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Effect of Minerals Salts in Fermentation Process using Mango Residues as Carbon Source for Bioethanol Production

[1]Marius Kounbèsiounè Somda, [1]Aly Savadogo, [1]Nicolas Barro, [2]Philippe Thonart and [1]Alfred Sabadénédyo Traore
[1]Research Center in Biological, Food and Nutritional Sciences University of Ouagadougou, 03 BP 7021 Ouagadougou 03, Burkina Faso
[2]Centre Wallon de Biologie Industrielle, Unité Bio-industries, Gembloux, Agro-Bio Tech, Université de Liège, Belgique 2, Passage des Déportés, B-5030, Gembloux, Belgium

Corresponding Author: Somda K. Marius, Research Center in Biological, Food and Nutritional Sciences, University of Ouagadougou, 03 BP: 7021 Ouagadougou 03, Burkina Faso Tel: (00226) 78805242

ABSTRACT
In this study, the use of mango (*Mangifera indica*) residues as substrate for bioethanol production was investigated. The batch cultures were realized to study the nutritional requirement of the yeasts strains and to optimize the fermentation conditions. Minerals salts play an important role in fermentation process and affecting microbiological stability. In the present study, the effects of diverse salts on the fermentation profiles were studied. Method was batch fermentation process in supplemented medium, using adapted yeast strain W1 (*Saccharomyces cerevisiae*). Different supplementations, such as yeast extract, $MgSO_4$, $MnSO_4$, $FeSO_4$ and KH_2PO_4 have been proposed. Effectively with mango hydrolysate supplemented a satisfactory bioethanol concentration like 21.75 g L^{-1} was obtained.

Key words: Residues, mango, substrate, supplementation, fermentation, bioethanol

INTRODUCTION
Bioethanol produced from renewable biomass, such as sugar, starch or lignocellulosic materials, is one of the alternative energy resources, which is both renewable and environmentally linked (Mojovic *et al.*, 2010). The commercial viability of ethanol production from agricultural residues is dependent both on the availability in great quantities at low cost. The bioconversion of waste to useable energy is also a part of utilization of waste, as by burning solid fuel for heat, by fermenting plant matter to produce fuel, as ethanol, or by bacterial decomposition of organic waste to produce alcohol (Prasad *et al.*, 2009). Ethanol is a desirable fuel additive because it allows fuel to burn more cleanly and lowers green house gas emissions. It is cost-effective to blend ethanol into gasoline in view of high crude oil prices in recent years (Louime and Uckelmann, 2008). Also bioethanol can be synthesized from cellulose and hemicellulose that originates from the many sources of biomass (Cheng *et al.*, 2007a,b; Nigam, 2001). Lignocellulose is the structural component of plant biomass and can be derived from trees, grasses, cereal, paper waste etc. Both the cellulosic and hemicellulosic portions of the material (which in case of plants may comprise 60-80% of the non-sugar and starch components) can be converted to bioethanol (Beer *et al.*, 2006).

The biochemical production of alcohol from agricultural residues involves conditioning the residues by physical treatment, hydrolysis of cellulosic components to sugars and fermentation of

these to alcohol which must then be concentrated for use as fuels or chemical reagents. The real difficult is the control of fermentation process in the way to optimize alcohol rate. Most of the time the physico-chemicals parameters of medium fermentation affect the yield of bioethanol production (Somda *et al.*, 2011a). So many experiments have been realized with different feedstock, but the research of new source of biomass and fermentation control always remains to explore. So in Burkina mango residues coming from industrial area, market and site stockage can attain, 50,000 tons in year. These residues generate annually environmental pollution (Ajila *et al.*, 2007; Somda *et al.*, 2011a). Mango contains such carbohydrates such: starch, glucose, fructose, cellulose, pectins and tannins, whose glucose is the majority component (Ajila *et al.*, 2007). Of this fact the mango residues contained an important rate of carbohydrate, can constitute a biomass source and have needed to investigate for bioethanol production using yeasts (Somda *et al.*, 2011a, b).

