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Improvement of Bioethanol Production using Amylasic Properties from *Bacillus licheniformis* and Yeasts Strains Fermentation for Biomass Valorization

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ABSTRACT

Saccharomyces is a potentially useful organism for the commercial production of ethanol as it is not capable of fermenting starch containing in the mango peel. To enhance this carbon source utilization and increase the rate of alcohol production, simultaneous saccharification and fermentation were conducted using *Bacillus licheniformis* and yeasts. The proposal of this research was to study amylasic activity, rate and amount of starch utilization and ethanol yields increasing several-fold in coculture due to the synergistic metabolic interactions between the species. The methods used, included: enzymatic hydrolysis with *B. licheniformis* and simultaneous saccharification and fermentation with S3 and A1. These two yeasts strains were isolated from wine and among local beer yeasts respectively. The enzymatic hydrolysis has allowed to obtain 78% (g g^{-1}) of reducers sugars released. The maximum concentration of ethanol produced from peel mango by S3 and A1 during 5 days were, respectively 16 and 14.4 g L^{-1}.

Key words: Biomass, amylase, fermentation, ethanol, yeast

INTRODUCTION

Saccharomyces strains were used widely and traditionally for industrial ethanol production because of its ability to produce high concentrations of ethanol from hexoses and its high tolerance to ethanol and other inhibitory compounds. However, *S. cerevisiae* is naturally unable to metabolize pentoses, such as xylose, arabinose and also polysaccharides (starch). Fribous (lignocellulosic) is the second major source of carbohydrates in hard woods and vegetable biomass, so its fermentation is essential for the economic conversion of starch to ethanol, which may provide an ideal alternative fuel source in the future (Lin and Tanaka, 2006). The use of renewable resources, such as lignocellulosic biomass, to produce ethanol offers several environmental benefits and averts competition (Kumar *et al*., 2009; Lin and Tanaka, 2006).

Many microorganisms, including *Saccharomyces cerevisiae*, are not able to degrade starch and they do not produce starch decomposing enzymes as amylase, pullulanase or isoamylase and glucoamylase (Gupta *et al*., 2003). The ethanol-fermenting microorganisms, such as *S. cerevisiae* are lack of amylolytic enzymes and unable to directly convert the starch into ethanol (Ang *et al*., 2001).

Considering that the main potential feedstock (biomass) for producing bioethanol is composed of carbohydrates, which include starch, cellulose and hemicelluloses, among others, the use of enzymes to break down these oligosaccharides into easily fermentable sugars is a requirement, previous to conducting the fermentation (Kim and Dale, 2004).

It is necessary either to use starch enzymes producing strains in order to get free carbohydrates monomers for using as carbon source (Altintas *et al*., 2003; Gupta *et al*., 2003; Nakamura *et al*., 2002). Starch fermentations with *S. cerevisiae* transformed with amylase and glucoamylase genes showed ethanol productivity similar to that observed when starch decomposing enzymes were added to the medium (Ulgen *et al*., 2002; Eksteen *et al*., 2003; Kang *et al*., 2003; Shigechi *et al*., 2004). Starch is converted into ethanol in starch plants where the raw material, is milled and then treated with a combination of heat and enzymes without prior separation of its constituents (Bothast and Schlicher, 2005). Industrial processes such as starch liquefaction demands the process to be carried out at high temperature so economical application of amylase to such process, its thermostability is of a prime importance (Joshi, 2011).

Several agricultural wastes have been tested for their bioethanol-producing potential. In the present study, the utilization of some agricultural residues (like mango residues) containing carbohydrates for the production of bioethanol was evaluated. Also, In Burkina Faso, enormous quantities of mango are lost per year and contribute to increase the environmental rate residues. In order to valorize these residues, it was important to research the possible ways. The aim of this study was to improve the bioethanol production by silmultaneous fermentation of yeasts using amylasic properties from *Bacillus licheniformis* in the way to valorize mango biomass.

MATERIALS AND METHODS

Collection and processing of samples: Mango residues (70 samples as 25 kg) were collected from waste dumping sites in the principals mango production areas. Three regions (Banfora, Houet, Orodara) and peripheral area of Ouagadougou (Capital of Burkina Faso) were concerned to the sampling. The sampling was done during, April to May, 2010.

Isolation and selection of microorganisms: The selection was carried out on a total of 8 yeasts strains. The strains codified as A1 to A4 were *S. cerevisiae* strains isolated from wine cultures. Also the strains codified as S1 to S4 the Baker's yeast microorganism commonly used in local drinking beer (dolo).

