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Citric Acid Production from Cellulase-digested Palm Oil Mill Effluent

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ABSTRACT

Palm Oil Mill Effluent (POME) cause serious pollutions of soil, water and environment but could be exploited as substrate for microbial citric acid production because of its rich mineral and high carbohydrate contents. Therefore in this study, *Aspergillus niger* ATCC 9642 was grown in the different concentrations (25, 50, 75 and 100%) of cellulase-digested POME for 7 days. Result showed that citric acid was highest (0.78±0.02 g L^{-1}) in the 75% POME but lower (0.32±0.04 g L^{-1}) in the undiluted POME. When supplemented with methanol (3%), citric acid production increased (1.02±0.03 g L^{-1}) only in the 100% POME. Citric acid was not detected in the 25% and the non-digested POME. Biomass measurement showed that the 100% POME gave the highest (10.2±0.6 g L^{-1}) mycelial weight among the digested treatments. As the dilution increased, the biomass concentration decreased proportionally. Biosolids from the non-digested POME supplemented with glucose (1%) weighed 13.4±0.15 g L^{-1}. The Chemical Oxygen Demand (COD) of the digested POME (100%) dropped by 47.4% but the presence of methanol did not significantly affect the COD kinetics. However, at higher POME dilutions, COD reduction rate was greater than 63% but in the non-digested batches, COD reduction was negligible. The sugar utilization patterns showed that glucose was completely used up in the different POME dilutions except the undigested and glucose (1%) supplemented batches which had 75 and 40% sugar uptake, respectively. The use of POME hydrolysates as substrate could lower the present cost of citric acid as well as save treatment costs and help solve environmental problems.

Key words: Palm oil mill effluent, citric acid, hydrolysis, enzymatic, *Aspergillus niger*

INTRODUCTION

Citric acid is a valuable microbial product that is mainly produced by submerged fermentation involving *Aspergillus niger* (Kumar *et al.*, 2003). It is used extensively as food and drink additives, preservatives, in hair drying, explosives, industrial pipe cleaning and photography. In 2004 alone, global production of citric acid was about 1.4 million metric tonnes (Soccol *et al.*, 2006). Like other bulk chemicals, the cost of substrate (raw materials) represents high percentage of citric acid total production cost. The growing global demand for this product presently underscores the need to examine other inexpensive and available raw materials and industrial by-products as alternative substrates for its production. In this regard, the use of corn cobs, sugarcane bagasse, beet molasses (Wang, 1998; Hamissa and Radwan, 1977; Abdullah-Al-Mahin *et al.*, 2012) and palm oil mill effluent (Jamal *et al.*, 2005) has been investigated for citric acid production.

POME is high strength organic waste slurry that is acidic, brownish and colloidal. It is the final liquid discharge after extracting palm oil from the fresh fruit bunch (Bek-Nielsen *et al.,* 1999). If discharged untreated into water courses, POME causes considerable environmental problems due to its high Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) values (Ma, 1995). Ponding is the most popular treatment method for POME due to its low capital cost (Tong and Bakar Jaafar, 2004), but ponding under anaerobic condition emits methane, a green house gas that is 21 times more damaging to the environment than carbon dioxide (Lam and Lee, 2011). Presently, owing to increasing amount of POME generated annually, the 5R concept of reduction, replacement, re-use, recovery and recycling (Wu *et al.,* 2009) is now advocated as a means of controlling the adverse effects of POME on the environment. POME contains significant amounts of carbohydrates, proteins, lipids, nitrogenous compounds and minerals (Habib *et al.*, 1997) and so can be exploited in the production of biomass as well as other valuable products such as citric acid.