So for bioethanol production the medium composition affects yeast performance. Yeast sugar metabolism is strongly influenced by the concentration of mineral components in growth media. Several metal ions are essential for optimal yeast growth and fermentation at millimolar concentrations (for example, Magnesium), (Birch *et al.*, 2003). The bioavailability of certain metal ions in grape must has been shown to be an important factor in governing fermentation performance by yeasts (Birch *et al.*, 2003). For example Magnesium is involved in numerous functions essential to yeast physiology, including: cell division and growth; mitochondrial structure and function; respiro-fermentative metabolism and responses to environmental stress (Birch and Walker, 2000). Premature cessation of yeast growth and alcoholic fermentation is a serious problem in bioethanol making because it produces a bioethanol with residual unfermented sugar and low alcohol content. These problems may be influence by the mineral compounds of growth media.

The present study has focused on the effect of minerals salts in fermentation capacity activation of yeast to optimize bioethanol production, using mango residues as carbohydrates sources.

MATERIAL AND METHODS

This present study entering in the project plan has been achieved to the laboratory of biotechnology in the CRSBAN (Research Center in Biological, Food and Nutritional Sciences [University of Ouagadougou, Burkina Faso]) into August to October 2010.

Screening and selection of yeast: The selection was carried out on a total of 10 yeasts strains. The strains codified as A1 to A4 were *Saccharomyces 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). And those codified W1-W2 come of the local palm wine (Bangui).

The yeast 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. These selected yeast, were adapted in medium containing mango residues hydrolyzed and minerals concentrations.

Adaptation of the yeast to the hydrolysate: Adaptation of the yeast was performed by sequentially transferring and growing cells in media containing 80% mango residues hydrolyzed; 10 glucose, 5; yeast extract, 1; $(NH_4)_2HPO_4$, 2; $(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot$(g L^{-1}), 0.5 and pH 5.0. The

sub-culturing was carried out 48 h to obtain 'adapted strains'. It was maintained on nutrient broth containing (g L^{-1}): yeast extract, 1.0; $(NH_4)_2HPO_4$, 2.0; $(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot 7H2O$, 0.5. The hydrolysis of mango residues has been achieved by enzymatic way using *Bacillus licheniformis*.

Preparation of the mango residue hydrolysate for fermentation: 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.

Mango residues was hydrolyzed at 55°C using *Bacillus licheniformis* for 5 h and the pH was adjusted to 4.5 with acetate tampon medium, with an initial solid to liquid ratio of 100 g L^{-1}, under shaking in Incubator shaker Innova™ 4000 stirred at 350 rpm. Before enzymatic hydrolysis process 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 suspension was then centrifuged to remove the supernatant residue and to extract the sugars. The performance during enzymatic hydrolysis was evaluated based on the reducing sugars production and the liquefaction yield for the substrate (peel mango). Total reducing sugars was determined colorimetrically using a Dinitrosalicylic (DNS) acid reagent (Miller, 1959).

Fermentation process for bioethanol production

Simultaneous Saccharification and Fermentation (SSF) in medium of fermentation: Six minerals salts have been choised for their effect positives on microbial growth and bioethanol production: $MgSO_4$, $MnSO_4$, $FeSO_4$, KH_2PO_4, Nitrogen source.

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). It means that in the same medium a step of enzymatic hydrolysis is achieved using *Bacillus* and followed by strain W1 yeast fermentation step. The reason was to permit the yeast strain to use directly the reducing sugar released in the medium by enzyme action under non fermentables carbohydrates as starch, tannins.

Medium of fermentation added $MgSO_4$: Different concentrations of $MgSO_4$, respectively: 0, 0.1, 0.2, 0.4, 0.6, 0.8 and 1 g L^{-1} contained in the mango residue hydrolysate have been tested.

Medium of fermentation supplement $MnSO_4$: The $MnSO_4$ of has been studied at different concentration: 0, 0.01, 0.02, 0.03, 0.04, 0.06, 0.08 and 0.1 g L^{-1}.

Medium of fermentation completed $FeSO_4$: The bioethanol production has been followed at various concentrations: 0, 0.01, 0.02, 0.03, 0.04, 0.06, 0.08 and 0.1 g L^{-1}.

Medium of fermentation containing KH_2PO_4: The effect of on rate bioethanol production has been studied at 0, 0.1, 0.2, 0.4, 0.6, 0.8 and 1 g L^{-1} of KH_2PO_4 concentration.

