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BIOETHANOL PRODUCTION FROM TWO VARIETIES OF MANGO KERNEL USING CO-CULTURE OF *Bacillus subtilis*, *Aspergillus niger* and *Saccharomyces cerevisiea*

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ABSTRACT
Production of bioethanol from solid waste such as mango kernel using microbial culture is an attractive option for waste management and substitute for fossil fuel. This study was carried out to produce ethanol from two varieties of mango kernel using cultures of Aspergillus niger, Bacillus subtilis and Sacchromyces cerevisiae isolated from soil, fermented African locust bean (daddawa), palm wine respectively. The isolates were identified and confirmed using phenotypic identification techniques. The proximate composition of the mango kernels were evaluated using AOAC (2012) methods. The mango kernels ground into powder were subjected to enzymatic hydrolysis using co-culture of B. subtilis and A. niger. Analysis of reducing sugar residue was performed using Dinitrosalicyclic acid (DNS). Fermentation of the hydrolysates was carried out for 96 hours and the ethanol produced was quantified. The optimum conditions for ethanol production such as pH, temperature, substrate concentration and time of fermentation were determined. The proximate analysis revealed carbohydrate contents of 50.79% and 52.54% in Banginapalli and Benue mangoes, respectively. There is no significant difference (p>0.05) in the proximate composition of the mango kernels. The enzymatic hydrolysis revealed the reducing sugar content of 5.36g/L in Benue mango and 5.34g/L in Banginapalli mango after 5 days of hydrolysis. The fermentation of the hydrolysate of Benue mango yielded the highest quantity of ethanol (19.15%) with a fermentation efficiency of 37.47% after 72 hours fermentation. The results of optimization revealed that pH 4.5, temperature of 30°C, 30g substrate concentrations and fermentation period of 72 hours were the optimum condition for production of the bioethanol. The result of this work has shown that the two varieties of mango could serve as good raw materials in the production of bioethanol, thereby turning the mango waste to potential wealth.
Keywords: Bioethanol, Fermentation, Substrate, Waste.

INTRODUCTION

The diminishing fossil fuel reserves and increased concerns over environmental pollution accelerated the need to look for renewable and environmentally sustainable energy sources. In this context, bioethanol derived from biomass is means to meet our energy needs. Bioethanol is a sustainable and renewable transportation fuel that is a promising substitute to gasoline and represents an environment-friendly fuel because it reduces the amount of greenhouse gas emissions, which is a major cause of global warming (Tripti *et al.*, 2013). The development of alternative fuel and energy from biomass has therefore, resurfaced as a research priority in recent years. It is expected that demand for fuel ethanol will rise from current estimates of 4 billion to 22.7 billion gasoline-equivalent gallons (or 20% market share) by 2020 (Biomass Research Development Initiative BRDI, 2006). The future risks of global warming and shortage of petroleum, as well as the superior environmental characteristics of ethanol as an oxygenated additive to gasoline that improves the knocking resistance of gasoline, promote the production and usage of bioethanol in the fuel market (Millati *et al.*, 2005). Bioethanol is fermentation alcohol, which refers to ethyl alcohol produced by microbial fermentation processes as opposed to synthetically produced ethanol from petrochemical sources. It is produced through distillation of fermented biomass. In general bioethanol can be extracted from every sort of carbohydrate material. The main cost element in bioethanol production is the feedstock. These can be divided into three main groups: sugary, starchy and lignocellulosic biomass.

First generation feedstocks for bioethanol production primarily refer to plant biomass sources that are also sources of human and animal nutrition, namely cereal starches and sugar crops. Sucrose based materials are predominantly derived from sugarcane and sugar beet; while starch based materials are predominantly derived from cereal crops such as maize, wheat and other cereals. Second generation raw materials for bioethanol production typically refer to non-food biomass sources, mainly lignocellulosic biomass.

