration on substrates produces the reducing sugars of 31.69, 30.09, 30.68 and 27.82 mg/gm respectively. The *Acacia arabica* pod has shown the maximum yield of reducing sugars of 31.69, 30.69, 30.68 and 27.82 mg/gm respectively. The *Acacia arabica* pods has shown the maximum yield of reducing sugar with 26.27 mg/g, whereas *Peltophorum pterocarpum* has shown a minimum of reducing sugar with 28.15 mg/g at 4% a amylase enzyme concentration, when compared with those of different substrates.

Optimization of temperature (Graph 2)

The experiment was designed to study the incubation period for the production of ethanol using different substrates Acasia, Delbergia, Peltophorum and Perkia using *Saccharomyces cerevisiae*. The enzymatic hydrolysis results indicated that 4% crude alpha – amylase enzyme showed maximum yield of reducing sugars by four different substrates Acasia, Delbergia, Peltophorum and Perkia using *Saccharomyces cerevisiae*.

The enzymatic hydrolysis results indicated that 4% crude a – amylase enzyme shoed maximum yield of reducing sugars by four different substrates. Hence 4% concentration of enzyme was used in optimization of temperatures in the production of reducing sugars. The reducing sugar yields at 20°c were 26.82, 26.94, 26.17 and 27.11 mg/gm respectively. The reducing sugars yields at 30°C were 34.86, 33.14, 32.26 and 31.28 mg/gm respectively. Further the reducing sugars yields

at 40°C were 18.86, 19.23, 18.23 and 19.21 mg/gm respectively. *Acasia arabica* pods has showed maximum yield of reducing sugar with 34.86 mg/gm. Whereas, *Perkia biglobosa* has showed the maximum yield of reducing sugar with 31.28 mg/gm, when compared with those of different substrates by a – amylase at 30°C.

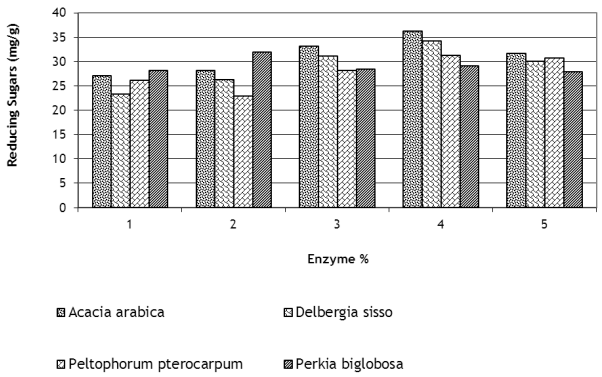
Optimization of incubation period (Graph 3)

This experiment was designed to study the optimum incubation time for the activity of α – amylase enzyme in the production of reducing sugar. In the production of reducing sugar by four different substrates used were Acasia, Delbergia, Peltophorum, and Perkia.

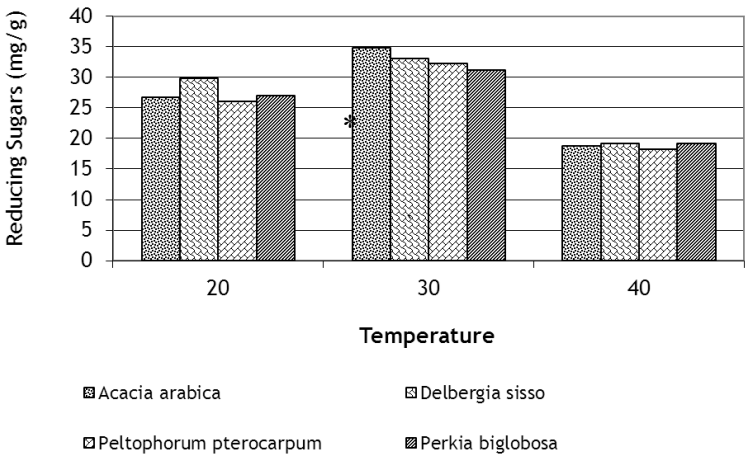
The various incubation time 8, 16, 24 and 48 hours were studied. The reducing sugars yield was 30.83, 28.27, 23.16 and 28.42 mg/gm for substrates of Acasia. Delberigia, Peltophorum and Perkia respectively, for the period of 8 hours incubation. The reducing sugar yields were 31.23, 30.20, 30.27 and 29.62 mg/gm for four different substrates respectively for the period of 16 hours incubation. The reducing sugars yields were 31.23, 30.02, 30.27 and 29.62 mg/gm for four different substrates respectively for the period of 16 hours incubation. The reducing sugars yields were 32.16, 31.63, 31.24 and 30 mg/gm for the different substrates respectively for the period of 24 hours incubation. Further the productions of reducing sugars were 31.46, 29.82, 28.87 and 28.63 mg/gm for the substrates respectively for the period of 48 hours of incubation. An *Acasia arabica* pod has showed the maximum yield of reducing sugar with 32 mg/gm, whereas *Perkia globosa* has showed the minimum yield of reducing sugar with 30 mg/gm for the period of 24 hours incubation.