Factors affecting the production of citric acid from POME include nutritional composition of POME, available carbon sources as well as the types and concentrations of sugars present (Alam *et al.,* 2008). Since, citric acid is produced principally by submerged fermentation of glucose or sucrose (Dasgupta *et al.,* 1994; Vandenberghe *et al.,* 1999), the absence of these major components in POME imposes severe limitations to the use of raw POME as substrate for citric acid production. Presently, this problem is solved by the direct addition of glucose, sucrose (El-Holi and Al-Delaimy, 2003; Kim *et al.,* 2006; Alam *et al.,* 2008) and/or crude natural products such as wheat flour (Jamal *et al.*, 2005).

On a large scale, the cost of these additives will significantly add to the final cost of production. POME contains 95-96% water, 0.6-0.7% oil, 4-5% total solids and 2-4% suspended solids (Ahmad *et al.,* 2003). The solids are made up of plant fibres which contain mainly cellulose (Bek-Nielsen *et al.,* 1999). Besides POME, the plant fibres are a major by-product of palm oil refining and due to their accumulation in the vicinity of processing mills; they often constitute public health hazards by promoting the breeding of fleas, rodents and other disease-bearing vectors. However, it can be harnessed by enzymatic degradation to release sugars which can be converted to various useful metabolites. In this study, the potential of using cellulase-digested POME as substrate for citric acid production was investigated.

MATERIALS AND METHODS

Palm oil mill effluent (POME): The POME used in this study was obtained from a local palm oil processing mill at Nsukka, Enugu State-Nigeria and stored at 4°C until use.

Microorganism and culture conditions: *Aspergillus niger* ATCC 9642 was obtained from the stock collection of the Department of Microbiology, University of Nigeria, Nsukka. The fungus was reactivated by streaking a loopful on Petri-dishes containing Potato Dextrose Agar (PDA) before incubating at 28±2°C for 5 days. Thereafter, culture was maintained at 4°C on PDA slants and sub cultured monthly. Mature spores for inoculation were harvested by gently washing the surface of 5 day old subculture with 5 mL of sterile distilled water containing 0.1% Triton X-100. The spore suspension was subsequently centrifuged at 4000 rpm for 10 min, washed and re-suspended in 5 mL distilled water. The resulting spore concentration was then adjusted to 1.5×10^7 spores mL^{-1} using a Neubeur haemacytometer.

Pre-treatment of POME by enzymatic digestion: POME was subjected to batch-wise enzymatic treatment in a temperature controlled water-bath fitted with a magnetic stirrer. A commercial cellulase preparation provided by Yakult Honsha Co., Ltd., Japan was used at a protein concentration of 0.3 mg mL^{-1} and specific activity of 1.82 (0.1684 IU) in filter paper assay according to the method described by Mandels *et al.* (1976). Hydrolysis was carried out for 24 h at 50°C and pH 4. Subsequently, the hydrolysates were transferred into a 500 mL capacity separatory funnel and left standing for 30 min to allow the oil-water interphases to separate out. The aqueous phase of the digest was then drained into a clean dried flask before both the digested and non-digested POME batches were supplemented with Prescott salt (NH_4NO_3, 2.23 g L^{-1}; K_2HPO_4, 1.00 g L^{-1} and $MgSO_4$, 0.23 g L^{-1}). Prior to inoculation and fermentation, enzymatic reaction was terminated by boiling for 10 min before sterilization at 121°C, 15 min and 15 psi.

Effect of POME concentration on citric acid production: The effect of POME concentration on citric acid production was investigated by diluting the POME with distilled water to 25, 50 or 75%. About 10 mL of the standardized spore suspension was inoculated into a 500 mL Erlenmeyer flask containing 90 mL of the diluted or undiluted POME. Incubation was carried out at 30°C for 7 days on a rotary shaker maintained at 210 rpm. Samples taken periodically were passed through Whatman No. 1 filter paper to remove mycelial fragments and medium components. The filtrates were evaluated for citric acid, COD and glucose.