Mango is ranked as the third most important tropical fruit crop after banana and plantain (FAO, 2011) with its production estimated at 39 million tonnes in 2010 (Jedele *et al*., 2003; FAO, 2011). Taking mango seed as 20 to 60% of the whole fruit, and the kernel as 45 to 75% of the seed (Maisuthisakul and Gordon, 2009), this represents between 4 and 18 million tonnes of mango kernel available per year. Due to its abundance and its limited use as a by-product in most parts of the world, there is economic advantage in investigating the use of mango kernel for bioethanol production since it has been reported to be a good source of carbohydrates (El Saadany *et al*., 1980; Jansman *et al*., 1995; Teguia, 1995; Anand and Maini, 1997; Diarra and Usman, 2008; Diarra *et al*., 2010; 2011). Mango kernel, on a dry basis, contains 65% starch, 2.9% reducing sugars, 5.7% proteins, 0.8% pectin, 9.3% fat and 1.1% tannins and rest is moisture (Garg, and Tandon, 1997). The present study was set up with aim of using mango kernel as a substrate for bioethanol production thereby turning the mango waste to potential wealth.

MATERIAL AND METHODS

Samples Collection

Two varieties of mango sample was collected from Na'ibawa fruit market, Kumbotso local government, Kano state Nigeria. Sample mango was packet in poly-ethene bag and was taken to laboratory for analysis. Soil samples were collected from Botanical Garden of Bayero University, Kano, using sterile spatula and sterile plastic bag. Freshly produced commercial *Dadddawa* was purchased from Kurmi Market, Kano and Fresh palm wine was purchased from Sabon Gari area of Kano state Nigeria.

Samples Processing

The mango seeds were air-dried by spreading on sacks in an aerated room. The outer shell was decorticated and kernels were washed thoroughly with the tap water, air dried under fan, and finally dried at 60^{o}C in oven (Kaushlesh *et al*., 2016). This was then ground into powdered form using pestle and mortar in the laboratory, then sieved and packed in a clean, sterile container as described by Farida *et al*. (2017).

Proximate Analysis of Mango Kernels

The proximate composition of the mango kernels (moisture, ash, fat protein, fibre and carbohydrate content) was determined using AOAC (2012) method

Isolation and Identification of Microorganisms used

Yeast

Yeast was isolated by serial dilution technique on PDA Medium (Cheesbrugh 2005) and all the inoculated plates were incubated at 28˚C for 48 hours. The isolates were purified by repeated subculturing and preserved in slants of the same medium and stored at 4˚C

Carbohydrate (sugar) Fermentation Using API 20C AUX

An ampoule of API suspension was opened and isolated colony was scooped with inoculating loop aseptically and transferred into the ampoule, mixed properly until the suspension turbidity was equal to McFarland standard.

An ampoule at API 20^{o}C medium was opened and approximately aliquot of 100µl was transferred into the C medium and was gently homogenized. The inoculation box (tray and lid) was prepared and 5ml of sterile distilled water was added into the honey combed wells while the strips 0 – 29 were placed in the box. The microtube (cupule) of the strips was filled with the yeast suspension obtained in the ampoule of API C medium with the inoculum level. The lid was placed onto the box and the preparation was incubated at 29^{o}C for 48hours. After 48 hours of incubation, the strips were read and the results were recorded. The isolates were confirmed using API web software.

Aspergillus niger

Aspergillus niger were isolated from soil sample by serial dilution technique on PDA Medium (Cheesbrugh 2005) and all the inoculated plates were incubated at 28˚C for 48 hours. The isolates were purified by repeated subculturing and preserved in slants of the same medium and stored at room temperature. Morphological appearance and microscope observations were made for the pattern of conidiation and hyphal branching of the pure fungal isolate were identified using standard reference manuals and appreciate publication by wet amount preparation (Frazier and Westhoff, 1995; Gams and Bissette 1998; Dubey and Maheshwari, 2004 McClenny, 2005).

Bacillus subtilis

Fermented African locust bean (*daddawa*) were prepared by homogenizing 25ml of each sample in 225 ml of sterile 0.1% peptone water. The homogenates were then subjected to 10 fold serial dilutions in sterile 0.1% peptone water. From these 10 fold dilutions, 1 ml of dilutions 10^{-1}, 10^{-3}, and 10^{-5} was each plated on sterile plates of Mannitol Egg yolk Polymyxin B Agar (MYP) (OXOID). MYP plates of the food samples were incubated at 37°C for 24-48 hours. The isolates were purified by subculturing and preserved in slants of Nutrient Agar Medium and stored at 4°C. Isolated bacteria were preliminary characterized on the basis of colonial morphological and biochemical characteristics (Cheesbrugh 2005).