Medium of fermentation containing Nitrogen source: In order to improve the bioethanol production by yeast strains (w1, w2), the mango residue hydrolysate has been enriched with different concentrations of yeast extract, respectively: 0, 5, 10, 20 and 30 g L^{-1}.

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. The hydrolysate and the basal media were autoclaved separately and mixed aseptically before fermentation. Yeasts performing strains W1 and W2 were each aseptically inoculated into each flask and incubated at 37°C. Two flasks of each sample (containing mango peel) were removed after every 24 h, up to 7 days for each medium of fermentation.

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 L^{-1}). 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.

Statistical methods: All analyses were carried out in triplicate and data sets shown are the average of the sum of these sets, unless otherwise stated. Graphs were plotted with Microsoft excel.

RESULTS

Selection of performed and adapted yeasts: Among 10 yeasts coming in different biotopes, five yeasts (S3, A1, A3, W1 and W2) were performed for ethanol fermentation process. But only yeast strains W1 was able to keep its fermentation properties and stability in hydrolysate medium. Their maximum specific growth rate (μmax) varies from 0.32 to 0.35 h^{-1}.

Saccharification of mango residues: The following of the amylasic activity of *B. licheniformis* show the kinetic of reducing sugars released at the end of 5 h. The optimal middle rate of reducing sugars attains 76% (g/g). These released sugars were available to yeasts strains for alcoholic fermentation.

Profile of bioethanol production from mango residue hydrolysate by Simultaneous Saccharification and Fermentation (SSF)

Effect of $MgSO_4$, KH_2PO_4 on bioethanol kinetic production: The ethanol concentration gradually increased during the fermentation with $MgSO_4$, KH_2PO_4 concentrations

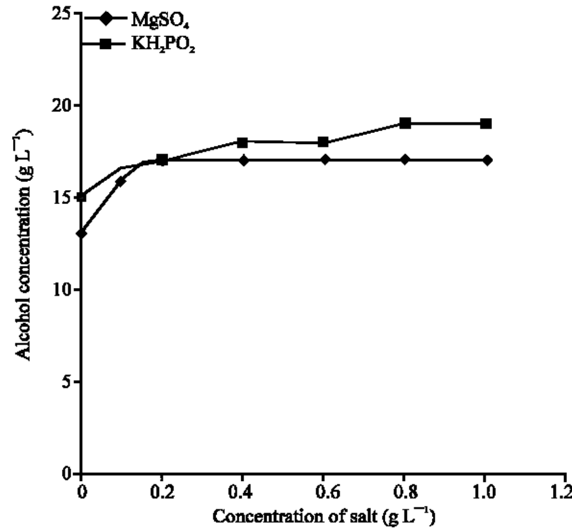

Fig. 1: Kinetic of bioethanol production in medium containing $MgSO_4$ or KH_2PO_4

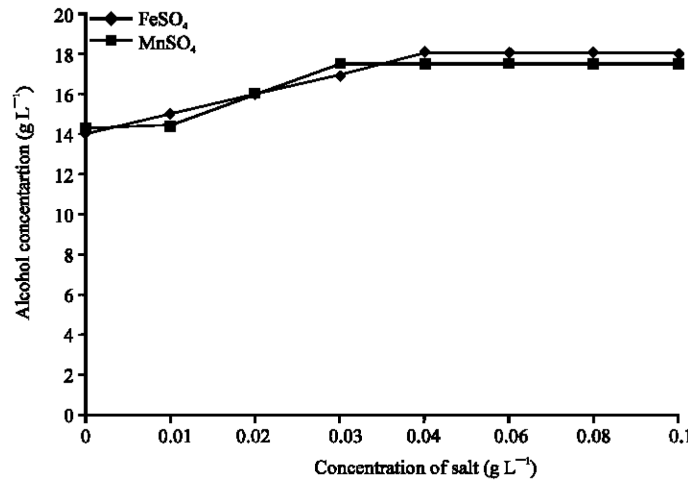

Fig. 2: Kinetic of bioethanol production in medium containing $FeSO_4$ or $MnSO_4$

gradient, respectively. The maximal values of ethanol concentration after 7 days were attained respectively 17 g L^{-1} for $MgSO_4$ and 19 g L^{-1} for KH_2PO_4. The profile of ethanol released in the medium was mentioned in Fig. 1.