Analytical methods: Citric acid concentrations were determined spectrophotometrically at 420 nm by the pyridine-acetic anhydride method (Marier and Boulet, 1958). pH, Oil/Grease (O and G) and Total Solids (TS) were determined according to Standard Methods (APHA, AWWA and WPCF, 2005). Chemical Oxygen Demand (COD) was measured by the Hach's Spectrophotometic method (DR/4000, Hach Co., Ltd., Tokyo). Reducing sugar (i.e., glucose) was monitored using Glucose C_2 Kit (Wako Pure Chemical Ind., Osaka). Total carbohydrate was analyzed by the Phenol-Sulphuric acid method (DuBois *et al.,* 1956) while the elemental composition of POME was estimated in a 10 mL filtrate using plasma emission spectrometer (Shimadzu ICPS-8100, Perkin Elmer Optima 7300 DV). Mycelial dry weight was determined by filtering the content of each Erlenmeyer, washing with distilled water and drying at 105°C to constant weight.

Statistical analysis: The presented data are means and standard deviations of triplicate determinations. Where appropriate, results were statistically analyzed by the two-way analysis of variance (ANOVA).

RESULTS

Effect of different treatments on citric acid production: The effects of cellulase-digested POME concentrations on citric acid production are shown in Table 1. The maximum concentration of citric acid (0.78±0.02 g L^{-1}) was obtained in the 75% POME. At lower POME concentration (50%), citric acid decreased to 0.47±0.06 g L^{-1} and was not detected at 25% POME. Citric acid concentration from the 100% POME was 0.32±0.04 g L^{-1} but in separate batches amended with 3% methanol, the concentration increased significantly ($p<0.05$) to 1.02±0.03 g L^{-1}. However, no such increment was observed when 75% and higher POME dilutions were amended with methanol. In the 75% POME for instance, citric acid concentration (0.74±0.08 g L^{-1}) was not statistically ($p>0.05$)

Table 1: Kinetic parameters of citric acid production from cellulase-digested and non-digested POME by *A. niger* ATCC 9142

Treatment	Sugar conc. (g L^{-1})	Citric acid (g L^{-1})	Biomass dry wt. (g L^{-1})	Citric acid yield (g g^{-1} glucose)	Biomass yield (g g^{-1} glucose)	COD reduction (%)	Sugar utilization (%)
Digested POME (%)							
25	0.5	0.00±0.00	5.4±0.12	0.000±0.00	10.80±0.02	63.00±2.0	100.0
50	1.0	0.47±0.06	6.5±0.10	0.470±0.08	6.50±0.05	63.00±2.0	100.0
75	1.5	0.78±0.02	8.5±0.18	0.590±0.10	5.67±0.03	66.00±1.0	100.0
75+3% methanol	1.5	0.74±0.08	9.2±0.05	0.560±0.14	5.72±0.04	64.00±1.5	100.0
100	2.0	0.32±0.04	10.2±0.60	0.160±0.11	5.10±0.06	47.40±2.5	78.5
100+3% methanol	2.0	1.02±0.03	9.4±0.40	0.590±0.18	4.70±0.08	49.20±1.0	74.0
Non-digested							
100% POME	0.0	0.0±0.00	4.0±0.14	0.000±0.00	4.00±0.04	4.60±1.5	0.0
Non-digested							
100% POME+1% glucose	10.0	0.26±0.05	13.4±0.15	0.026±0.19	1.34±0.07	5.40±1.5	40.0

*Values are reported at the 7th day of fermentation

different from the value obtained (0.78±0.02 g L^{-1}) without methanol. Although, citric acid production in the 100% POME hydrolysate was promoted in the presence of methanol, the final yield (0.59 g citric acid g^{-1} substrate) of the metabolite was comparable to the value obtained from the 75% POME due to variation in the initial substrate concentrations. Also, the yield obtained from the 75% POME was also identical to the citric acid value (0.56 g citric acid g^{-1} substrate) in the presence of methanol. In the non-digested (100%) POME, citric acid was undetected but when supplemented with glucose (1%), the production increased to a maximum concentration of 0.26±0.05 g L^{-1}.