Standardization of the Inoculum

Fungal cultures were inoculated onto potato dextrose agar (PDA) medium in the Petri dish, after 72 hours, the spores were harvested using sterilized water with 0.1% Tween 80 (Ajeet *et al*., 2014). Spore count was measured with haemocytometer (Zakpaa *et al*., 2009). A loopful of the overnight broth culture of bacteria was diluted in 4ml of sterile physiological saline (0.8w/v), such that its turbidity matches that of 0.5 McFarland standard i.e. 1.5 x 10^8 cfu/ml (Cheesbrough, 2005).

Microbial Hydrolysis of the mango kernels

Microbial hydrolysis was carried out according to the method described by Gupta *et al*. (2009). Five hundred milliliter capacity conical flasks were used for the enzyme hydrolysis of the substrate. The conical flask were labeled BA and BE (BA= Banginapalli mango, BE=Benue Mango) and 30g of each mango sample was put in the flask and 100ml of distilled water added. The flasks were plugged with cotton wool and aluminium foil and then sterilized at 121^{0}C for 30min. Each flask was inoculated with 0.5ml suspension each of the standardized inocula (co-culture of *B. subtilis* and *A. niger*) and uninoculated flasks were used as control. The flasks were incubated at 37^{0}C for 5 days with frequent shaking to provide aeration and agitation. After the 5 days, the samples were filtered through Whatman filter paper No-1. All the experiments were done in triplicate.

Determination of Reducing Sugar

The reducing sugar content of the hydrolyzed substrate was determined using DNS method of Miller (1959) with glucose as standard. It was assayed by adding 3ml of 3, 5 – Dinitrosalicylic acid (DNS) reagents to 3ml of the sample. The mixture was heated in boiling water for 10min to develop the red – brown colour. Then 1ml of 40% potassium tartarate solution was added to stabilize the colour and cool to room temperature under running tap water. The absorbance of the sample was measured at 491nm using ultraviolet (uv – vis) spectrophotometer. The reducing sugar content was subsequently determined by making reference to a standard curve of glucose concentrations.

Fermentation of Hydrolysates

One hundred milliliters of the hydrolyzed sample was added into 500ml capacity conical flask. The flasks were covered with cotton wool wrap in aluminium foil and autoclaved at 121^{o}C for 15min. The flasks were allowed to cool and 10 ml of the inoculum culture of yeast containing 1.5x10^8 cells/ml cell of *Saccharomyces cerevisiae* was added (Zakpaa *et al.*, 2009). The pH of the fermentation medium was adjusted to 5.5. All the flasks were incubated at 35^{o}C for 4 days according to the procedure of Iruolaje *et al*. (2015). The hydrolysates were then distillated according to standard method.

Optimization of Experimental Conditions

The fermentation of the hydrolysates was carried out under different conditions of fermentation time (24, 48, 72 and 96hours), pH (4.5, 5.0 and 5.4), temperature (30, 35 and 40^{o}C) to determine the optimum condition for maximum ethanol yield via the one factor at a time technique.

Estimation of Bioethanol Production

The percentage bioethanol yield was calculated based on the ethanol produced (g/L) from the amount of fermentable sugar (g/L) x 100, while the fermentation efficiency was calculated using the following formula below (Sharma *et al.*, 2004; Hamed *et al*. 2015).

$$\text{FE}\ (\%) = \frac{\text{Ethanol yield obtained}}{\text{Theoretical maximum yield from sugar}}\ x\ 100$$

Statistical Analysis

The data generated were subjected to statistical analysis using One-Way Analysis of Variance (ANOVA) and significant difference was considered when $p>0.05$.

RESULTS AND DISCUSSION

The successful isolation and identification of *Saccharomyces cerevisiae* from the palm wine using morphological characteristics and its ability to ferment glucose, sucrose and lactose (Table 1) is in line with the findings of Chilaka *et al*. (2010) who reported the isolation of *Saccharomyces cerevisiae* and other yeasts from palm wine.

Aspergillus niger colonies consist of a compact white to yellow basal felt covered by a dense layer of dark-brown to black conidial heads. Conidial head were large, globose, dark brown, becoming radiate and tending to spilt into several loose columns with age.

