Journal of Agricultural Science; Vol. 8, No. 9; 2016
ISSN 1916-9752 E-ISSN 1916-9760
Published by Canadian Center of Science and Education

Cassava Biomass Transformation by *Aspergillus oryzae*

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Received: March 10, 2016 Accepted: May 4, 2016 Online Published: August 15, 2016

doi:10.5539/jas.v8n9p37 URL: http://dx.doi.org/10.5539/jas.v8n9p37

Abstract

The cassava spirit, known as *tiquira*, is produced mainly in the north region of Brazil and it is regulated by Brazilian legislation. This study aimed to evaluate the traditional method of cassava spirit production, using a controlled process with application of fermentation technology in a laboratory scale production. *Aspergillus oryzae* was used for liquefaction and saccharification of cassava starch. It was obtained wort with 13.03 °Brix and 6.80% of reducing sugars. Commercial *Saccharomyces cerevisiae* yeast was used for alcoholic fermentation. After alcoholic fermentation, it was obtained fermented wort with very low volatile acidity. The cassava spirit was obtained by a double distillation process, with separation of the fractions head, heart and tail. The heart fraction of the distillate showed alcohol content of 51.56°GL. The contents of aldehydes, esters, methanol and higher alcohols (n-propyl, isobutyl and isoamyl) were determined in cassava spirit using gas chromatography. According to the results, the cassava spirit showed methanol and higher alcohols contents above the limits imposed by Brazilian legislation.

Keywords: cassava, spirit, distillate, *Aspergillus oryzae*, amylases

1. Introduction

Cassava (*Manihot esculenta* Crantz) is a root native of tropical America (Amazon region) and even before the arrival of Europeans in America it was already widespread for food crop, with several varieties. Brazil is the second largest cassava producer in the world and cassava processing in Brazil includes the production of flour (which is basically produced by the peeled grated roots, pressed and followed by dried or toasted process) and of starch, also known as *polvilho* (Cereda, 2003; Demiate & Kotovicz, 2011; FAO, 2004).

The higher value-added ingredient in cassava is the starch (the fresh roots contain about 30% of starch) and cassava can become the raw material base for an array of processed products (Abera & Rakshit, 2003; Charles & et al., 2005). The cassava spirit, known as *tiquira*, is a product derived from processed cassava little described in the literature. *Tiquira* is produced mainly in the north region of Brazil and it is regulated by Brazilian legislation, although its production is mainly artisanal (Anonymous, 2008).

In the traditional method of cassava spirit production, the wet mass of cassava is placed to mold, so the starch hydrolysis into fermentable sugars is made by native molds naturally present in the environment. The starch must be converted into simple fermentable sugars to produce ethanol as product. This is due to the fact that in the process of alcoholic fermentation, yeasts such as *Saccharomyces* are not able to produce amylolytic enzymes. After that, the mold cassava mass is mixed with water and the wort is placed to ferment. The alcoholic fermentation is also made by yeast and bacteria naturally present in the fermentation environment. In the traditional method of cassava spirit production there are no quality and technical control in the process, causing disadvantages such as low yield and a final product without quality standard (Cereda, 2003; Cereda & Carneiro, 2008; Venturini Filho, 2005).

Amylases can be obtained from several microorganisms; however the *Aspergillus* species are considered a significant source of these enzymes, once it can be obtained in higher yield. *Aspergillus oryzae* is generally recognized as a safe (GRAS) microorganism by the Food and Drug Administration and has been widely used to obtain amylases. *Aspergillus oryzae* amylases have a high efficiency in saccharification of starch (Pandey et al., 2005; Norouzian et al., 2006; Sivaramakrishnan et al., 2007).

This study aimed to evaluate the traditional method of cassava spirit production, using a controlled process with application of fermentation technology in a laboratory scale production. *Aspergillus oryzae* was used for liquefaction and saccharification of cassava starch and commercial *Saccharomyces cerevisiae* yeast for alcoholic fermentation.

2. Method

2.1 Fungal Spore Preparation

The filamentous fungi *Aspergillus oryzae* (Ahlburg) Cohn nº CCT 3940, acquired in the Foundation André Tosello (Collection of Tropical Crops). *Aspergillus oryzae* spores were Sabouraud dextrose (Prodimol® Biotecnologia S/A). Inoculum was prepared on 250 mL flasks with 50 mL Sabouraud dextrose medium. After 5-7 days of incubation at 24 °C, 30 mL of sterile distilled water was added and spores were suspended and 15 μL of the suspension were added in a Neubauer chamber, containing 5×10^6 spores. A volume of 10 mL of suspension were added per flask was used.