Effect of different POME treatments on biomass production: Among the digested treatments, biomass concentration decreased with increase in POME dilution. The mycelial weight obtained from the 100% POME was 10.2±0.6 g L^{-1} (Table 1). At the 75 and 50% POME, the final weights of the biosolids were 8.5±0.18 and 6.5±0.10 g L^{-1}, respectively. The least concentration (5.4±0.12 g L^{-1}) obtained from the 25% POME was found to be higher than the biomass (4.0±0.14 g L^{-1}) from the non-digested POME. However, when supplemented with glucose (1%), the highest biomass concentration (13.4±0.15 g L^{-1}) was obtained. When methanol (3%) was added to the different POME hydrolysates, especially the 100 and 75% batches, differences in biomass concentration among the respective batches was not statistically ($p>0.05$) significant. In contrast, when the yield coefficient was calculated, it was found that the 25% POME gave the highest (10.8±0.02 g g^{-1} glucose) biomass yield while the glucose (1%) supplemented POME had the least (1.34±0.07 g g^{-1} glucose). Differences in the biomass yield among the digested treatments were also statistically ($p>0.05$) insignificant.

Effect of different treatments on COD and other POME characteristics: COD was affected to various extents in the different treatments (Table 1). In the digested POME, COD decreased with increase in dilution. COD of the 100% POME decreased by 47.4±2.5% which is comparable to the value obtained (49.2±1.0%) in the presence of methanol (3%). At lower POME concentrations (i.e., 75, 50 and 25%), COD reduction was approximately 65% but changes in COD of the non-digested treatments were negligible. Besides the COD reduction from 11.890-6.260 mg L^{-1} (47.4%), several other changes were obvious in the 100% POME hydrolysates following fermentation. Results (Table 3) indicate that the pH dropped significantly ($p<0.05$) from 4.00-2.5

while the glucose concentration reduced to 430 mg L^{-1} from an initial concentration of 2000 mg L^{-1}. The total carbohydrate concentration declined significantly ($p<0.05$) from 3870-2380 mg L^{-1} (61.5%) while no variation ($p>0.05$) was found in values obtained for the 'total solids'. It is noteworthy to remark the modifying impact of hydrolysis on key physicochemical components of the undiluted hydrolysate. Besides the release of 2 g L^{-1} of glucose, COD dropped significantly ($p<0.05$) from 60400-11890 mg L^{-1} (80.3%) while the total carbohydrate decreased from 4470-3870 mg L^{-1} (13.4%). 'Total solids' was nearly completely eliminated as it dropped from 27267-210 mg L^{-1} (99.2%) while the oil/grease increased from 2798-16225 mg L^{-1} (580%).

Effect of different POME treatments on sugar utilization: The glucose content of the diluted POME was completely exhausted before the end of fermentation (Table 1). In the non-diluted batches, the presence of methanol did not affect sugar utilization significantly because sugar-uptake in both digested treatments was above 75%. In the glucose (1%) supplemented POME, only 40% of the sugar was used-up by the end of the experiment while in the non-digested POME (without glucose), sugar was not detected in the medium.