Conidiophores were smooth-walled, hyaline or turning dark towards the vesicle. Conidial head were biseriate with the phialides borne on brown, often septate. Conidia are globose to subglobose, dark to black and rough-welled. The *Bacillus subtilis* isolated from *daddawa* is shown in Table 2. The isolate was gram positive, rod, catalase positive and spore formers with irregular shape. These characteristic allowed preliminary identification of Bacillus (Harrigan and MacCance, 1976 ; Harrigan, 1998).

The proximate composition of Benue and Banganipalli mango kernel is shown in Table 3. The highest carbohydrate content of 56.54% was observed in Benue mango and the lowest was in Banganipalli mango (50.79%).This is in line with the result of Kittiphoom (2012) who reported carbohydrate content of 48.19% in Alphonso mango kernels. This result is also in agreement with the result of Manisha and Sikdar (2015) who reported that the proximate composition of Mango kernels with Carbohydrate content of 59.06% in Sindhori and 48.42% in Banginapalli mango. This result is also in line with the literature finding by Kaushlesh *et al* (2016) who reported the carbohydrate content of 69.22% in Totapuri mango kernels.

The reducing sugar yield from the Benue and Banginapalli mango kernels by enzymatic hydrolysis are shown in Table 4. A maximum yield of 5.36gm-L of reducing sugar was obstained from Benue mango and Banginapalli mango produced the yield 5.34gm-L after 120 hours of incubation. The result was compared with those obtained with non inoculated medium (non-inoculated control) incubated in the respective media (0.18gm-L). Farida *el al*. (2017) reported maximum glucose production (2.81gm-L) from the highest substrate concentration (15g) after 120 hours of incubation. Singh *et al*. (1984) has reported that the fermentation of enzymatic hydrolysate showed better fermentation efficiencies in comparison to acid hydrolysate of agricultural residue.

The fermentation studies showed a maximum ethanol production of 17.32% with fermentation efficient of 33.58% and minimum yield of 9.22% in Benue mango (Figure 1). The amount of bioethanol increased with increase in time of fermentation. This might be attributed to the increase in the number of the microbial cells that utilize the available substrate to produce bioethanol as waste product. The production began to decrease after the maximum concentration was reached. The decline in the ethanol production beyond 72 hours of incubation in hyrodrolysate might be probably due to decrease in substrate concentration or due to decrease in the number of viable yeast cells or because of the denaturation of enzyme by the ethanol produced during fermentation. The fermentation was essentially completed in 96 hours with maximum yield of 17.32% in Benue and16.85% in Banginapalli mango kernels respectively. A maximum concentration of ethanol from Totapuri mango kernels (9.14%) was reported by Kaushlesh *et al.*, (2016) using yeast fermentation. It is comparably low with the current findings on ethanol production from Banginapalli and Benue mango kernels. This result is in line with the result of Srichuwang *et al*. (2009) who reported a maximum ethanol yield of 16.61% (v/v) in bench scalle SSF process carried out under the optimal condition in 61 hours from potato mash.

The optimum pH for ethanol production was found to be 4.5 at which the highest production (18.15%) of ethanol in Benue mango was recorded (Figure 2). The ethanol concentration decrease with increased in pH the decrease in ethanol concentration could be due to lesser enzyme activity at pH above the optimum. This is in line with the report of Roukas (1994) who studied the effect of pH on ethanol production from carob pod by *S. cerevisiea* and found that the maximum ethanol yield was obtained at pH of 4.5.

The maximum ethanol yield of 19.15% in Benue Mango was produced at temperature of 30^{o}C. The ethanol yield decreased with the increase in temperature from 30 to 40^{o}C (Figure 2). However, temperatures beyond 30^{o}C showed a fall in ethanol production which is in line with the findings of El-Refai *et al*. (1992) who reported maximum ethanol productivity from beet molasses by *S. cerevisiae* Y-7 after 72 hours of incubation at 30°C. Verma *et al*. (2000) also reported 30°C as the optimum temperature for maximum ethanol production using starch employing co-culture of amylolytic yeast and *S. cerevisiae*. Thus, optimum temperature for fermentation of mango kernels was found to be 30°C. At temperatures higher than optimum, less ethanol production was observed. Decline in ethanol yield at increased temperature might be due the inactivation of enzymes involved in ethanol production pathways (Sharma *et al.*, 2008). These observations are consistent with findings of Sanchez *et al*. (2004), El-Abyad *et al*. (1992), Farida *et al*. (2017).

