Optimization of Bio-Fuel Production by *Saccharomyces cerevisiae* Isolated from Sugar Cane Bagasse

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Abstract: Twenty yeast isolates were tested for ethanol productivity, *Saccharomyces cerevisiae* isolated from sugar cane bagasse was the potent producer. Fresh *Saccharomyces cerevisiae* was grown overnight on YEPD medium and was tested to determine the optimum conditions for both biomass and ethanol production. The maximum production of ethanol was obtained at 30°C, pH 6, 35% sugar cane molasses as fermentation medium, 1% corn steep liquor, 1ml of 1 O.D. YEPD broth and shaking at 200 rpm. Different microelements also were tested.
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1. Introduction:

Due to the diminishing fossil fuel reserves, alternative energy sources are needed to be renewable, sustainable, efficient, cost-effective, convenient and safe. An eco-friendly bio-ethanol is one of such alternate fuel that can be used in unmodified petrol engines with current fueling infrastructure and it is easily applicable in the present day combustion engine, as mixing with gasoline *(Hansen et al., 2005).* In 2005 Brazil produced 3.8 billion gallons of ethanol, represents 40% of the country`s consumption *(Baez et al, 2008).* Also as a result, they have become 80% independent from foreign oil. Most of the new cars that were sold in Brazil are flexible-fuel vehicles that can run on ethanol, gasoline, or any blend of the two. The United States fuel ethanol industry is based largely on corn. Thailand, India, China and Japan have now launched their national gasohol policies.

The 1st generation bio-fuel faced many problems, the most known is Food Vs Energy crisis, due to the dependence on edible crops as feedstocks. Thus, there was a need for a 2nd generation which depends on non-food sugary materials as feedstocks. Various raw materials like sugarcane juice and molasses *(Morimura et al, 1997; Agrawal et al, 1998),* sugar beet, beet molasses *(EI-Diwany et al, 1992; Agrawal et al, 1998),* Sweet sorghum *(Bulawayo et al 1996)* and starchy materials like sweet potato *(Sree et al, 2000),* Corn cobs and hulls *(Beall et al, 1992; Arni et al, 1999),* cellulosic materials like cocoa, pineapples and sugarcane waste *(Othman et al, 1992)* and milk, cheese, and whey using lactose hydrolyzing fermenting strains *(Silva et al, 1995; Ghaly and Ben-Hassan , 1995)* have been reported in ethanol production.

In fermentation, of the various ethanol producing micro-organisms yeast belonging to *Saccharomyces cerevisiae* have been used most commonly *(Mike and Kavin, 2006).* production from molasses using *Saccharomyces cerevisiae* & *Zymomonas mobilis.* yeast was found to be more ethanol tolerant and produced more ethanol at sugar concentration above 15% (v/v). The following table below lists some of the yeast strains used in distilleries and the amount of alcohol they produce.

Several reviews of literature *(Dale, 1987; Ferrari et al., 1992; Nigam, 1999; Olsson and Hahn, 1996; Beatriz et al., 2005; Martin et al., 2006)* available for the production of bio-ethanol from various sources, only a very few authors *(Doelle and Green-field, 1985; Huertaz et al., 1991; De Vasconcelos et al., 1998)* have studied optimization of ethanol production from sugar cane using yeast cells (*Saccharomyces cerevisiae*). Hence, this work aimed to enhance ethanol production through screening for a good producing yeast, screening for suitable non-food feedstock, and optimization of fermentation conditions to reach maximum production.

Table: Different types of ethanol producing strains

Strain	Ethanol produced (%)
Saccharomyces cerevisiae	5.8-11.16
Zygosaccharomyces sp.	4.2
Saccharomyces ellipsoids	9.7
Schizosaccharomyces pombe	8.7
Schizosaccharomyces mallaeri	7.8

2. Materials and Methods:

Isolation

Samples were collected aseptically in sterile containers, processed and were cultured on acidified malt extract medium ***(Spencer et al, 1995)***. Isolated yeasts were purified and maintained on YEPD agar

(1% Yeast extract, 2% Peptone, 2% Glucose, and 1.5-2% Agar) slants ***(Atlas, 2004)***.