Effect of $MnSO_4$, $FeSO_4$ on bioethanol kinetic production: The profile of bioethanol production on medium containing separately $MnSO_4$ and $FeSO_4$ has shown in the Fig. 2. In presence of $MnSO_4$, alcohol rate increased and ranged at 14.2 to 17.5 g L^{-1}. Also $FeSO_4$ bioethanol rate was attained 18 g L^{-1} after 7 days.

Effect of nitrogen source on bioethanol kinetic production: It was tested the influence of nitrogen source on cell growth and the bioethanol yield. The concentration of bioethanol increased concomittly with nitrogen source in fermentation medium and to be stabilised at 21.75 g L^{-1} during 7 days. The kinetic of bioethanol production has shown in Fig. 3.

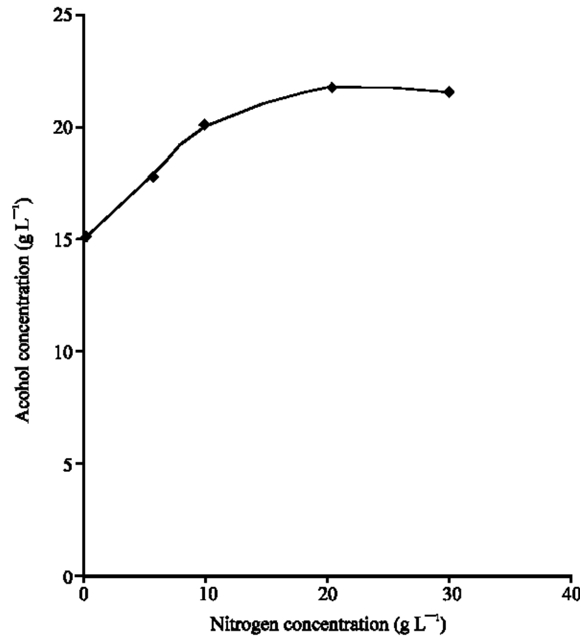

Fig. 3: Kinetic of bioethanol production in medium containing Nitrogen

DISCUSSION

Selection of performed and adapted yeasts: The yeast strain W1 was retained among other according to it specific capacity to growth on mango residue hydrolysate. So it has been selected for his possibility to adapt in hydrolysate medium which will be used for fermentation process. The maximum specific growth rate (μmax) of yeast strain W1 attained 0.35 h^{-1} is acceptable for his using to alcoholic fermentation. Compared to some data as those of Ezeronye (2004) on the starter yeasts, our strain W1 has a good growth capacity and is located in the interval determines by this author (0.18 to 0.22).

Saccharification of mango residues: Saccharification using *B. licheniformis* permit to obtain 76% of reducing sugars. It is approximative with values found by Somda *et al.* (2011b). Prasad *et al.* (2009) 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. 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.

Also same phenomenon of repression catabolic has been observed at *Clostridium thermohydrosulfuricum* and *Bacillus* sp. (Kiran *et al.*, 2005). So the reducing sugars released in the medium could be used by yeast W1 for fermentation alcohol.

Effect of salts $MnSO_4$, $FeSO_4$, $MgSO_4$, KH_2PO_4 on bioethanol kinetic production: The effect of salts was studied separately of interest from the medium formulation. Minerals salts at all concentrations tested were showed to give a significant effect on bioethanol production of yeast

strain W1. Figure 1 and 2 have showed the effect of different salts on the fermentations of yeasts using mango residues sugars.

Bioethanol achieved after 7 days of cultivation for salts cultures was range of 13 to 19 g L^{-1}. This value is superior to which reported by Somda *et al.* (2011b) using uncontrolled concentration salts in medium of fermentation. They found 16 g L^{-1} of bioethanol produced with mango residue hydrolysate. The reason of this value superior can be explain to the medium composition in minerals. It activates the membrane permeability and increase the intra-cellular glucose uptake. So our results are below compared to those found by Birch *et al.* (2003). They found 50 g L^{-1} of ethanol with a ratio 0.5 L^{-1} of magnesium and calcium in fermentation medium. And yet they explained that elevation of magnesium levels resulted in decreased yeast doubling times, together with increased ethanol yields and faster rates of sugar consumption. However, very high magnesium/calcium ratios had a deleterious effect on fermentation performance, indicating inhibitory effects on yeast sugar consumption and ethanol production.