2.2 Processing and Fermentation of Cassava

Cassava roots ((low-cyanide variety or sweet cassava), were obtained in a market of local farmers of Distrito Federal (Brazil) in July 2014. Cassava roots were acquired washed and peeled. In the laboratory, the roots were processed using a blender and the grated cassava mass was packaged and stored in a freezer at -18 °C until ready to use. The low-cyanide or absence or cyanide was assumed based on the literature and the regulation of cassava commercialization, which states that for commercial purpose cassava must have the cianogenic glucoside's bellow 2 mg/Kg. Also, the fact of gridding and autoclave decreases the HCN concentration as well.

For gelatinization of cassava starch, 250 g of grated cassava mass were added into 1000 mL erlenmeyer flasks and autoclaved for 15 min at 121 °C. For liquefaction and saccharification of cassava starch, amylolytic enzymes (α-amylase and amyloglucosidase) were produced inoculating 5 μM *Aspergillus oryzae* spores (10 mL inoculum) per flask. Erlenmeyers containing cassava mass and inoculated *Aspergillus oryzae* were incubated at 24 °C for 7 days. After that, the moldy cassava mass was mixed with 250 mL of distilled water. The mixture was homogenized and incubated in a water bath at 45 °C for 1 hour to complete the enzymatic hydrolysis of starch. This process gave rise to the wort, a sugary solution. The wort was autoclaved for 15 min at 121 °C to inactivate fungi. Alcoholic fermentation of wort was performed using Saccharomyces cerevisiae (Fermentis ® Yeast S-04). The dosage used was 5.75 g of dry yeast to 9 L of wort. Fermentation was carried out for 5-6 days at 24 °C. The fermented wort was filtered to separate the bagasse.

2.3 Distillation Procedure

Upon completion of alcoholic fermentation, the fermented wort was immediately distilled in a laboratory distiller. The temperature of the distillation process was maintained between 80 °C and 94 °C. The first distillation was made without separation of fractions. In the second distillation, the distillate was separated into 3 fractions. The first fraction (head fraction) was collected separately and corresponded to a volume about 10% of the total volume. The intermediate fraction (heart fraction) was then collected and corresponded to a volume about 80% of the total volume. The last fraction (tail fraction) corresponded to 10% of the final volume of spirit produced. The fractions of cassava spirit were stored in bottles and maintained at 8 °C for physico-chemical and chromatography analysis.

2.4 Physico-Chemical and Gas Chromatography Analysis

Analysis of pH in cassava mass, wort and fermented wort were measured using a digital potentiometer. Total acidity in wort and fermented wort were determined by titration with 0.1 N NaOH. The soluble solids were determined in wort through a benchtop refractometer at 20 °C and expressed in °Brix (AOAC, 2006). Volatile acidity in fermented wort was determined after distillation and subsequently titration with 0.1 N NaOH. The dry extract in fermented wort was determined by evaporation at 105 °C to constant weight (IAL, 2008). Reducing sugars in wort were determined using the 3,5-dinitrosalicylic acid method (Miller, 1959). Each parameter was analyzed in triplicate. Results are shown as mean values and standard deviation of the independent determinations and all data were treated with the aid of the MICROSOFT EXCEL® programme (version 2010).

The contents of aldehydes, esters, methanol and higher alcohols were determined using gas chromatography. Samples were spiked with the internal standard (4-methyl-2-pentanol). Aliquots of 1.0 μL were automatically injected into the gas chromatographic system (Shimadzu, QP-2010 PLUS, Tokyo, Japan) equipped with a Stabilwax-DA column (crossbond carbowax polyethylene glycol, 30 m × 0.18 mm × 0.18 μm film thickness) and a flame ionisation detector (FID). The analyses were performed at a 1:25 split ratio, in triplicate. Nitrogen was used as the carrier gas (flow rate of 1.5 mL min^{-1}, total flow of 27 mL min^{-1} and pressure of 252.3 kPa). The

temperatures of both the injector and the detector were set at 250 °C. The oven temperature program was 40 °C for 4 min, followed by an increase to 120 at 20 °C min^{-1}, kept for 1 min, and then up to 180 °C at 30 °C min^{-1} and maintained for 4 min more (Bortoletto & Alcarde, 2013).