The yeasts strains were isolated in maintenance medium (used in agar plates) contained 20 g of glucose, 20 g of agar, 5 g of peptone, 5 g of $MgSO_4 \cdot 7H_2O$ per liter. In the way to select the strains having best growth; the in the liquid inoculation contained (growth medium) 50 g glucose, 5 g of yeast extract, 1 g of KH_2PO_4, 0.3 g of NH_4Cl and 2 g of $MgSO_4 \cdot 7H_2O$ per liter has been utilized. The fermentation medium contained 0.05 M citrate buffer pH 4.8; 1,5 g peptone, 5 g of yeast extract, 1 g of KH_2PO4, 0.3 g of NH_4Cl and 2 g of $MgSO_4 \cdot 7H_2O$ per liter. It was used to select the best strains producing alcohol.

Carbohydrate (sugar) fermentation: The ability of the yeasts to ferment various carbohydrates using glucose, fructose, sucrose, maltose, lactose and arabinose was determined by growing the isolate in liquid standard medium containing 1% (w/v) of the particular carbohydrate. Durham-tubes were inverted into the culture tubes for gas collection. The incubation was at 30°C for 24 h and uninoculated broths were used as control. The standard medium used for fermentation was it recommended by Konlani *et al*. (1996).

***Bacillus licheniformis* isolation:** *Bacillus licheniformis* has been isolated from and characterized in laboratory (CRSBAN).

Also it was performed to improve amylolitic property using different physiologicals conditions (Temperature, pH, percentage of starch). This process is driven to select the *Bacillus licheniformis* strains having a best enzymatic hydrolysis activity.

Bioethanol production: Methods used for production of bioethanol include enzymatic hydrolysis, fermentation and fractional distillation.

Enzymatic hydrolysis: An optimization of temperature and enzyme activity was performed. The performance during starch hydrolysis was evaluated based on the reducers sugars production and the liquefaction yield for the substrate (peel mango) (Miller, 1959).

One hundred grams of mango peel was weighed into seven 1 L conical flasks and 2 mL of solution containing amylase from *B. licheniformis* was added to each conical flask.

The flasks were covered with cotton wool, wrapped in aluminum foil, heated for 2 h in a water bath and then autoclaved for 30 min at 121°C. The Flasks were allowed to cool, filtered trough Whatman filter paper and the pH was adjusted to 4.5 with acetate tampon medium.

The amylasic hydrolysis activity of *B. licheniformis* was setting in evidence by following of kinetic of reducers sugars released through carbohydrates hydrolysis during 5 h.

Fermentation: The fermentation was carried out along with saccharification (Simultaneous Saccharification and Fermentation (SSF)), as described by Kroumov *et al.* (2006) and Oghgren *et al.* (2006). The flasks containing the hydrolyzed samples were covered with cotton wool, wrapped in aluminium foil, autoclaved for 15 min at 121°C and allowed to cool at room temperature. Yeasts performing strains A1 and S3 were each aseptically inoculated into each flask and incubated at 30°C. Two flasks of each sample (containing mango peel) were removed after every 24 h, up to 7 days.

Fractional distillation: The fermented broth was dispensed into round-bottom flasks fixed to a distillation column enclosed in running tap water. A conical flask was fixed to the other end of the distillation column to collect the distillate. A heating mantle with the temperature adjusted to 78°C was used to heat the round-bottomed flask containing the fermented broth.

Determination of quantity of ethanol produced: The distillate collected over a slow heat at 78°C was measured using a measuring cylinder and expressed as the quantity of ethanol produced in g L^{-1} by multiplying the volume of distillate collected at 78°C by the density of ethanol (0.8033 g mL^{-1}). The g L^{-1} is equivalent to the yield of 100 g of dried substrate (Humphrey and Okafoagu, 2007).

Determination of percentage ethanol: A standard ethanol density curve was prepared by taking series of percentage (v/v) ethanol solutions, which were prepared in volumetric flasks and the weight was measured. The density for each of the prepared ethanol solutions was calculated and a standard curve of density against percentage ethanol was plotted. The percentage ethanol concentration of ethanol produced was obtained by comparing its density with the standard ethanol density curve.

RESULTS

Selection of performed yeasts: Among 8 yeasts coming in different biotope, only two yeasts (S3, A1) were retained for ethanol fermentation process.