The osmotic properties of yeast cell are due to selective permeability of the cell wall with regard to solutions. This selectivity plays an important role in controlling the movement of nutrients into a cell. The permeability of the cell wall also permits the release of alcohol and carbon dioxide from the cell during fermentation.

Therefore, it was concluded that Mn^{2+}, K^{+}, Mg^{2+}, Fe^{2+} were the ions that Yeast needed to alcohol fermentation process. Fitzpatrick *et al.* (2001) have showed the beneficial effect of Mn^{2+} for metabolites production by *Lactobacillus casei*, due to its role as a constituent of lactate dehydrogenase. Jin-Bong *et al.* (1990), Gawande *et al.* (1998) and Owades (1991) have also found Mg^{2+} to be essential for enzyme production and consequently alcohol released. Mori *et al.* (1985) showed that Magnesium acts as activator of some enzymes (phosphatidyl transferase and decarboxylase). Ion K^{+} is indispensable for the growth and Fe^{2+} stimulates the breathing and the cellular multiplication (Shockey and Barta, 1991).

These different authors confirm the importance of the minerals salts on the microbial metabolism. It comes to reinforce our results finding.

Effect of Nitrogen source on bioethanol kinetic production: The kinetic of bioethanol production with nitrogen positive influence within 7 days is reported in Fig. 3. So the optimal for nitrogen concentration, created bioethanol increasing range from 17 to 21.75 g L^{-1}, also it was indicated that increasing concentration of this component is less critical for enzyme (alcohol dehydrogenase) production.

Then the maximal bioethanol rate found in this work (21.75 g L^{-1}) with using nitrogen source in medium is very higher than result found by (Somda *et al.*, 2011b) in their previous research (16 g L^{-1}), also those found by Humphrey and Okafoagu (2007) like 11.2 g L^{-1} at 96 h and 12.9 g L^{-1} at 216 h. Otherwise our alcohol maximal value is lower than these reported by Tasic *et al.* (2008). They have conducted fermentation on potato tuber mash and found during 18h to 33 h of incubation, an ethanol concentration growing at 31.2 to 32.9 g L^{-1}.

During fermentation, nitrogen is taken up from the medium by the cell and directly incorporated into proteins or transformed into other cellular nitrogenous constituents (Ferchichi *et al.*, 2005). By contrast, the cell spends more energy and time in synthesizing amino acids for protein synthesis from inorganic nitrogen sources (Ferchichi *et al.*, 2005; Kalil *et al.*, 2008).

Among organic nitrogen sources, differences in protein and amino acid composition could have accounted for the differences in the production rates and yields observed. Kalil *et al.* (2008) have

showed that microflora requires a proper nitrogen supplement for metabolism during fermentation and suggested 70% the C/N ration. They found that yeast extract nitrogen source at concentration of 13 g L^{-1} was the best organic source and resulted in 85% metabolites yield of 308 mL g^{-1} glucose utilized.

While referring to our results obtained in fermentation process, it can be noticed that high concentrations of sugars, salts and other solubles inhibit yeast fermentation as a result of effects produced by high osmotic pressures.

Basically, all fermentable sugars begin to exert an inhibiting effect on yeast when their concentration exceeds about 100 g L^{-1}, with the degree of inhibition becoming progressively greater as the concentration of the sugar rises (Konlani *et al.*, 1996; Somda *et al.*, 2011a).

This inhibitory effect is more pronounced with such sugars as sucrose, glucose and fructose than with maltose. The sensitivity of yeast to osmotic pressure varies with different yeast strains, with some being better suited than others for fermenting sweet doughs with their high sugar contents. Some mineral salts in excess and are capable to inhibit yeast growth (Rose, 1987).

D'Amarc and Stewart (1987) and Xu *et al.* (1996) have showed that 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. Finally it can be retained that the presence of minerals salts at the adapted concentration increased rates of ethanol production, reduced fermentation times and faster yeast growth rate.

CONCLUSIONS

The results obtained from the tests of fermentation on minerals medium has showed that the yeast strain W1 can produce until 13 g L^{-1} of bioethanol on mango residue hydrolysate broth containing 76% of reducing sugars. And yet the maximal production like 21.75 g L^{-1} of bioethanol has been reached after optimization of the chemical condition.

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