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Full Length Research Paper

Optimization of Fermentation Parameters for Production of Ethanol from Coffee Pulp Waste Using *Pichia anomala* M4 Yeast Isolated from Coffee Environment in Tanzania

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Abstract

Coffee pulp waste (CPW), an abundant agro-waste available in Tanzania was studied as a potential substrate for bioethanol production. Selected yeast Pichia anomala M4 previously isolated from coffee environment was tested against three fermentation parameters namely pH, temperature and soy flour supplementation. Maximum ethanol of 4.7% (v/v) and 4.07% was produced at an optimum pH 4.5 and temperature of 30 °C respectively. There was a significant increase (ANOVA, P< 0.05) in ethanol production on addition of soy flour as a supplement with a maximum yield of 6.04% (v/v) ethanol at a soy flour concentration of 2.0% g/l. A slight increase on ethanol production (6.3% v/v) was recorded when all the three parameters were put together. Appreciable amount of sugar content (8.2 g/l) found in CWP and significant levels of ethanol produced by yeast from the agricultural waste, calls for more research on how best to utilize this untapped bioresource.

Key words: *Coffee pulp waste, Ethanol, Optimization, Yeast.*

Introduction

Considerable attention has been focused on biomass as an alternative energy biosource due to an increasing demand over fossil fuels. The increasing in air pollution from exhaust emissions (Koç *et al.,* 2009), depletion of the worldwide fossil fuel reserve (Festel, 2008) and continuously rising petroleum costs (Ayhan, 2005) has lead to technological innovation on alternative renewable fuel production (Festel, 2008) and in engines technology (Celik, 2008). Agro-waste raw materials could substantially reduce the cost of bioethanol production (Mutreja *et al.,* 2011). In this context, some researchers have conducted studies on the use of banana and pineapple wastes (Itelima *et al.,* 2013(a)), cassava and sweet potato peels (Oyeleke *et al.,* 2012), pawpaw waste (Dhanaseli and Balasubramanian, 2014), corn cobs (Itelima *et al.,* 2013(b), Zakpaa *et al.,* 2009) and coffee waste (Gouvea *et al.,* 2009).

Tanzania is producing a huge quantity of approximately 143,259 metric tons of coffee processing industrial wastes and effluents (TCB, 2010). Usually these wastes are disposed with appreciable amount of sugars (Chanakya and De Alwis, 2004) and hence favor quick growth of microorganisms (Roussos *et al.,* 1995) which might be unattractive to the environment. Nevertheless, these waste may serve as an important biotechnological feedstock and have previously been used for production of mushroom from coffee leaves, spent ground and coffee husk (Pandey *et al.,* 2000), methane gas from coffee pulp (Kivaisi *et al.,* 1996, Boopathy, 1988) and ethanol from coffee husks (Gouvea *et al.,* 2009). Efficient use of coffee waste however, is problematic due to the presence of caffeine and polyphenols (Zuluaga, 1989, Roussos *et al.,* 1995) and therefore optimal parameters for yeast fermentation processes to achieve high yield would be important.

The potential of coffee pulp waste (CWP) produced in Tanzania for ethanol production using locally isolated yeast strains has not been evaluated before. The present study therefore, focuses on development of optimal conditions for ethanol fermentation by a locally isolated yeast strain through optimization of key parameters such as temperature, pH and supplementation with soy flour.The investigation reported in this study was focused on the effect of some fermentation parameters and supplementation in the fermentation of CPW by indigenous yeast identified as *Pichiaanomala* M4. This fungi is a non-saccharomyces yeast associated with wine fermentation (Spagna *et al.,* 2002, Naumov *et al.,* 2001) and has previously been used for bioethanol production from shatian pummelo peels in China (Tao *et al.,* 2011).

Materials and Methods

Coffee pulp waste collection

Coffee pulp waste was collected from Mbinga in Ruvuma and Moshi in Kilimanjaro, the regions where there is high production of coffee in Tanzania. In both regions C*offee arabica* is mostly grown and coffee processing is done using wet method. The samples were collected at industrial sites from the fermentation tanks, frozen, kept in ice box and transported to the laboratory at the Department of Molecular Biology and Biotechnology, University of Dar es salaam. To avoid microbial contamination, CPW was sterilized at 121.1°C for 15 min, cooled to room temperature (28- 32 °C) and then kept in the fridge at 4°C before use.