Table 1: The Sugar Fermentation Pattern of the Yeast Isolate (API 20 CHL).

S/N		
1.	Glucose	+
2.	Glycerol	-
3	Calcium 2-keto-gluconate	-
4	Arabinose	-
5	Xylose	-
6	Adonitol	-
7	Xylitol	-
8	Galactose	+
9	Inositol	-
10	Sorbitol	-
11	Methyl D-glucopyranoside	+
12	N-acetyl-glucosamine	-
13	Cellobiose	-
14	Lactose	-
15	Maltose	+
16	Saccharose	+
17	Trehalose	+
18	Melezitose	+
19	Raffinose	+
20	Identified isolate using API web	*S.cerevisiae*

Key: + = positive, - = negative.

Table 2: Morphological and Biochemical Characteristics of *Bicillus subtilis* isolated from *Daddawa*.

Morphological, physiochemical parameters and biochemical tests of isolates	
Cell morphology	Cream colored, gram positive, central spore forming rods.
Cell colony	Short rods, sparse colonies
Gram's reaction	+
Endospore test	+
Catalase test	+
Motility test	+
Citrate utilization test	+
Methyl red test	-
Voges proskeur test	+
Starch hydrolysis	+
Oxidase test	-
Indole test	-
Eggyolk reaction test	+
Identity of organism	*Bacillus subtilis*

Key:
+ = Positive
- = Negative

Table 3: Proximate Composition of the Two Varieties of Mango Kernel.

Parameter (%)	**Banginapalli Mango**	**Benue Mango**	**p-value**
Moisture	10.30±0.03	10.30±0.00	NS (P>0.05)
Ash	1.80±0.06	1.81±0.03	NS (P>0.05)
Protein	14.93±0.05	13.93±0.03	NS (P>0.05)
Fiber	1.44±0.08	1.42±0.05	NS (P>0.05)
Carbohydrate	50.79±0.22	52.54±0.08	NS (P>0.05)
Fat	20.97±0.03	19.97±0.00	NS (P>0.05)

Each value represents mean of three independent test ± standard error. NS- not significant

Table 4: Reducing Sugar Concentration (g/L) after hydrolysis of Mango Kernel using *B. subtilis* and *A. niger*.

Sample	Substrate (g)	Amount of Reducing Sugar (g/L)
Banginoppali Mango	30	5.34
Benue Mango	30	5.36
Control (Uninoclated Mango Kernel)	30	0.18

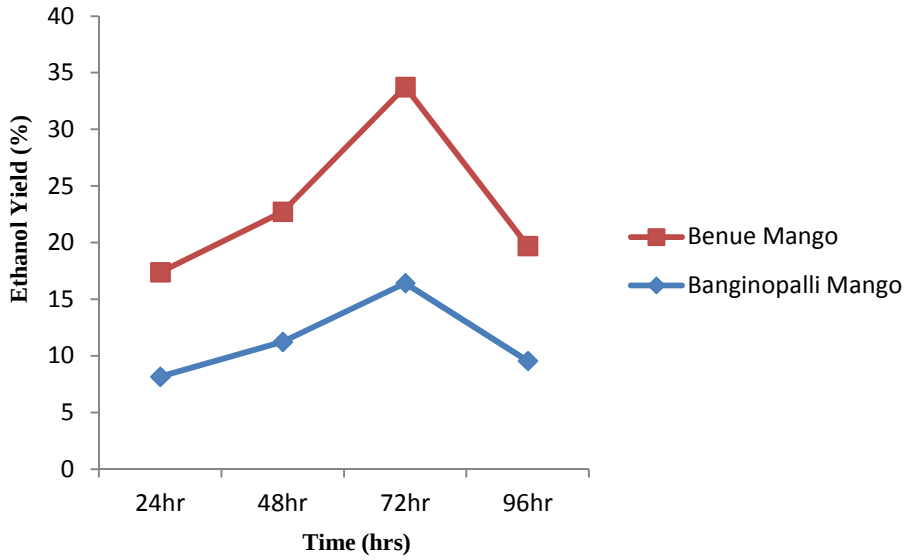

Figure 1: Percentage (%) Ethanol Produced from Hydrolysate of Mango Kernel at Varying Fermentation Time at Temperature of 35°C, pH of 5.5 and 30g of substrate