3. Results and Discussion

3.1 Processes of Hydrolysis and Fermentation of Cassava

In this study, enzymatic hydrolysis of cassava starch was made using a total of 4.5 kg of grated cassava mass that presented 36.69% of moisture (Table 1). According to Cereda (2003) in the traditional method of *tiquira* production, *Aspergillus niger*, *Aspergillus oryzae* and *Neurospora sitophila* are the species commonly observed in the cassava mass.

After liquefaction and saccharification of cassava starch by amylases produced by *Aspergillus oryzae*, it was obtained 9 liters of wort with 13.03 °Brix and 6.80% of reducing sugars. Cereda and Carneiro (2008) proposed a modern method for tiquira production using commercially thermotolerant α-amylase and amyloglucosidase produced by *A. niger*. Proceeding hydrolysis by these commercial amylases, Bastos (2013) obtained 13-16 °Brix in the hydrolysed cassava mass hydrolysed using these commercial amylases for the modern process of *tiquira* production. In distillates production, the best results in alcoholic fermentation are obtained with worts in concentrations of 13-18 °Brix (Venturini Filho, 2005).

Table 1. Physico-chemical analysis of grated cassava mass, wort and fermented wort

Analysis	Values
Moisture (g 100 g^{-1}) of grated cassava mass	36.69 ± 1.35
pH of grated cassava mass	6.50 ± 0.01
pH of wort	5.89 ± 0.21
Soluble solids (°Brix) of wort	13.03 ± 0.05
Reducing sugars (g 100 g^{-1}) of wort	6.80 ± 0.30
pH of fermented wort	5.33 ± 0.01
Total acidity (meq L^{-1}) of fermented wort	9.67 ± 0.57
Volatile acidity (meq L^{-1}) of fermented wort	2.00± 0.01
Dry extract (g L^{-1}) of fermented wort	11.35 ± 0.28

Note. Results are reported as means ± standard deviation of three measurements.

The grated cassava mass showed pH of 6.50, the wort showed pH of 5.89 and the fermented wort showed a pH of 5.33. *Aspergillus oryzae* is a producer of kojic acid (5-Hydroxy-2-(hydroxymethyl)-4H-pyran-4-one) (Bentley, 2006) and this explains the reduction in pH of the wort. The reduction in pH of the wort caused by the production of organic acids by fungi reduces the risk of contamination of wort with acetic or lactic bacteria, which increase the volatile acidity (acetic acid) and is undesirable in the fermentation process. The variation in pH between the wort and the fermented wort is due to organic acids formed as by-products of alcoholic fermentation by the yeast *Saccharomyces cerevisiae* (Asquieri et al., 2009).

After alcoholic fermentation, it was obtained 4.5 liters of filtered fermented wort. The alcoholic fermented cassava presented low values of total acid (9.67 meq L^{-1}) and volatile acidity (2.00 meq L^{-1}). The low value of volatile acidity shows that fermented was technologically well prepared. Although *Saccharomyces* can produce acetic acid, excessive concentrations of acetic acid in wine are largely the result of the metabolism of ethanol by aerobic acetic acid bacteria. The volatile acidity in fermented cassava is far below the limit established by Brazilian legislation for wines, which is a maximum of 20.00 meq L^{-1} (Anonymous, 1988).

3.2 Cassava Spirit Production and Analysis

The first distillation of fermented wort resulted in 400 mL of distilled (yield of 8.9% in comparison to the initial quantity of raw material, 4.5 kg of cassava mass) and presented an alcohol content of 24% (v/v). After the second distillation, the heart fraction of cassava spirit showed an alcohol content of 51.56% (v/v) (Table 2). This value is within the limit established by Brazilian legislation for *tiquira*, which is 38-54% (v/v) (Anonymous, 2008).

Table 2. Concentration of volatile compounds (mg.100 mL anhydrous ethanol) in the cassava spirit

Compounds	Head fraction	Heart fraction	Reference values*
Ethanol % (v/v)	80.75	51.56	36-54
Volatile acidity (acetic acid)	8.37	30.70	0-100
Aldehydes (acetic aldehyde)	31.91	0.41	0-20
Esters (ethyl acetate)	69.30	4.65	0-200
Methanol	99.88	127.15	0-20
1-Butanol	3.73	2.58	0-3
2-Butanol	1.19	0.37	0-10
Isobutyl alcohol	415.33	166.41	-
Propyl alcohol	224.82	172.46	-
Isoamyl alcohol	260.69	123.62	0-300
Superior alcohols (sum of isobutanol, propanol and isoamyl alcohol)	900.84	462.49	0-300
Furfural	0.00	0.00	0-5
Cooper (mg/L)	0.00	0.02	0-5
Total volatile congeners (sum of aldehydes, esters, superior alcohols, volatile acidity and furfural)	1010.43	498.25	250-650

Note. * Reference values established by Brazilian legislation (Brazil, 2008) to the quality of *tiquira*.