Organism and culture media

The *Pichia anomala* M4 non-saccharomyces yeast isolated from the coffee environment was used for ethanol production optimization study. The yeast strain was identified based on D1/D2 region of 26s rDNA by INQBA, SA. This strain had proven to be the best in ethanol fermentation from coffee pulp among 8 isolated yeast strains screened by producing 3.56% (v/v) ethanol compared to less than 1.2% (v/v) ethanol produced by the others. The yeast strain was maintained on agar slants (1% glucose, 0.5% peptone, 3% malt extract, and 2% agar-agar) was used to inoculate pre-fermenting media which contained 2% sugar and 3g/L yeast extract and incubated for 12 hours before use in the optimization studies (Viegas *et al., 1985)*.

Determination of sugar content of the CPW

Total carbohydrate/sugar content of the CPW was determined in order to assess the initial concentration of sugars before fermentation and the residual amount of sugars after fermentation, using phenol-sulphuric acid method described previously by Dubois *et al*. (1956). A standard graph was constructed using glucose, and used to find the concentration of total sugar in coffee pulp waste. Determination of residual sugars on each of the optimized parameter for maximum ethanol production was done using samples taken at every 24 hours for 4 days.

Determination of selected minerals in CPW

Nitrogen and trace elements are essential elements in the production of ethanol by yeasts (Deesuth *et al*., 2012).Thus, their presence in the fermentation media (CPW) was determined according to the procedure described by Allen, (1989). The minerals of the CPW were obtained by dry ashing method. About 5g of CPW was placed in a porcelain crucible and dried in an oven (Gallenkamp®, Sanyo OMT Oven, UK) at 105 ^{o}C until a constant weight was obtained. The dried CPW was then placed in a muffle furnace (Tanco, Muffle Furnace, Model PLT 240, INDIA)and was ignited at 550 ^{0}C for 2hours to get the ash. The crucible with ash was left to cool while covered with petridishes to prevent ash loss and brought into a dessicator. The obtained ash was used for analysis of the minerals following the procedure described by Allen, (1989), by the dissolution of the ash using concentrated hydrochloric acid and water, and their concentrations determined by the Atomic absorption spectrophotometer (UNICAM, Model 939, Solaar system, UK) . Nitrogen content was determined by Kjeldahl method as described by Persson, (1995).

Optimization of initial pH

The pH is claimed to be very essential in the fermentation because it affects the ionic state of its mineral components and cellular surface of fermenting yeast (Munene *et al.,* 2002). Eight sets of pH values were used to provide a wide range of selection of an optimum pH condition for ethanol production. 100 mls of CPW were distributed into 250 ml conical flasks and pH was adjusted by using HCl and NaOH to 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5 using digital pH meter (BOECO pH meter BT-600, Germany). The flasks were then sterilized at 121.1°C for 15 minutes (WiseClave®, WACS-2060, Daihatin Scientific, Korea)

Upon cooling, the pre-fermentative yeast (M4) measuring 5ml was inoculated aseptically to the sterilized media and there after incubated (without shaking) at an ambient temperature, which was in the range of 28- 32 ^{0}C for 4 days. After every 24 h, 1.5ml of the fermenting media was withdrawn to eppendorf tubes for four consecutive days according to the procedure described by Periyasamy *et al.* (2009). The eppendorf tubes were centrifuged at 13000 rpm (Mikro 220- Hettich machine: CH 8806 Bäch, Germany) for 4 minutes to get supernatant for ethanol analysis.

Optimization of temperature

Suitable temperature for maximum production of ethanol by the yeast isolate was studied by incubating the CPW yeast in various temperatures namely 28, 30, 35, 37 and 39 ^{0}Cin incubators (Memmert, Omnilabo, Netherlands). These temperatures are within the range of temperatures in the factories and the surrounding environment. This experiment was done using optimum pH value of 4.5 obtained in section 2.5 above. The cultures were incubated at different fermentation temperatures in ovens.