Peel saccharification optimization: The amylasic activity of *B. licheniformis* show the kinetic of reducing sugars released as shown in Fig. 1. The optimal rate of reducing sugars attains 78% (g g^{-1}).

Ethanol production from peel mango sugar performed by simultaneous saccharification and fermentation (SSF): The kinetic of ethanol production by two yeasts strains (S3; A1) is shown in Fig. 2.

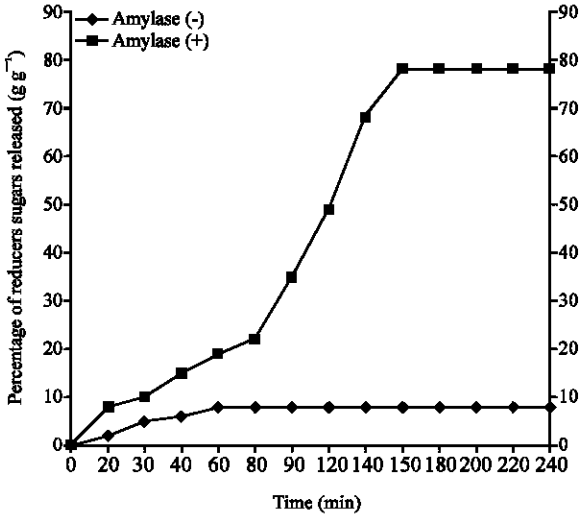

Fig. 1: Following of reducers sugars percentage releasing through carbohydrates hydrolysis on peel mango, incubated with α-amylase of *B. licheniformis*

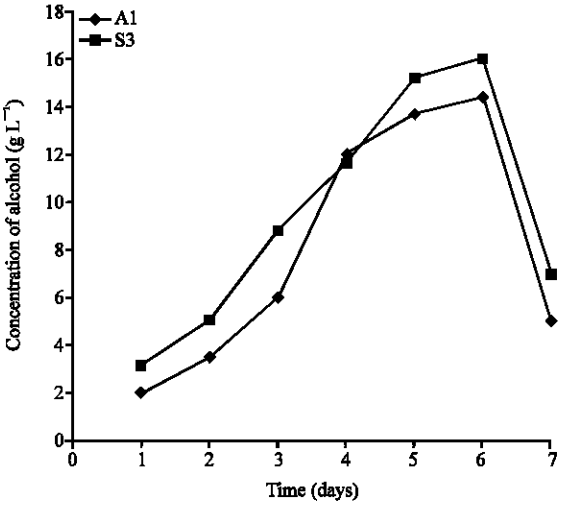

Fig. 2: Ethanol produced (g L^{-1}) from mango peel using yeasts strains S3 and A1 separately and each with amylase presence simultaneously

DISCUSSION

Selection of performed yeasts: The yeasts strains S3, A1 were selected according to their specific growth performance, that varies respectively at 0.31 to 0.24. And yet it can be noticed that specific growth parameters must not be directly link or attach to the ethanol high production.

Peel saccharification optimization: On mango peel containing 22.62 % (g g^{-1}) of carbohydrates (polysaccharides), the hydrolysis was conducted up to 4 hours and the results presented in Fig. 1, shows that releasing of reducing sugars increased with enzymatic activity of *B. licheniformis*. This result is demonstrated while varying the volume of the inoculum (contained the strains). Likewise it is noticed that there has not significant change or variation of reducers sugars rate without enzyme presence. Kim and Hamdy (1985) showed that the same trend was observed when the hydrolysis of potato was studied.

So liquefaction is a preliminary step for saccharification, by which large quantities of D-glucose can be produced from inexpensive sources.

The liquefaction conduct at middle temperature (55°C) result a higher yield of reducing sugars released (78% g g^{-1}), which might be related to the type of enzyme utilized. This result was greater than that obtained by Lazic *et al.* (2004) in the two-step enzyme hydrolysis (61 %).

Thus, fermentescibles sugars percentage obtained (78% g g^{-1}) by using *B. licheniformis* enzymatic activity is relatively very higher than at which found in *Bacillus* absence.

This value is slightly superior to which reported by Somda *et al.* (2010) using other species of *Bacillus* on peel mango (62% g g^{-1}). It can be explained that the enzyme has the capacity of decomposing into hexose, all polysaccharides which are built up of glucose residues united by α-1, 4 glycosidic bonds and also it is thermostable. And yet the incomplete utilization of polysaccharides (starch) by enzymes may due to lack of enough oxygen or feedback inhibition of amylase activity by glucose released, as reported by Abouzied and Reddy (1986). Others authors have explained mechanism of polysaccharides hydrolysis with some microorganisms.

