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Preparation of very high gravity cassava mashes and subsequent fermentation to ethanol using *Saccharomyces bayanus*

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Abstract

Fuel ethanol was produced from very high gravity (VHG) cassava mashes (30-40% w/v dry matter content) using flocculating yeast Saccharomyces bayanus. The total reducing sugars (TRS) concentrations of 30%, 35% and 40% mashes after starch liquefaction and saccharification were 29.0%, 31.64% and 37.15% (w/v), respectively. After 72 h fermentation, final ethanol concentrations reached 101.8 and 94.6 g/L with fermentation efficiencies of 75.8% and 57.8% in 30% and 35% mashes, respectively. A complete inhibition of yeast growth and fermentation was observed in the mash containing 40% dissolved solids.

Keywords: Cassava, very high gravity mash, *Saccharomyces bayanus,* yeast nutrients

1. Introduction

Ethanol has become an immediate viable alternate to fast depleting fossil fuels. Also, it is a renewable, environmentally friendly fuel. Therefore, the demand for fuel ethanol has been increasing dramatically over the last few years. A wide range of substrates can be used as feedstock for ethanol production, ranging from readily fermentable sugary substrates (cane juice, cane molasses, beet molasses, sweet sorghum juice, etc.) to starchy substrates (corn, wheat, cassava, sorghum, rice, etc.) to complex lignocellulosic biomass (corn stalks, rice straw, wheat straw, bagasse, wood chips, etc.). However, cost of feedstock is crucial in determining the profitability of fuel ethanol production as it typically represents more than 50% of the total production cost (F.W. BAI [1]). Because of this reason, potentials of low-cost lignocellulosic biomass for ethanol fermentation are being explored all over the world.

Although a significant progress has been made towards development of cellulosic ethanol in recent years, it is still economically problematic to replace sugar and starch materials in the near future (H.R. BUNGAY [2]). On the other hand, process technologies which allow rapid fermentation and accumulation of high ethanol concentrations in fermented mashes are desirable for existing molasses or starch-based ethanol distilleries. Very high gravity (VHG) ethanol fermentation is one such technology that allows preparation and fermentation of media containing sugar in excess of 250 g/L in order to achieve more than 15% (v/v) ethanol compared with 10–12% (v/v), the range that is generally being observed in most distilleries all over the world (P. PULIGUNDLA & al. [3]). However, when the sugar level or total dissolved solids level of medium increases above 30% (w/v), yeast cells are subjected to high

osmolarity stress at initial stages of fermentation, therefore sluggish fermentation occurs, and ultimately results in decreased efficiency of ethanol production. Several researchers suggested various strategies to overcome osmo-stress in yeast cells, including nutritional supplementation (G.P. CASEY & al. [4]; P. PRADEEP and O.V.S. REDDY [5]; T.N.L. PHAM & al. [6]), mutant yeast strains, and immobilized yeast (D. SMOGROVICOVA & al. [7]; P. PULIGUNDLA & al. [8]).

Suitability of different starches for VHG ethanol fermentation has been tested by several researchers and reviewed (P. PULIGUNDLA & al. [3]). Cassava is one of the potential feedstocks for fuel ethanol fermentation as it is cheap, drought resistant, and abundant in starch. B. YINGLING & al. [9] have reported the production of ethanol from VHG cassava mash by *Saccharomyces cerevisiae* during simultaneous saccharification and fermentation (SSF). However, there are fewer reports on ethanol production capability of yeast strains other than *S. cerevisiae* under VHG conditions. Therefore, in the present study, ethanol fermenting ability of flocculating yeast strain *Saccharomyces bayanus* is tested using VHG cassava mashes.

2. Materials and Methods

2.1. Yeast strain and maintenance

A flocculating, non-amylolytic yeast *Saccharomyces bayanus* was generously provided by Dr. Roberto Ambrosoli, university of Turin, Italy. A non-flocculating *Saccharomyces cerevisiae* (baker's yeast) was procured from a local store. The *S. bayanus* strain was preserved at 4°C by regular subculturing (once every 3 months) over malt extract, peptone, yeast extract, dextrose (MPYD) agar. The MPYD agar contained (g/L): malt extract, 3; peptone, 5; yeast extract, 3; dextrose, 20 and agar, 15.