Supplementation with soy flour

Soy is very important source of proteins and some unsaturated fatty acids which are easily incorporated into cellular materials and enhance bioethanol production (Balakumar and Arasaratnam,2012, Bajpai*et al*., 1988). Optimization of soy flour supplement was carried out according to the procedure described by Viegas *et al.* (1985). Soy flour was added in various flasks at final concentrations of 0.25 %, 0.5 %, 1.0 %, 1.25 %, 1.5 %, 2.0 %, 3.0 % and 4.0% (v/v).

Ethanol analysis

Samples analyzed for ethanol production were those taken at the end of fermentation after 96 hours of incubation. Ethanol analysis was done by using gas chromatography (Varian model CP-3800, USA) which is fitted with an auto-injector and auto-sampler system. Flame ionization detector (FID) was set at 280 ^{0}C and an injector at 250 ^{0}C with split state at ratio of 50. Separation was effected in a 30 m, 0.25 mm and 1 µm column type CP- SIL 8 CB, with the temperature maintained at 45- 55 ^{o}C at a rate 2^{0}C/min. for 10 min then at 10 ^{0}C/min to 200 ^{0}C. Column flow was employed at 1.5ml/min with nitrogen as a carrier gas and hydrogen as a combustion gas at 20 psi; linear velocity was 39.0 cm/sec and a total flow of 116.2 ml/min. Samples were diluted with deionized water and to all samples and standards, butan-1-ol was added as internal standard at 0.25%. Concentrations of ethanol were calculated by relating the peak areas of the sample and standards to their corresponding peak areas of the internal standards using the following formula:

$$\text{Ethanol Concentration \% (v/v)} = \frac{Ms/(As \times W)}{Mi/Ai}$$

Where; Ms = peak area of a ethanol in a sample, As = peak area of internal standard in the sample, Mi = peak area ethanol in the standard, Ai = peak area of internal standard in the standard and W = concentration of ethanol in the standard.

Statistical analysis

All analyses were performed in triplicates and data was presented as mean standard deviation. Differences in performance between treatments were analyzed using analysis of variance (ANOVA). Differences at $P<0.05$ were considered statistically significant.

Results and Discussion

Sugar and mineral content of CPW

The results on sugar and mineral content of the studied CPW are presented on **Table 1.** CPW contained appreciable amount of minerals including nitrogen which are important in supporting growth of yeasts. As a result, no additional elements were used in the CPW during fermentation. The amount of sugars in coffee pulp used for optimization study was 8.2 g/l which was considered sufficiently high to serve as a raw material for ethanol production. It has been reported that sugar concentration in industrial fermentation hardly exceed 5-7% w/w and very low sugar in less than 2% w/w are attained in the fermentation process (Basso *et al.,* 2011). Similar studies by Navia P. *et al.* (2011) reported total sugar of 8.2% in Colombian coffee pulp and Kefale *et al*., 2012 reported varying sugar content from 2.6 – 31.26 g/L in Ethiopian coffee pulp. Nevertheless, other agricultural wastes like mango peels can contain up to 40% (w/v) amount of reducing sugars (Reddy *et al.,* 2011).

Table 1. Mineral and Sugar content of CPW

Mineral	Amount (mg/kg)
Manganese	1.74
Magnesium	136.5
Zinc	5.28
Iron	45.52
Copper	4.02
Nitrogen content (%)	0.056%
Sugar content (g/l)	7- 9 g/l