Growth conditions

To prepare the inocula, a loopful of the test organism was inoculated into 25 ml of YEPD medium in a 250 ml Erlenmeyer flask containing the same components as in the maintenance medium, except that agar was not added. The flasks were incubated in a shaking incubator at 30°C of 200 rpm for 24 h.

Identification of isolated yeast strains

Yeast isolated were identified according to their morphological and biochemical characteristics *(Barnett et al, 2000).*

Screening

Batch fermentation in 250 ml Erlenmeyer flask containing 100 ml fermentation medium (30% Glucose, 0.3% $(NH4)_2SO_4$, 0.2% KH_2PO_4, 0.1% $MgSO_4$.$7H_2O$, 0.01% $CaCl_2$. $2H_2O$, 0.01% NaCl, and 0.3% Yeast extract), inoculated with 1 ml of 24 h. old yeast culture (0.01 at 600 nm), and incubated in a shaking incubator at 30°C of 200 rpm for 48 h.

Optimization of fermentation conditions

Raw Materials:

Fermentation media prepared as 10% of sugar cane molasses, 10% corn steep liquor, and 10% whey. Erlenmeyer flask (250 ml) containing 100 ml fermentation medium was inoculated with 1 ml of 24 h. old yeast culture (0.01 at 600 nm), and incubated in a shaking incubator at 30°C of 200 rpm for 48 h.

Molasses concentration:

Different concentrations of molasses (5%, 10%, 15%, 20%, 25%, 30%, 35%, and 40%) were prepared as fermentation medium in 250 ml Erlenmeyer flasks each contained 100 ml and inoculated with 1 ml of 24 h., 1 O.D. (at 600 nm) yeast culture, and incubated in a shaking incubator at 30°C and 200 rpm for 48 h.

Inoculum size:

Fermentation media with 35% molasses were prepared. Erlenmeyer flasks (250 ml) each contained 100 ml media were inoculated with different volumes (0.5, 1, 1.5, 2, 2.5, and 3 ml) of 24 h. old yeast culture (0.01 at 600 nm), and incubated in a shaking incubator at 30°C and 200 rpm for 48 h.

Incubation period:

Fermentation media contain 35% molasses were prepared. Erlenmeyer flasks (250 ml) each contained 100 ml media were inoculated with 1 ml of 24 h. old yeast culture (0.01 at 600 nm), and incubated in a shaking incubator at 30°C and 200 rpm for different periods (12, 24, 36, 48, 72, and 84 h.).

Initial pH Value:

Fermentation media contain 35% molasses were prepared at different pH values (3,4,5,6,7,8, and 9). Erlenmeyer flasks (250 ml) each contained 100 ml media were inoculated with 1 ml of 24 h. old yeast culture (0.01 at 600 nm), and incubated in a shaking incubator at 30°C and 200 rpm for 72 h.

Temperature:

Fermentation media contain 35% molasses were prepared. Erlenmeyer flasks (250 ml) each contained 100 ml were inoculated with 1 ml of 24 h. old yeast culture (0.01 at 600 nm), and incubated in a shaking incubator at 200 rpm and different temperatures (25, 28, 30, 35, and 40°C) for 72 h.

Shaking rate:

Fermentation media contain 35% molasses were prepared. Erlenmeyer flasks (250 ml) each contained 100 ml media were inoculated with 1 ml of 24 h. old yeast culture (0.01 at 600 nm), and incubated in a shaking incubator at 30°C and different shaking rates (100, 150, 200, and 250 rpm) for 72 h.

Nitrogen sources:

Fermentation media contain 35% molasses were prepared with equimolecular weights of Yeast extract, Peptone, Urea, Casein and Corn steep liquor, separately, Each Erlenmeyer flasks (250 ml) contained 100 ml media were inoculated with 1 ml of 24 h. old yeast culture (0.01 at 600 nm), and incubated in a shaking incubator at 30°C and 200 rpm for 72 h.

Corn steep liquor (CSL) concentration:

Fermentation media (35% molasses) were supplemented with different concentrations of CSL (0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4%) in 250 ml Erlenmeyer flasks, each contained 100 ml and inoculated with 1 ml of 24 h yeast culture (0.01 at 600 nm), and incubated in a shaking incubator at 30°C and 200 rpm for 72 h.