2.2. Enzymes for cassava starch hydrolysis

For starch liquefaction, the enzyme Biotempase L (Biocon India Limited) was applied. It is a heat-stable-amylase preparation with an activity of 1,00,000 BAA units/g, specific gravity of 1.18 g/ml, and optimal activity in the pH range of 5.5-6.5 Amylo 300L, a mixture of glucoamylase (260 GAU/g) and pullulanase (390 ASPU/g) having specific gravity of 1.10 g/ml was used for saccharification of liquefied starch.

2.3. Determination of ethanol tolerance

The ethanol tolerance of the yeast strains was determined through ethanol shock experiments as described by F.W. BAI [1]. The 250 ml flasks containing 100 ml medium composed of 30 g/L glucose, 5 g/L yeast extract, and 3 g/L peptone were prepared and sterilized at 121 °C for 15 min. After cooling, 10 ml fresh culture of each strain was inoculated and incubated overnight at 30 °C and 150 rpm. Then, ethanol shock was exerted onto the cultures by adding 20 ml ethanol into each of these cultures, making the ethanol concentration about 15% (v/v). The cultures were incubated at 30 °C and 150 rpm for two hours, and the percentage of viable cells was counted by the methylene blue stain and chamber counting technique.

2.4. Preparation of very high gravity cassava mashes

Pure dried (moisture <12%) cassava starch (100 g each) was taken in different conical flasks of 500 ml capacity. An amount of hot water (60 °C) was added to starch flour to maintain final dry matter content in the range 30%-40% (w/v). pH was adjusted to 6.5 with dil. HCl. The α-amylase (Biotempase) was added in the dose range of 0.3-0.5% (v/w), stirred well, liquefaction was carried out by using steam under increased pressure conditions (autoclaving) for 20-30 min at 105-110°C. Liquefaction was completed in a single step. All the flasks were

then cooled to room temperature and tested to determine the progress of liquefaction using starch-iodine reaction.

For saccharification of the liquefied starch, pH of the slurries was lowered to 4.2 with dil. HCl. Then the saccharifying enzyme, glucoamylase (Amylo 300), was added in the dose range of 0.4-0.8% (v/w). Mixed well, allowed for incubation at 60 °C in a hot air oven for 24 h duration.

2.5. Yeast pre-culture

Cassava mash (hydrolysate) was used for preparing pre-culture. Hydrolysate containing 10% reducing sugars, 1% urea and 0.2% yeast extract was taken in a flask and autoclaved (121 °C for 15-20 min). After cooling to room temperature, one loopful of yeast colony from YPD plate was transferred to flask. The preculture was propagated at 30˚C on a rotary shaker (130 rpm) for 24 h.

2.6. Fermentation conditions

For the fermentation of cassava mashes, different concentrations of yeast nutrient substrates such as urea, ammonium sulphate, yeast extract, and peptone were added to the saccharifying mashes. Stirred well and allowed to saccharify further for 2 h at 60 °C. Cooled to 30 °C temperature, pH was adjusted to 5.0 with NaOH. An amount of preculture yielding a concentration of 2.5×10^7 cells/ml medium was added to the fermenting flasks. Total reducing sugar concentrations in fermenting mashes were not adjusted. Fermentations were allowed to run for 72 h.

$$\text{Fermentation efficiency} = \text{ethanol produced in fermentation/ethanol produced in theoretical}\times 100\%$$

2.7. Determination of ethanol

Ethanol and congeners concentration was determined by gas chromatography (GC) equipped with a flame ionization detector (FID) (J.C. ANTHONY [10]). Agilent systems model 6890 GC was used under the following conditions: Graphitized packed column 5% carbowax 20 M phase, matrix 80/120 carbopack-B, and Length 6 ft (1.83 m) × 2 mm ID × 1/4-inchOD. Nitrogen at flow rates of 20 ml/min was used as carrier gas. Hydrogen was used as fuel gas, at flow rate 40 ml/min, along with air at a flow rate of 400ml/min. GC yields determined using sec-butyl alcohol as internal standard.

3. Results and Discussion

3.1. Evaluation of ethanol tolerance

To avoid struck fermentation under VHG conditions, ethanol-tolerant yeast strains are prerequisite (C.-G. LIU & al. [11]). The ethanol tolerance of the self-flocculating yeast strain, *Saccharomyces bayanus,* was examined by applying 15% and 18% ethanol shocks to their cultures for two hours, and followed by the count of their viable cells. Higher the percentage of the viable cells, better the ethanol tolerance of the strain will be. The experimental results are illustrated in the table 1.

Table 1. Ethanol tolerance of *Saccharomyces bayanus* in comparison with *Saccharomyces cerevisiae* (baker's yeast) at 30°C.