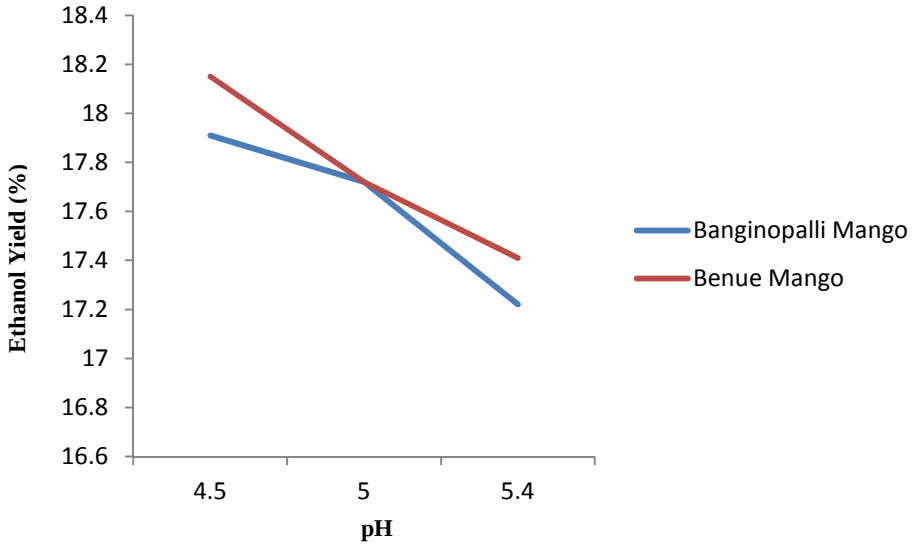

Figure 2: Effect of pH on Percentage (%) Ethanol Production from Hydrolysate of Mango Kernel at 72 hrs and 30g of the Substrate

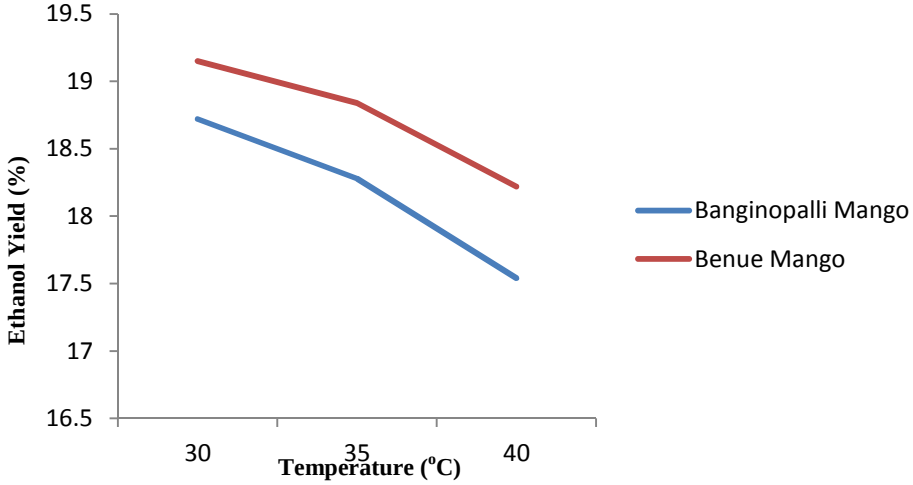

Figure 3: Effect of Temperature on Percentage (%) Ethanol Production from Hydrolysate of Mango Kernel at 72 hrs and 30g of the Substrate

CONCLUSION

The study revealed that palm wine is a good medium for growth of *S. cerevisiae*. The mango kernels were found to have a substantial amount of carbohydrate content of 52.54%. The results also revealed that Benue mango kernel produced the highest concentration of reducing sugar (5.36g/L) and subsequently produced the highest bioethanol yield of 19.15% with fermentation efficiency of 37.47%. Fermentation of mango kernel using *S. cerevisiae* under optimized condition of pH 4.5, temperature of 30^0C, sugar concentration (5.36g/l) and fermentation period of 72 hours produced the maximum yield of bioethanol.

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