Microelements:

Fermentation media containing 35% molasses and 1% CSL with equimolecular weights of $FeSO_4.7H_2O$, $MgSO_4.7H_2O$, $ZnSO_4.7H_2O$, and $(NH_4)_2SO_4$ were supplemented, separately, in 250 ml Erlenmeyer flasks, each contained 100 ml and inoculated with 1 ml of 24 h yeast culture (0.01 at 600 nm), and incubated in a shaking incubator at 30°C and 200 rpm for 72 h.

Analytical methods

Growth*:*

Growth was measured by Optical Density (O. D.) at wavelength 600 nm.

Estimation of reducing sugars:

The DNS method of *Miller (1959)* was used to estimate reducing sugars.

Ethanol determination:

One ml of the fermented wash was taken in 500ml pyrex distillation flask containing 30 ml of distilled water. The distillate was collected in 50 ml flask containing 25 ml of potassium dichromate

solution (33.768 g of $K_2Cr_2O_7$ dissolved in 400 ml of distilled water with 325 ml of sulfuric acid and volume raised to 1 liter). About 20 ml of distillate was collected in each sample and the flasks were kept in a water bath maintained at 62.5ºC for 20 minutes. The flasks were cooled to room temperature and the volume raised to 50 ml. Five ml of this was diluted with 5ml of distilled water for measuring the optical density at 600nm using a spectrophotometer. A standard curve was prepared under similar set of conditions by using standard solution of ethanol containing 2 to 12% (v/v) ethanol in distilled water. Ethanol content of each sample was estimated and graph was made *(Caputi et al, 1968).*

Statistical analysis:

The data obtained were subjected to statistical analysis according to the procedure outlined by *sendecor and Cochran (1981)* and the means were compared using Duncan`s multiple range test *(Duncan, 1988).*

3. Results and Discussion

Isolation and identification:

Table 1 shows 5 yeast species and 10 genera isolated from different sources. *Candida sp* (*Candida albicans or Candida dubliniensis*), *Cryptococcus laurentii*, and *Saccharomyces cerevisiae* from sugar cane bagasse which matches results obtained by *Luciana et al, 1998. Candida guilliermondii, Saccharomyces cerevisiae,* and *Saccharomyces kluyveri* from banana matching the results obtained by *Brooks, 2008. Candida kruisii, Candida tamarandei, Candida sp*[1] from date. *Candida guilliermondii, Candida kruisii, Candida sp,* and *Debaryomyces hansenii* from grapes. *Trichosporon mucoides* was isolated from both Mediterranean sea and El-Nasr Solar salterns. *Auerobasidium sp.* was isolated from the nectar of Crimson bottle brush flowers.

Table 1: Screening for ethanol production by isolated yeast strains

Source	Yeast isolated	Ethanol produced (%)
Sugar cane bagasse	***Candida sp (albicans or dubliniensis)***	Not Tested
	Cryptococcus laurentii	1.56
	Debaryomyces hansenii	7.84
	Saccharomyces cerevisiae	10.95
Banana fruits	***Candida guilliermondii***	4.53
	Saccharomyces cerevisiae	9.68
	Saccharomyces kluyveri	6.32
Date fruits	***Candida kruisii***	7.12
	Candida tamarandei	2.06
	Candida sp(albicans or dubliniensis)	Not Tested
Grapes	***Candida guilliermondii***	4.00
	Candida kruisii	6.79
	Candida sp(albicans or dubliniensis)	Not Tested
	Debaryomyces hansenii	8.01
Mediterranean sea	***Trichosporon mucoides***	Nil
Solar salterns	***Trichosporon mucoides***	Nil
Crimson bottle brush	***Auerobasidium sp.***	Nil

Screening:

Germ tube forming *Candida sp* (*Candida albicans or Candida dubliniensis*) were avoided because of their known pathogenic behavior. Very high gravity ethanol fermentation *(Petra Bafrncova et al, 1999)* was used as screening method. Table 1 shows that *Saccharomyces cerevisiae* isolated from sugar cane bagasse produced (10.95%) highest ethanol concentration as compared to others, followed by *Saccharomyces cerevisiae* isolated from banana fruit (9.68%).