Strains	conditions	viability
Saccharomyces cerevisiae (baker's yeast)	15% ethanol shock for 2 hours	87.7%
	18% ethanol shock for 2 hours	0.0
Saccharomyces bayanus	15% ethanol shock for 2 hours	97.6%
	18% ethanol shock for 2 hours	28%

At 15% volume ethanol shock, both the tested strains, *Saccharomyces bayanus* and *Saccharomyces cerevisiae,* exhibited better ethanol tolerance. However, the *S. bayanus* strain was found to be superior as it exhibits 11% more viability after 2 hour ethanol shock than baker's yeast strain. On the other hand, at 18% volume ethanol shock, significant difference in the viable cell counts between the two strains was observed. Therefore, *S. bayanus* strain was used for further studies using VHG cassava mashes. High ethanol tolerance of *S. bayanus* could be due to its flocculating nature. It has been reported that yeast flocculation creates a physiological shelter to protect yeast cells from ethanol toxicity (C. HU & al. [12]).

3.2. Optimization of VHG cassava mash preparation

Cassava starch slurries having total solids in the range of 30% to 40% (w/v) were prepared by enzymatic hydrolysis procedure. Total reducing sugars levels in mashes were increased with increase of alpha-amylase and glucoamylase concentrations. The dosage levels of alpha-amylase, which is required for liquefaction, were decreased significantly with addition of calcium chloride to the enzyme (Table 2). It has been reported that calcium ions addition can increase the amylase stability at temperatures over 50°C (D.W.S. WONG & al. [13]; A.V. PRESECKI & al. [14]). And also, gelatinization-cum-liquefaction of slurries by autoclaving at 105-110 °C for 30 min showed beneficial effect instead of heating (at 90-100 °C for 1 h) at atmospheric pressure. Continuous stirring of slurry or mash during liquefaction was improved the yield significantly, and also in terms of energy conservation. Glucoamylase concentrations in the range of 0.4-0.8% (v/w) were found optimal for saccharification step. A maximum concentration of 29% (w/v) total reducing sugars (TRS) was observed in 30% starch slurries. Increased dosage volume of alpha-amylase (0.6% v/w) and glucoamylase (1.0% v/w) in 40% (w/v) slurry has increased the final total reducing sugars level to more than 37% (w/v) (Table 2).

Table 2. Total reducing sugars level of 30%, 35% and 40% cassava mashes prepared under different hydrolysis conditions

Conditions	Liquefaction	Saccharification	Total reducing sugars
Gelatinization-cum-liquefaction for 60 min at 60-65 °C with stirring	a-amylase 0.3%(v/w) + 1mM $CaCl_2$	Glucoamylase-0.4%(v/w); pre-saccharification at 60°C for 24 h	26.29%(w/v)
Gelatinization at 65 °C for 1 h. Then, liquefaction at 90-100 °C for 1 h	a-amylase 0.3%(v/w) + 1mM $CaCl_2$	Glucoamylase-0.4%(v/w); pre-saccharification at 60 °C for 24 h	27.1% (w/v)
Gelatinization-cum-liquefaction 30 min at 105-110 °C (autoclaving)	a-amylase 0.5%(v/w)	Glucoamylase-0.8%(v/w); pre-saccharification at 60 °C for 24 h	29.0% (w/v)
35 % mash Gelatinization-cum-liquefaction for 1 h at 90-100°C	a-amylase 0.3%(v/w) + 1mM $CaCl_2$	Glucoamylase-0.4%(v/w); Pre-saccharification at 60 °C for 24 h.	31.64%(w/v)
40 % mash Gelatinization -cum-liquefaction for 30 min at 105-110 °C (autoclaving)	a-amylase 0.6%(v/w) + 1mM $CaCl_2$	Glucoamylase-1.0%(v/w); pre-saccharification at 60 °C for 24 h.	37.15%(w/v)

3.3. Fermentation of VHG cassava mashes

Yeast nutrients including peptone, yeast extract, malt extract, urea, magnesium sulphate and ammonium sulphate were supplemented to the media in the beginning of fermentation.