Lagzouli *et al.* (2007) showed that the production of glucoamylase in presence of starch and of glucose suggests that the glucoamylase produced by *Candida guilliermondii* is an enzyme greatly led by the starch. And that its activity is probably under the effect of a glucose repression catabolic.

The same phenomenon of repression catabolic has been observed at *Clostridium thermohydrosulfuricum* (Hyun and Zeikus, 1985) and *Bacillus* sp. (Kiran *et al.*, 2005).

As *B. licheniformis* expressing both α-amylase and glucoamylase activities. Therefore this potentiality *Bacillus* could be used to fermentation process in medium containing polysaccharides substrates like mango peel.

Kinetic of amylasic hydrolysis of peel starch and simultaneous fermentation to ethanol: The hydrolyzate obtained by amylase of *B. licheniformis* was used as substrates for bioethanol production respectively by yeasts strains A1 and S3. This effect is shown in Fig. 2. Peel mango was used to produce ethanol through enzymatic hydrolysis and SSF with respectively two yeasts strains (S3, A1).

In SSF, the two different microorganisms behaved differently, according to their nutrient requirements, but synergistically in the degradation of organic substrate. An enzyme (carbohydrate hydrolases produced by *Bacillus licheniformis*) was able to hydrolyze peel mango. The saccharification products were simultaneously utilized by yeasts strains (S3, A1) for ethanol production.

These yeasts are able to produced ethanol due to the presence of Pyruvate Decarboxylase (PDC) and alcohol dehydrogenase (ADH), which are key enzymes in ethanol formation, as reported by Gunasekaran and Chandra (2007). Figure 2 shows that the maximum volume of ethanol (16 g L^{-1}) produced from peel mango by S3 and B1 (14.4 g L^{-1}) in this study at the 120th h is lower than the results found by Agulejika *et al.* (2005) who also reported maximum ethanol yield at 120th hour from fresh fruit (64.01 g L^{-1}) and waste fruits (21.14 g L^{-1}) using *Z. mobilis*. The higher ethanol yield from fresh fruit was due to higher presence of fructose and glucose in fresh fruits, as stated by Micheal and Rosaline (2000). The maximum volume of ethanol produced from peel mango is lower than the 59 g L^{-1} reported by Gunasekaran and Chandra (2007) at 120th h from cassava starch hydrolysate. This is due to cassava containing more carbohydrates, which could be fermented to ethanol. Thus, Sree *et al.* (2000) reported about the ethanol production by SSF of wheat products using Saccharomyces cerevisiae. Those authors were able to produce up to 44.2 g-ethanol L^{-1} when, fine wheat flour was used as substrate and 34.1 g L^{-1} using damaged wheat flour. Present results are in agreement with those found previously by these authors quoted.

Also, Tasic *et al.* (2008) have conducted fermentation on potato tuber mash and found during 18 to 33 h of incubation, an ethanol concentration growing at 31.2 to 32.9 g L^{-1}.

Ethanol produced from treated sample (bitter kola, pulp agrowaste) by Humphrey and Okafoagu (2007) was 11.2 g L^{-1} at 96 h and 12.9 g L^{-1} at 216 h. These values were lower than our values.

The decline and stabilization in ethanol noticed at some stages may be due to the inhibitory effect of ethanol on growth and transport metabolism of the yeast (D'Amarc and Stewart, 1987; Xu *et al.*, 1996).

Present results revealed that ethanol could be produced from agricultural residues, such as peel mango using performed yeasts strains (S3, A1) as fermenting organisms.

Considering the cost-effectiveness, in addition to being a means to control environmental pollution, the use of peel mango for ethanol production is concluded as a worthwhile venture.

CONCLUSIONS

Present results demonstrate that the simultaneous saccharification and fermentation of mango peel carbohydrates with *B. licheniformis* and yeasts (S3 and A1) is sufficient to increase bioethanol production. This led us to suggest that it would be interesting to use this carbohydrates fermentation process in repeated fed-batch or continuous fermentation for further improvements of bioethanol production.

Finally, studies using *B. licheniformis* expressing α-amylase are promising and will lead to valuable applications in ethanol production from renewable feedstocks and the conversion of mango peel into ethanol.

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