Optimization of fermentation conditions

Raw Materials:

A huge backlash against using food crops for energy has developed in 2008 ***(Mostafa, 2010)***. As a result, scientists now are looking to harvest energy

from nonfood crops and industrial wastes. Hence, this study tried 3 different industrial wastes; sugar cane molasses, corn steep liquor, and whey. Sugar cane molasses was found to be optimum for ethanol production, however, corn steep liquor was optimum for growth. Statistical analysis showed significance of results, and the optimum relationship between growth and ethanol production was achieved when sugar cane molasses was the fermentation medium (Table 2). The obtained results matches the results obtained by *Doelle and Green-field, 1985; Huertaz et al., 1991; Morimura et al 1997; Agrawal et al 1998; and De Vasconcelos et al., 1998.*

Table 2: Effect of different raw materials on growth and ethanol production

Raw material	Corn Steep L.	Molasses	Whey
Ethanol (%)	0.19 e	2.27 a	0.01 f
Growth (O. D.)	2.12 b	1.33 d	1.48 c

Means followed by the same letter are not significantly different

Different Molasses concentration(Sugar Concentration):

Different concentration of sugar was tested in term of molasses concentration in the fermentation medium. Table 3 shows that 35% molasses which contains 20% reducing sugars was optimum for ethanol production and optimum for the statistical relation among ethanol production, growth, and sugar concentration however, 5% molasses which represent 2.9% sugars was optimum for growth. This results are in agreement with that of *Kadambini, 2006 and Sivakumar et al, 2010.*

Table 3: Effect of sugar cane molasses different concentration on growth and ethanol production

Molasses Conc. (%)	5%	10%	15%	20%	25%	30%	35%	40%
Ethanol (%)	1.0 j	2.6 g	4.2 f	5.9 e	7.0 d	9.7 c	11.7 a	10.0 b
Growth(O. D.)	1.2 hij	1.1 ij	1.0 ij	1.5 h	1.4 hi	1.2 hij	0.95 j	0.57 k

Means followed by the same letter are not significantly different

Inoculum size:

Sivakumar et al, 2010 noticed that as the concentration of yeast increases, the yield of bio-ethanol increase up to specific point and then it starts to decrease. Mostly the same results were obtained and the specific point was at 1 ml of 24 h yeast culture (0.01 at 600 nm), then ethanol concentration decreased. Figure 1 shows that as the concentration of yeast increased, the produced yeast biomass and reached maximum production at 2.5 ml then declined at higher concentrations.

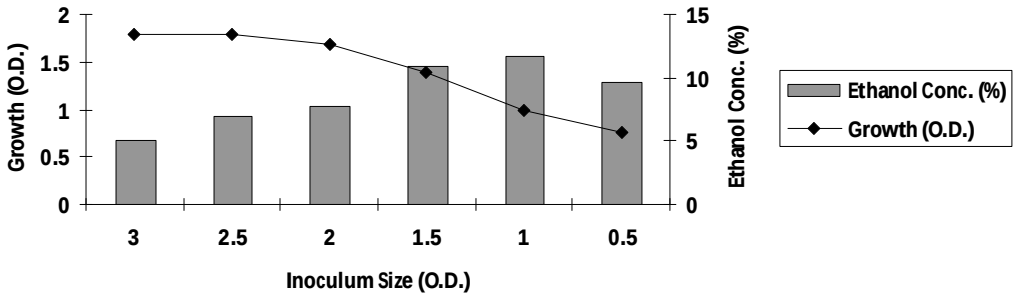

Figure 1: Effect of different inoculum size on both growth and ethanol production

Incubation period:

Most studies on ethanol production by Saccharomyces cerevisiae reported that the maximum productivity was at range from 48 to 84 hours (*Doelle et al, 1985; Huertaz et al., 1991; Morimura et al 1997; Agrawal et al 1998; De Vasconcelos et al., 1998; Kadambini, 2006; Brooks, 2008 and Sivakumar et al, 2010).* In the present study the optimum incubation period for ethanol production was 72 hours, while 48 hours for biomass (Table 4).