DISCUSSION

Effect of different treatments on citric acid production: Citric acid was produced from different POME concentrations as a result of the release of sugars following digestion of the plant components. Citric acid production is known to be affected by the concentration of available sugars (Roukas and Kotzekidou, 1997). In a previous study, Jamal *et al.* (2005) obtained a maximum citric acid concentration of 0.28 g L^{-1} from 1% (w/w) of substrate (POME) with wheat flour (2% w/w) added as co-substrate. The present findings indicate that the concentration of citric acid produced from digested POME (1.02 g L^{-1}) was higher (3.5 folds) than previously reported. Citric acid from enzyme-hydrolyzed POME offers more yield and economic benefits than when supplemented with organic compounds. The addition of glucose, sucrose or wheat flour as co-substrates might be unacceptable due to concerns that the materials could complicate waste treatment efforts. The practice might also be unsustainable on a large scale. Citric acid production was inhibited in the digested 100% POME and the non-digested batches perhaps due to the presence of heavy metals. Heavy metals such as iron, copper and zinc are known to be present in POME (Habib *et al.,* 1998). However, their composition and concentration in POME vary with geographical origin, climatic conditions, palm variety and the palm oil extraction methods (Mercade *et al.,* 1993). In the present study, other heavy metals like chromium, cobalt, nickel and lead were not detected (Table 2) but the concentration of iron, copper and zinc (18.74, 7.49 and 12.24 mg L^{-1}, respectively) were found to be higher than the inhibitory levels reported in previous studies (Al-Obaidi and Berry, 1979; Wang, 1998). Many mineral elements such as magnesium, phosphorus, potassium and calcium were found in the effluent in higher concentrations. Although, they are not considered toxic metals by definition, their presence in excess amounts could interfere with normal microbial metabolic functioning since they are required in very minute quantities by micro-organisms for growth. Many studies indicate that heavy metals inhibit the growth of microorganisms, influence the ionic strength and pH of the medium and are involved in the inactivation of the enzymes associated with citric acid metabolism in the TCA cycle (Oderinde *et al.,* 1986).

However, in the presence of methanol, citric acid production was promoted. This is in agreement with Roukas and Kotzekidou (1997) which found a beneficial relationship between methanol and

Table 2: Elemental composition of POME used in the study

Elements	Concentrations (mg L^{-1})
Boron	0.426±0.00
Lithium	0.114±0.00
Sodium	23.24±0.07
Magnesium	86.58±0.15
Aluminium	2.26±0.00
Phosphorus	36.99±0.16
Potassium	154.9±0.36
Calcium	71.38±0.26
Chromium	ND
Manganese	0.498±0.00
Iron	18.74 ±0.10
Cobalt	ND
Nickel	ND
Copper	7.49±0.00
Zinc	12.24±0.00
Arsenic	0.045±0.00
Selenium	0.021±0.00
Cadmium	ND
Tin	10.09±0.03
Lead	ND

ND: Not detected

citric acid production from a metal-rich industrial by-product. The exact mechanism by which methanol stimulates citric acid production has not been satisfactorily explained. However, according to Maddox *et al.* (1986), methanol acts by altering the permeability properties of the cell membrane leading to excretion of higher concentration of citrate from the cell. Subsequently, the cell responds by increasing its citric acid production through repression of 2-oxoglutarate dehydrogenase in an attempt to maintain an adequate intracellular level of the metabolite. At higher POME dilutions (Table 1), it was found that the addition of methanol did not positively affect citric acid production. The high stimulation effect of methanol on citric acid production has also been attributed to increase in the organisms' tolerance to high levels of toxic metals (Wang, 1998). Thus as the POME got more diluted, the reduction in the concentration of metals might indicate a lowering of the inductive effect of the additive on cultures.

Effect of different POME treatments on biomass production: The highest biomass concentration was obtained from the undigested POME supplemented with glucose. This could be on account of the high concentration of fermentable sugars present in the medium. Fermentable sugars usually serve as substrates for the production of microbial protein. The digested 100% POME amended with methanol also promoted higher biosolid accumulation than the diluted concentrations for a similar reason. At higher POME dilutions, biomass decreased as a result of decrease in the concentration of sugars present. It is indicated that biomass from the glucose supplemented non-digested POME was higher (13.4±0.15 g L^{-1}) than data obtained from the digested treatments despite having low citric acid concentration. The organism might have appropriated the glucose sorely for biomass development due to the limitations imposed by the presence of the inorganic cations on synthesis of citric acid.