The volatile acidity of heart fraction of cassava spirit (30.70 mg acetic acid.100 mL anhydrous ethanol) was approximately 3 times lower than the tolerance limit established by Brazilian legislation (Anonymous, 2008) to the *tiquira* (100 mg acetic acid.100 mL anhydrous ethanol). This result was expected due to the low volatile acidity of the fermented wort. Duarte et al. (2011) obtained similar result of 37 mg acetic acid.100 mL anhydrous ethanol for jabuticaba spirit (a tropical fruit from Brazil) and Asquieri et al. (2009) also obtained low value of volatile acidity (30 mg acetic acid.100 mL anhydrous ethanol) for jabuticaba spirit. Bortoletto and Alcarde (2013) studied the aging of sugar cane spirits in casks of different woods. After a period of 36 months, these authors obtained high values of volatile acidity (62.4-143.9 mg acetic acid/100 mL anhydrous ethanol) in the aged spirits. The presence of high concentrations of acetic acid may be an indicative of contamination of the wort by acetic and aerobic bacteria that could metabolize ethanol into acetic acid. (Asquieri et al., 2009; Bortoletto & Alcarde, 2013; Duarte et al., 2011; Swiegers et al., 2005). Acetaldehyde is the predominant aldehyde in spirits, corresponding to approximately 90% of total aldehydes in the distillate (Bortoletto & Alcarde, 2013). The heart fraction of cassava spirit presented very low concentration of acetic aldehyde (0.41 mg.100 mL anhydrous ethanol). Duarte et al. (2011) result of 1.12 mg acetic aldehyde/100 mL anhydrous ethanol for jabuticaba spirit. Hernandez-Gomez et al. (2005) obtained results of 20.4-57.5 mg acetic aldehyde.100 mL anhydrous ethanol in melon distillate beverages, above the tolerance limit established by Brazilian legislation (20 mg/100 mL anhydrous ethanol) (Anonymous, 2008). Acetic aldehyde is an undesired compound in distillate; mainly due to the fact that at high concentrations has a pungent odour and chemical reactivity (García-Llobodanin et al., 2008; Bortoletto & Alcarde, 2013; Soufleros et al., 2005). The presence of high concentrations of acetaldehyde in spirits is an indicative of oxidation of the ethanol during alcoholic fermentation (Asquieri et al., 2009; Bortoletto & Alcarde, 2013; Swiegers et al., 2005).

Ethyl acetate is another compound that may affect the quality of distillate due to its unpleasant flavor in high concentrations (Asquieri et al., 2009; Duarte et al., 2011; Hernandez-Gomez et al., 2005). The heart fraction of cassava spirit presented low concentration of ethyl acetate (4.65 mg/100 mL anhydrous ethanol). García-Llobodanin et al. (2008) obtained similar results of total esters (2.1-5.5 total mg/100 mL anhydrous ethanol) for pear distillates. According to Hernandez-Gomez et al. (2003), low concentrations of ethyl acetate (5 to 8 mg/100 mL anhydrous ethanol) contribute to the distillate flavor complexity and has a positive impact on product quality. Ethyl acetate is generally the predominant ester in spirits, corresponding to approximately 80% of total esters in the distillate. Ester formation occurs during fermentation mainly due to esterification reactions between acids and alcohols of the spirit. Ethyl acetate originates from the esterification reaction between ethanol and acetic acid and its amount depends on the relative abundance of the corresponding alcohols and the acyl-coA radicals involved in yeast metabolism (Asquieri et al., 2009; Bortoletto & Alcarde, 2013; Soufleros et al., 2005).

The cassava spirit showed lower concentrations of aldehydes and esters in the heart fraction compared to the head fraction. This results show that the distillation process and separation of the head fraction was corrected

made and have a positive influence on the final distilled beverage.The head is the fraction of distillate consisting mainly of volatiles components that distill first, or have low boiling point (BP) and are soluble in alcohol. Most are separated at the beginning of the distillation and their concentrations are relatively higher in the first distillate fractions. Acetaldehyde (BP = 21 ºC), methanol (BP = 64.6 ºC) and ethyl acetate (BP = 77 ºC) are included in this group (Alcarde et al., 2010).