Table 4: Effect of Different Incubation periods on growth and ethanol production

Incubation Time (hours)	0	12	24	36	48	72	84
Ethanol (%)	0.00 i	3.56 e	7.60 d	8.85 c	10.91 b	11.62 a	11.46 a
Growth (O. D.)	0.00 i	0.75 h	1.45 g	1.85 f	2.15 f	2.21 f	2.25 f

Means followed by the same letter are not significantly different

Initial pH Value:

Wide initial pH range was tested (Table 5), at pH value 3 no growth observed and no ethanol was produced, while pH 6 was the optimum for both biomass and ethanol production. The results are in agreement with that of *Kadambini, 2006*, but it doesn`t match results of *Sivakumar et al, 2010*, who found pH 4 optimum for ethanol production and this is due to difference in the tested strains.

Table 5: Effect of different pH values on growth and ethanol production

pH Values	3	4	5	6	7	8	9
Ethanol (%)	0.00 k	1.69 f	11.6 b	12.03 a	11.24 c	10.04 d	8.82 e
Growth (O. D.)	0.00 k	0.22 j	0.94 i	1.52 g	1.26 h	1.50 g	1.05 i

Means followed by the same letter are not significantly different

Temperature:

Temperature is one of the major constraints that determines the ethanol production. To know the optimum temperature for ethanol fermentation, the fermentation media were kept at 25, 28, 30, 35 and 40°C. Two parameters were studied, the growth and the ethanol yield (Figure 2). The maximum ethanol production and biomass was obtained at 28-30°C. this result are in agreement with most previous studies on *Saccharomyces cerevisiae*. Temperature tolerance was found to depend upon sugar concentration of the medium as *Morimura et al, 1997* observed that fermentation of molasses at 35°C was possible when sugar concentration was 20%(w/v), while no fermentation when sugar concentration was 22%(w/v).

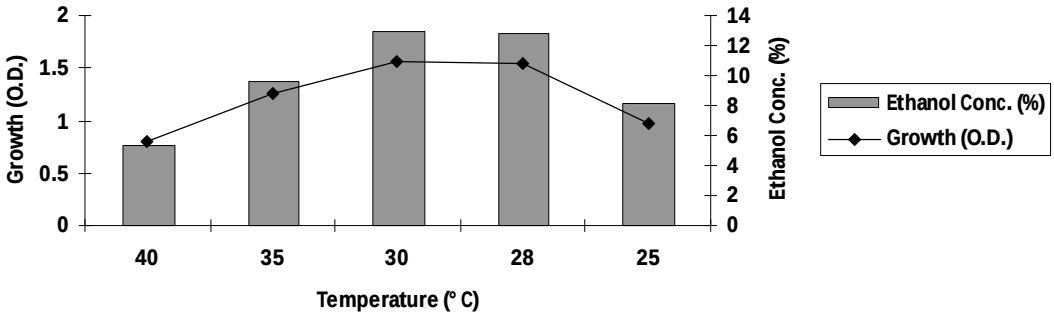

Figure 2: Effect of different temperature on both growth and ethanol production

Shaking rate:

Shaking is a vital factor that influence ethanol fermentation, so this study was interested to determine the optimum shaking rate for ethanol production through incubating the fermentation media at different shaking rates (0, 50, 100, 150, 200,

and 250 rpm). The optimum shaking rate for ethanol production was at 200 rpm, while growth was increased by increasing the shaking rate as shown in Figure 3.

Nitrogen sources:

Petra Bafrncova et al, 1999 noticed that the final ethanol concentration achieved was increased when excess assimilable nitrogen was added to the batch ethanol fermentations by *Saccharomyces cerevisiae*. Results obtained in this study assure the results of *Petra Bafrncova* and his team. Table 6 shows that most supplemented nitrogen sources improve ethanol production specially Urea and Corn Steep Liquor.