Initial pH optimization

The natural pH of CPW was observed to vary between 5.3 and 5.5. The results from this study show that the concentration of ethanol produced after 96 hours of fermentation differed significantly ($P<0.05$) with the variation in initial pH values (Figure 1). The lowest ethanol concentration of 2.04% (v/v) was found in pH 3 but increased with increase in pH to a maximum concentration of 4.7% (v/v) at pH 4.5, beyond which it started to show a slight decreasing trend. According to these results, pH 4.5 provides optimal condition for ethanol production. However, ethanol concentrations above pH 4.5 were equally high, suggesting that the natural pH of CPW (pH 5.3 to 5.5) can support yeast growth and ethanol production at appreciable levels. Thus CPW at its natural pH can be used in the production of bioethanol with little or no cost related to pH adjustments. Similar studies by Geetha *et al.* (2013) reported significant increase in ethanol yield from pH of 4.5 to 5.5, beyond which the levels did not increase much. Izmirlioglu and Demirci (2012) reported optimum pH for ethanol fermentation from west potato mash with a maximum production of 30.99 g/l at pH 5.5. Pramanik and Rao (2005) and Kourkoutas *et al.,* 2002 also reported pH 4.5 as optimum in fermentation of whey. Slightly lower optimal pH values (pH 4.25) have been reported by Pramanik, (2003). However, at lower pH (3-4) the production of ethanol was slightly lower compared to pH 4.5. This could be attributed to the fact that at lower pH the yeast was unable to activate the enzymes due to its metabolic sensitivity (Pramanik 2003, Munene *et al.,* 2002).

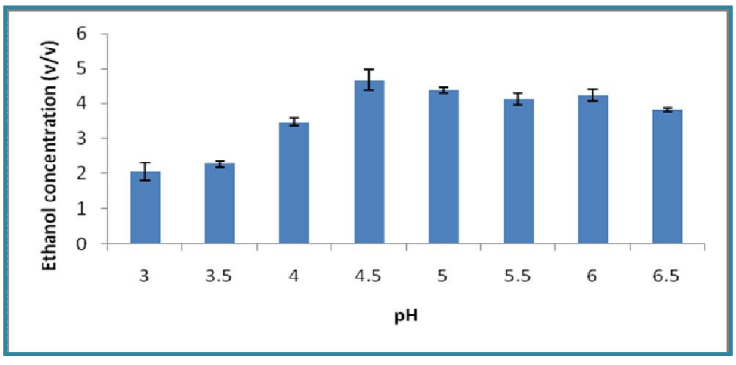

Figure 1. Ethanol production by M4 yeast at different initial pH in CPW
(Values are means of three different measurements; Mean ± SD, n=3)

The amount of sugar residue in g/l after fermentation at various pH values is inversely proportional to the alcohol produced. The sugar residue was highest at pH 3 (5g/l) and lowest at pH 4.5 with less than 1 g/ L of the sugar remained in the CPW (Fig 2).

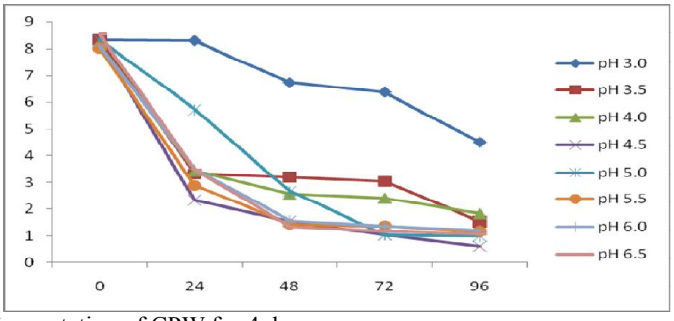

Figure 2. Residual sugar after fermentation of CPW for 4 days

Temperature optimization

The results on the impact of temperature on ethanol production are reported on Figure 3. The highest ethanol produced after 4 days incubation (4.07% v/v) was obtained at 30 ^{0}C followed by 3.66% (v/v) at 37 ^{0}C and the lowest was 1.85% (v/v) at 28 ^{0}C.The optimum fermentation temperature was thus 30 ^{0}C which was the same as natural temperature of CPW at the period of this study and is also within the range of temperature optima for alcoholic fermentation by conventional yeasts (Pramanik, 2003, Pramanik and Rao 2005). Neelakandan and Usharani (2009) reported maximum ethanol yield by *Saccharomyces cerevisiae* at 32 ^{0}C to be 8.53% after 24 hours of fermentation time. However, a slightly higher temperature (33.2 ^{0}C) was reported by Chin *et al.* (2010) to be the optimum temperature. Temperature tolerance for growth and fermentation is said to be strongly strain dependent (Rousseau *et al.,* 1992). Ethanol production using the yeast strain studied will be economically viable because it does not require a lot of adjustment of production temperatures.