Optimization of incubation period of *Saccharomyces cerevisiae* (Graph 4)

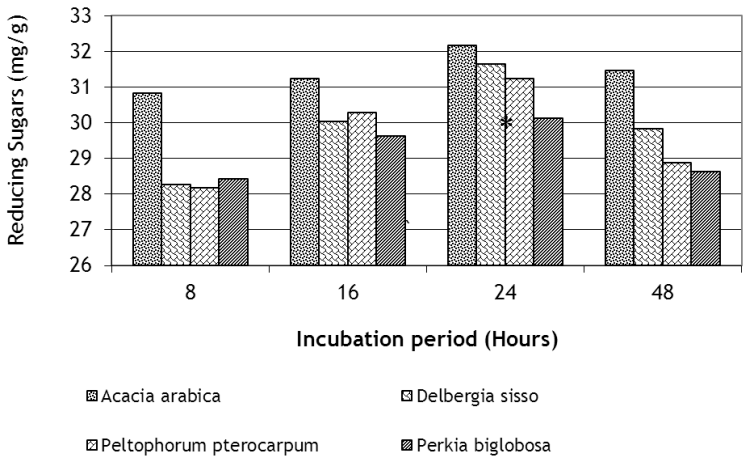
This experiment was designed to study the incubation period of Saccharomyces cerevisae in the production of ethanol using different substrates Acasia, Delbergia, Peltophorum, and Perkia. The various incubation periods like 2, 3, 5 and 7 days were studied in the production of ethanol. The production of ethanol was 21.10, 20.38, 27.94 and 28.08 mg/gm for four different substrates respectively for the period of 2 days incubation. The ethanol production for 3 days incubation period was 27.40, 27.14, 26.07, and 26.07 mg/gm for four different substrates respectively.



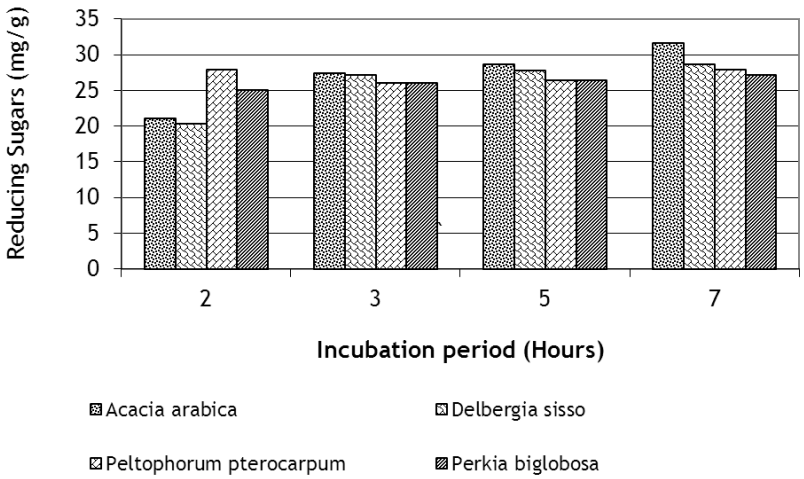
Graph 1 : Optimization of α-amylase in the production of reducing sugars by different substrates



Graph 2: Optimization of temperature on the activity of α-amylase in production of reducing sugars by different substrates



Graph 3: Optimization of incubation period on the activity of α-amylase in the production of reducing sugars by different substrates



Graph 4 : Optimization of incubation period of Saccharomyces cerevisiae in the production of ethanol by different substrates

The ethanol production was 28.68, 27.82, 26.42, and 26.42 mg/gm for different respectively for the period of 5 days incubation. Further, the yield of ethanol was 31.63, 28.62, 27.97 and 27.16 mg/gm for different substrates respectively for the period of seven days incubation. *Acasia arabica* has showed the maximum yield of ethanol, whereas the *Perkia globosa* has showed the maximum yield of ethanol for the period of seven days incubation.