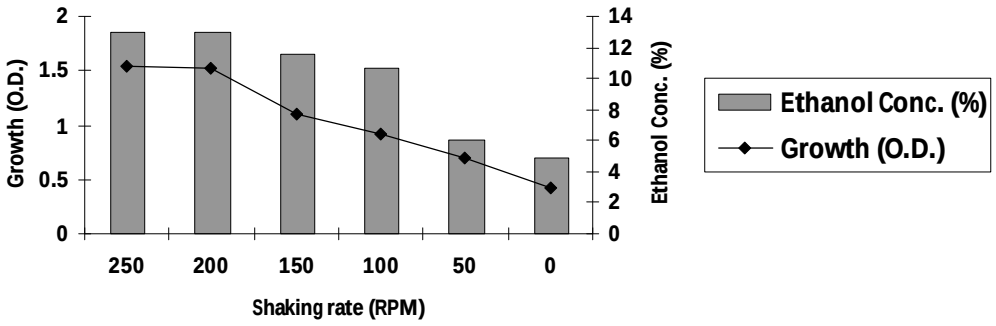

Figure 3: Effect of different shaking rate on both growth and ethanol production

Table 6: Effect of different nitrogen sources on growth and ethanol production

Treatment	Control	Peptone	Yeast extract	Casein	Urea	Corn Steep L
Ethanol (%)	11.60 c	12.03 b	12.04 b	10.17 d	13.08 a	13.02 a
Growth(O. D.)	1.56 h	1.88 g	2.13 f	1.60 h	2.47 e	2.49 e

Means Followed by the same letter are not significantly different

Corn steep liquor concentration:

Available, cheap industrial waste, nitrogen source, and improve ethanol production; these characters pushed us towards studying corn steep liquor different concentrations and its effect on ethanol production and biomass. The influence of different concentration of corn steep liquor on ethanol and biomass production represented in figure 4, and the optimum concentration for ethanol production was 1% (V/V) corn steep liquor, while statistical analysis tells that no great difference in biomass among concentration above 1% (V/V) corn steep liquor.

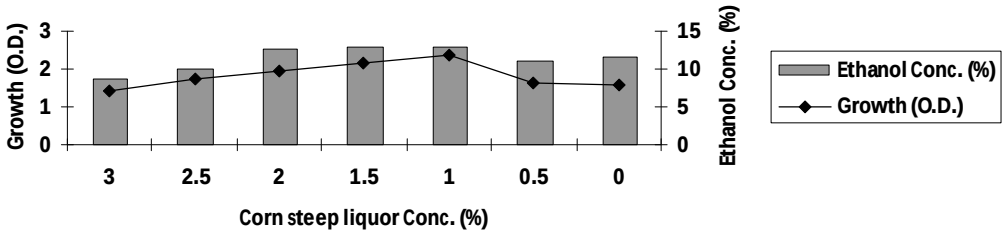

Figure4: Effect of different concentrations of CSL on both growth and ethanol production.

Microelements*:*

Jones et al. (1981), have listed out the various cations that may be used as supplements and their stimulatory effect on the physiology of fermenting organism. Iron, Zinc and Manganese are required as cofactors for several metabolic pathways *(Morris, 1958).* However, *Mary et al, 2008* observed that ethanol yield has increased when supplemented with microelements, statistical analysis of our provided results (Table 7) show no significant difference among used microelements ($FeSO_4$, $MgSO_4$, $ZnSO_4$, and $(NH_4)_2SO_4$) and the control. This contrast may be due to difference of used feedstock as molasses are known to contain metals and *Dhamija et al (1986)* showed that the removal of metal ions from molasses enhanced ethanol production.

Table 7: Effect of Different Microelements on growth and ethanol production

Treatment	Control	$FeSO_4.7H_2O$	$MgSO_4.7H_2O$	$ZnSO_4.7H_2O$	$(NH4)_2SO_4$
Ethanol (%)	12.99 a	12.68 a	12.95 a	12.04 b	13.02 a
Growth (O. D.)	2.35 cd	1.87 ef	1.56 f	2.13 de	2.49 c

Means Followed by the same letter are not significantly different

4. Conclusion:

Biomass and ethanol (biofuel) production by *Saccharomyces cerevisiae* isolated from sugar cane bagasse was investigated in this study. Optimum conditions for ethanol production was 30°C temperature, 6 pH value, fermentation medium of 35% sugar cane molasses (20% reducing sugars) supplemented with 1% corn steep liquor as nitrogen source, 1 ml of 24 h yeast culture (0.01 at 600 nm) and shaking rate 200 rpm. Finally, around 13% ethanol was detected under optimum conditions by batch fermentation.

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