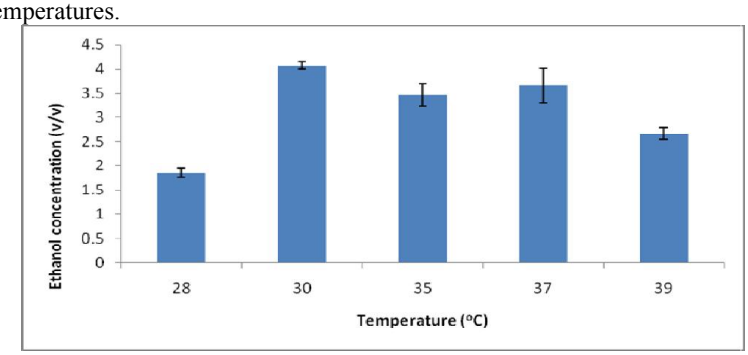

Figure 3. Ethanol production in varying temperatures with initial pH of 4.5
(Values are means of three different measurements; Mean ± SD, n=3)

As shown in Figure 3, temperatures above 35 ^{0}C are accompanied with lower ethanol production which might be due to decreased amount of yeast and thus induced slower ethanol production (Torija *et al.*, 2003). At 39 ^{0}C there was a significant decrease in final ethanol production (2.67% v/v) probably due to reduced yeast viability at high temperatures (Casey *et al.,* 1984). The optimum temperature for the isolate used in this study (30 ^{0}C) was the same as local ambient temperatures (28 – 32^{0}C) hence might reduce the cooling cost that would have been required to maintain the fermentor. Sugar residues in CPW after fermentation measured over a period of time (Figure 4) showed a reverse trend, highest amount of sugars in 28^{0}C (3.082g/l) and 39^{0}C (2.267g/l) and lowest in 30 ^{0}C (less than 0.5 g/l).

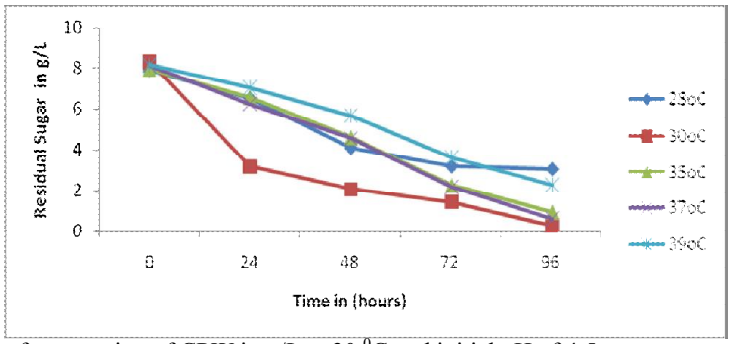

Figure 4. Residual sugar after fermentation of CPW in g/L at 30 ^{0}C and initial pH of 4.5

Soy bean flour optimization

Amongst the affordable industrial protein supplements is soy bean flour. It has the potential of increasing batch and continuous ethanol fermentation rate (Bafrncova *et al.,* 1999, Bajpai *et al.,* 1988) and previously shown to enhance yeast osmo-tolerance,

thermo-tolerance and ethanol- tolerance thus increasing growth rate and viable cell count (Balakumar and Arasaratnam, 2012, Nwachukwu *et al.,* 2008, Nwachukwu *et al.,* 2006, Viegas *et al.,* 1985).The soybean optimization results of this study are presented in Figure 5. There was a remarkable difference in ethanol production between un-supplemented and soy supplemented cultures. For instance, final ethanol concentration in un-supplemented medium was 1.64% (v/v) compared to 4.98% (v/v) and 6.04% (v/v) in media supplemented with 0.25% and 2.0% of soy, being 67% and 73% increase respectively. Notably, there were minor differences in ethanol yield among the different soy concentrations used.