Methanol is an important compound to control in the spirits and its regulation is based on associated health hazards. The concentration of methanol in cassava spirit was found to be 99.88 mg/100 mL anhydrous ethanol in head fraction and 127.15 mg/100 mL anhydrous ethanol in heart fraction. These values are superior to the limit established by the Brazilian law (20 mg/100 mL anhydrous ethanol) (Anonymous, 2008); however they are in accordance with the USA and European Union regulations. The United States legal limit on methanol in distilled fruit spirits is 0.35% (v/v) or 700 mg/100mL of anhydrous alcohol. The EU general methanol limit for spirits made of fruits and marc brandy is 1000-1200 mg. 100 mL of anhydrous alcohol (European Union Rules No.1567/89, 1989). Some studies in literature obtained high concentrations of methanol in fruit spirits. Duarte et al. (2011) obtained a result of 85 mg methanol/100 mL anhydrous ethanol in jabuticaba spirit, Hernandez-Gomez et al. (2005) obtained results of 150-206 mg methanol/100 mL anhydrous ethanol in melon distillates and Soufleros et al. (2005) obtained mean value of 744 mg methanol/100 mL anhydrous ethanol in a traditional Greek fruit distillate called *koumaro*.

Although methanol occurs naturally, at a low level, because it is a side product of the fermentation process, it may reach larger concentrations in some cases. In fruits spirits with high content of pectin (like plums, apples and pears) methanol is formed mainly from interaction of pectinmethylesterases (enzymes present naturally in fruits) with the pectin of the fruit. Pectinmethylesterases catalyze the hydrolysis of the methoxyl group from pectin and thus release methanol and consequently increases the concentration of methanol in fruit spirits (Bortoletto & Alcarde, 2013; Duarte et al., 2011; Soufleros et al., 2005; Zhang et al., 2011). Methanol is metabolized in the body to formaldehyde and formic acid. Formic acid is a toxic metabolite that acts on the retina, optic nerve and central nervous system. Humans are uniquely sensitive to the toxicity of methanol as they have limited capacity to oxidize and detoxify formic acid. Thus, the toxicity of methanol in humans is characterized by metabolic acidosis, blindness or serious visual impairment and even death (Paine & Davan, 2001; Zhang et al., 2011).In the case of cassava spirit, the increase concentration of methanol probably occurred in the process of liquefaction and saccharification of cassava starch with inoculation of fungi *Aspergillus oryzae.* Cassava roots contain pectin (2.19 to 2.46% of total pectin in fresh weight basis) (Feniman, 2004) and *A. oryzae* is a producer of pectinolytic enzymes (Hoa, 2013; Sabika, 2012). These facts suggest that the pectinolytic enzymes of fungi catalyzed the hydrolysis of the methoxyl group from pectin of cassava and increased the concentration of methanol in wort, before the alcoholic fermentation process.

Higher alcohols are responsible for imparting complex sensory attributes to spirits and refer to the sum of isobutyl, propyl and isoamyl alcohols according to Brazilian legislation (Anonymous, 2008). The heart fraction of cassava spirit presented a high content of higher alcohols (498.25 mg/100 mL anhydrous ethanol) and this value was superior to the limit established by the Brazilian law (300 mg/100 mL anhydrous ethanol). Higher alcohols originate from the metabolism of nitrogen-containing compounds by yeast. The alcohols containing up to 5 carbon atoms, such as amyl and propyl alcohols, contribute to the formation of the flavoring aroma. However, the excess of higher alcohols interferes negatively in the quality of spirits (Bortoletto & Alcarde, 2013; Duarte et al., 2011; Soufleros et al., 2005).

4. Conclusion

Cassava spirit production was viable on a controlled laboratory scale process using Aspergillus oryzae CCT 3940 for cassava starch liquefaction and saccharification instead of the commercially available enzymes. The method here describes must to be adequate to reduce the levels of methanol and higher alcohols generated during the process because the negative impacts of such compounds in product quality and human health. Further evaluation and methodological modification must be conducted aiming to improve cassava spirit production yield, and to reduce the amount of undesired toxic by-products. Our results, point out the potential hazards of the production of traditional distillates for human consumption.

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