Total reducing sugars concentrations were not adjusted after hydrolysis step. Fermentation of all samples was allowed to occur for a period of 72 h. Maximum conversion of sugars to ethanol was observed in case of 30% cassava mash having 27.1% reducing sugars after saccharification. Fermentation efficiency higher than 75% was observed in this case. Supplementation of higher concentrations of yeast nutrients and osmoprotectants has not shown positive impact on ethanol yield. Beyond 30% w/v reducing sugars, reduced fermentation efficiencies were observed even with sufficient yeast nutrients supplementation (Table 3). Yeast extract provides nitrogenous compounds, carbon, sulfur, vitamin B complex and other important growth factors for yeast, including trace nutrients such as magnesium which is essential under VHG conditions. A complete inhibition of yeast growth and fermentation was observed in the medium containing 37% reducing sugars concentration.

Table 3. Ethanol production profile from fermentation of cassava mashes containing various levels of TRS, at different concentrations of nutrients supplementation.

Total reducing sugars	Nutrients added	Ethanol yield	Ethanol productivity (g/L/h)	Fermentation efficiency
26.3% (w/v)	Peptone-1.5% Yeast extract-1.5% Glycine -0.25% $MgSO_4$-0.25%	94.7 g/L	1.31	71.7%
27.1% (w/v)	Urea-1.0% Yeast extract-0.1% Malt extract-0.2% $MgSO_4$-0.1%	101.8 g/L	1.41	75.8%
29.0% (w/v)	Yeast extract-1.5%	105.7 g/L	1.46	71.4%
31.6% (w/v)	Yeast extract-0.2% $(NH_4)_2SO_4$-2.0% $MgSO_4$-0.1%	82.0 g/L	1.13	51.9%
32.0% (w/v)	Yeast extract-1.5% Peptone-1.5%	94.6 g/L	1.31	57.8%
37.15% (w/v)	Yeast extract-1.5% Peptone-1.5%	--Yeast growth and fermentation was not observed --		

3.4. Congeners profile

Aldehydes are produced during fermentation and are unpleasantly pungent but are reduced by 50% during distillation. These compounds can be distilled over and concentrated, mainly in the more volatile, first fraction (heads) of the distillate. The major carbonyl compound (90% of total aldhydes) produced during degradation of sugars by yeasts is acetaldehyde. Like many other aldehydes, it is formed by decarboxylation of the corresponding 2-keto acid, 2-oxopropanoic acid, produced as an intermediate in the metabolism of amino acids. Lack of nitrogenous nutrients in medium can lead to formation of increased aldehydes. Increased levels of aldehydes and methanol are observed in medium containing 32% TRS compared to 26% TRS containing medium (Table 4). It could be due to insufficient nitrogen nutrients in higher gravity medium. This indicates that with increase of medium gravity nitrogenous nutrients requirement for yeast also increases. Fusel Oil (bad spirit) predominantly consists of higher alcohols-a mixture of amyl alcohols, propanol and butanol, esters, fatty acids and some specific aldehydes formed from distillation. They are produced through the metabolisation of nitrogenous compounds by yeasts. Formation of

reduced levels of fusel oils was observed in 32% w/v TRS containing medium compared to 26%w/v TRS medium.

Table 4: Congeners' profile in the mashes with 26% w/v and 32% w/v total reducing sugars (TRS) after 72 h fermentation

Congeners	Concentration (mg/100 ml @100% alc.).	
	Medium with 26% w/v TRS	Medium with 32% w/v TRS
Acetaldehyde	36.31	51.68
Methanol	30.91	42.48
Total esters		
Ethyl acetate	5.10	4.72
Total fusel oils		
n-Propanol	21.39	18.11
Butanol	0.00	0.00
iso-Butanol	71.68	64.12
act-Amyl Alcohol	26.56	18.21
iso-Amyl Alcohol	165.53	117.97

4. Conclusions

From the results obtained, it can be concluded that cassava mashes having total dissolved solids level greater than 300 g/L can be fermented to ethanol. Heat-stable amylase was effective in nearly complete liquefaction of very high gravity cassava (30-40% w/v) mashes. Also, for maximum reducing sugars yield, the mixture of glucoamylase and pullulanase enzymes can be used for saccharification of liquefied cassava starch. A maximum fermentation efficiency of 75.8% with 30 % mash indicates that there is still a room for further improvement in ethanol yield. However, the yeast strain used in this study has performed well (15.6% v/v ethanol; 86.6% fermentation efficiency) using VHG finger millet mash ethanol fermentation (P. PRADEEP & al. [15]). Relatively low final ethanol yields compared to finger millet mashes could be due to toxic cyanogen compounds and antinutrients, which are commonly present in cassava. Enhanced efficiencies could be achieved through removal of toxic compounds by processing, supplementation of yeast nutrients/osmoprotectants, and optimization studies.

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