Effect of different POME treatments on COD: Although, citric acid yield from the various treatments were low, the enzymatic conversion and microbial activity brought about significant reduction in the COD of the effluents. POME is a highly polluting wastewater because of its high organic matter content; therefore, its discharge without appropriate treatment could cause serious ecological problems (Ma, 1995). A simple and sustainable method for the treatment of the effluent could be developed around the model highlighted in the present study. The COD of the 100% POME decreased by 47.4% (11890-6260 mg L^{-1}) after fermentation but the initial hydrolytic process and oil removal reduced the original COD of the raw effluent by 80% (60400-11890 mg L^{-1}) while the total loss in COD calculated by the end of fermentation was 90% (i.e., 60400-6260 mg L^{-1}) (Table 3). The removal of oil before fermentation was imperative because oil was not a major nutritional resource for the cultures in this case. Secondly, the enzymatic treatment caused the release of so much oil from the plant fibres (2798-16225 mg L^{-1}) that biomass quantification became difficult. The recovered oil was mixed with dried palm inflorescences to make a traditional fire starting material called flint. Therefore, the COD value of 6260 mg L^{-1} obtained after the fermentation could be accounted for by other polysaccharide polymers unaffected by the activity of the enzyme. Table 3 reveals that 86% (3870 mg L^{-1}) of the total POME carbohydrate (4470 mg L^{-1}) remained after cellulase treatment. Out of this, 38.5% (1490 mg L^{-1}) was utilized by the fermenting microbes. The remainder 2380 mg L^{-1} implies that 53% of the total POME carbohydrate was in non-utilizable forms and confirms the presence of other substances with chemical properties different from cellulose. As the POME got more diluted, it contained lower concentration of such elements which increased COD reduction to over 60% (Table 1). Data from the methanol amended POME showed that methanol was not beneficial to COD reduction. COD changes in the non-digested treatments were very low because of the low nutrient content of the medium and the inability of the fermenting microbes to metabolise the carbohydrate polymers in the effluent. In the present study, enzymatic treatment of the effluent liberated 2 g L^{-1} of glucose and decreased the initial carbohydrate concentration by less than 15% (Table 3). Besides cellulose, POME solids also contain hemicellulose and lignin (Kwon *et al.,* 1989; Alam *et al.,* 2006). The lignins form a complex with cellulose which are resistant to conversion by enzymes and many chemical agents (Balat *et al.,* 2008). Thus the rate of COD removal could be significantly enhanced to discharge characteristic by implementing a pre-treatment stage in order to promote the physical disruption of the lignocellulosic matrix and facilitate the formation of sugars before fermentation. Several pre-treatment methods such as mechanical pre-treatment (Rivers and Emert, 1987), steam explosion (Brownell and Saddler, 1987), ammonia fibre explosion (Alizadeh *et al.,* 2005), alkali or acid pre-treatment (Silverstein *et al.,* 2007) have been attempted but the suitability of any of the techniques on POME need to be further verified.

Table 3: Effect of digestion and fermentation on key physico-chemical components of the undiluted POME

Parameters (mg L^{-1})	Before digestion	After digestion	After fermentation
pH*	3.98±0.02	4.00±0.04	2.50±0.04
Glucose	ND	2000±100	430±40
Oil/grease	2798±300	16225±170	**
COD	60400±784	11890±120	6260±40
Total carbohydrate	4470±230	3870±80	2380±65
Total solids	27267±644	210±30	165±27

ND: Not detected, *Not measured in mg L^{-1}, **Oil was removed before fermentation

Effect of different POME treatments on sugar utilization: Generally, citric acid is a glucose fermentation product. Being the major substrate and a readily utilizable energy source, it is not surprising that glucose was virtually used up from the different POME media at higher dilutions. In the 100% POME, residual glucose level was 0.43 g L^{-1} (data not shown) indicating a 78.5% uptake while in the diluted media, glucose was completely eliminated. It may be inferred, therefore that the concentration of citric acid produced was limited by the amount of sugars present in the medium.

CONCLUSION

The present study clearly shows that cellulase treatment promoted the use of POME as substrate for citric acid production and also brought about significant (≥80%) reduction in the COD of the effluent. An integrated mechanism for simultaneous citric acid production and POME treatment could be developed around this method. Further detailed study is however needed to determine the appropriate POME pre-treatment methods that could enhance the conversion of more lignocellulosic materials into substrate sugars.

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