DISCUSSION

Fossil fuel has the main drawbacks, which limits their production and usage. One among them is that, they are fast approaching depletion and the other is the environmental problem associated with their continued and; increasing use. In the past few years, the prices of the petroleum production and natural gas have been inflated. A long-term practical solution to this problem is, to identify product and convert as major source of continuously renewable non-fossil carbon like organic biomass.

A renewable non-depleting raw material that can be converted into fuel would assume a perpetual energy supply. As time passes and the fossil fuel shortage intensifies, renewable energy source could eventually assume position of dominance. Ethanol is one of the biofuel, which can substitute diesel up to 20 percent it is has been implemented in few states in India, 5 percent of ethanol is with diesel for transportation indicating the demand for ethanol. Ethanol can be produced from various biomass such Sudan grass, sweet sorghum, sugarcane, sugar beets, corn, wheat barley, potato

etc., which are available abundantly. Apart from these, some of the alternate biomass tried successfully are the pineapple, cannery waste; starch[6]. The starch containing substrates include sweet potato, cassava starch, potato etc., which do have the potential for production of ethanol as they contain considerable amount of sugars of sugar bounds in the form of starch.

There are several attempts to produce ethanol from different substrates. It has been reported that ethanol was produced from sugar beet and got maximum of 0.5 mg/gm with fermentation time of 20 hours. Czamecki in 1991 reported that, the influence of temperature and incubation time on starch gelatinization in wheat, rye, maize grain and found that rye starch has showed most susceptible to enzymatic hydrolysis and subsequent highest production of alcohol of 65%[7]. The findings reported from Shreenath and Jeffries (2000) showed that the maximum production of ethanol of 0.41 – 0.49 gm/g using *Pichia stipitis* and Candida sheathe on the substrates of total 43 forest products[8]. A report from Ramanathan (2000) showed that the substrate of Cassava plant has produced the ethanol of 42 liters / 100 kg of fees stock using Saccharomyces cereviceae[9].

The present investigation was undertaken to know the extent of possible yield of ethanol by utilizing maximum reducing sugars from *Acacia arabica* , *Dalbergia sisoo*, *Peltophorum pterocarpum* and *Parkia biglobossa*. The enzymatic pretreatment method was adapted to hydrolyze the substrate for the fermentation by yeast to produce ethanol. The results obtained in present study were revealed that *Acasia arabica* has shown maximum yield of ethanol compared to other substrates. It has been reported that the hydrolysis methods most commonly used are acid (dilute and concentrated) and enzymatic. Sun and Cheng (2002) have reported that, the a-amylase enzyme was used for hydrolysis of liquid hemicellulose content of the substrate has given good result[10]. An accurate description of enzymatic hydrolysis could be suitable for better control of the process. Enzyme dosages, temperature, incubation time of hydrolysis predominantly determine the conversion process, the temperature and enzyme concentration showed the interactive effect on the observed rate of hydrolysis[11]. In present study the crude amylase enzyme extracted from raagi malt is used for optimizing hydrolysis parameter like enzyme dilution, temperature and time of interval for hydrolysis. The 4% enzyme was effective on substrate at 30^{o}C in 24 hours of incubation has given maximum hydrolysis in the production of reducing sugars. The enzymatic pretreatment was effective because the enzymes are the biocatalyst specific action besides, enzymes are target oriented.

It has been revealed that, in case of crude enzyme there might be presence of other enzymes like nucleases which degrade the enzyme and there may be inhibition of reaction[12]. In the present study, the period of incubation of seven days was optimum and the observation is in accordance with the findings of previous studies in other substrates. Suresh et al., (1999) have obtained maximum ethanol yield of 2.9% in days of fermentation from damaged sorghum and rice grain[13]. Marakis and Marakis (1996) studied the effect of six different fermentation period viz., 0, 24, 48, 72, 90 and 100 hours on ethanol production from aqueous carob extract and achieved maximum alcohol concentration of 4.75 percent at 100 hours of fermentation period[14]. Similarly Jeffries (2000) recorded maximum ethanol concentration of 3.4 ± 2.42 g^{-1} during 3 to 5 days of fermentation of wood hydrolysates[15]. In present study the production of ethanol using four different substrates *Acasia arabica* has yielded maximum ethanol 31.63 mg/g using *Saccharomyces cerevisiae*.

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