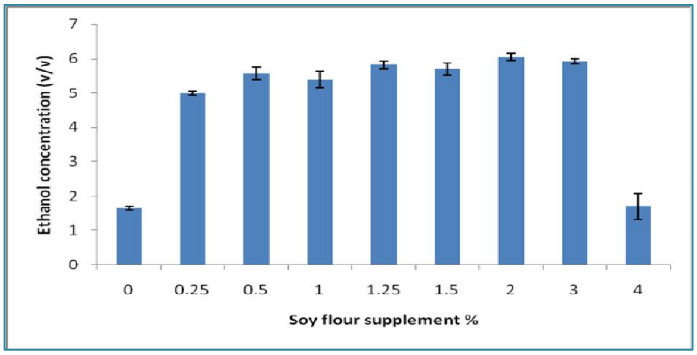

Fig. 5. Effect of soy supplement in CPW on ethanol production by *M4* yeast at 30 ^{0}C and initial pH 4.5
(Values are means of three different measurements; Mean ± SD, n=3)

Equally high ethanol concentration was produced when the lowest soy flour (0.25%) was applied compared to the maximum yield which was attained at a soy flour concentration of 2.0%. This difference in ethanol yield is a clear indication that the yeast isolate was lacking some important nutrients essential for its fermentation metabolism. However, there was drastic drop of ethanol concentration at a soy supplementation of 4%, suggesting that an overload of some nutrients may interfere negatively with ethanol production by yeasts. Addition of soy flour supplement has been reported to enhance sugar utilization and increase ethanol production by 50% by *Saccharomyces cerevisiae* (Bajpai *et al*., 1988, Bafrncova *et al*., 1999). As observed in other parameters reported in this study, the amount of ethanol produced and the residual sugar obtained after fermentation were negatively correlated (Fig 6). The highest amount of ethanol produced was6.04% (v/v) and sugar remaining was less than 0.5g/l.

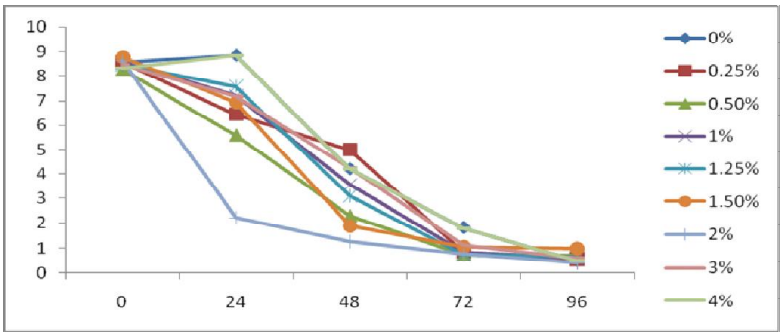

Figure 6. Residual sugar g/l after fermentation of CPW with varying concentrations of soy flour, at initial pH 4.5 and incubation temperature of 30 ^{0}C (n=3)

Fermentation of CPW using all optima parameters derived from this study
When ethanol fermentation optima parameters derived from this study namely, pH 4.5, temperature, 30 ^{0}C and soy flour supplementation, 2 g/l were put together, the ethanol concentration increased significantly from day one, 1.16 % (v/v) to day two, 3.5% (v/v), day three, 6.3% (v/v) and slightly decreased on day four, 6.0% (v/v) (Figure 7). None of the single parameter or a combination of few parameters had such a high yield suggesting that maximum production of ethanol by the studied yeast strain require a balanced combination of growth factors and nutrients. The observed decreasing trend on day 4 could probably be due to suppressed activity in high alcohol and sugar depletion (Cai and Nip, 1990) or assimilation of ethanol as source of energy (Zayed and Foley, 1987). Kefale *et al.,* 2012 reported slightly higher value of ethanol production (7.4 g/l) from Ethiopian coffee pulp. Other agro-waste residues have been reported to have optimum ethanol production of 7.14% (w/v) from mango peels (Reddy *et al.,* 2011), 8.637% (v/v) from pineapple peels (Hajar *et al.,* 2012) and 7.1% (v/v) from banana waste (Hossain *et al*., 2011).However, ethanol production value reported in this study (6.3% v/v) is appreciably higher than 5.19% (v/v) and 5.5% (v/v) reported from pawpaw waste and Henequen (*Agave fourcroydes Lem*), respectively, (Caceres-Farfan *et al.,* 2008, Akin-Osanaiye *et al*., 2005), in support of making CPW a potential feedstock for bioethanol production in Tanzania.

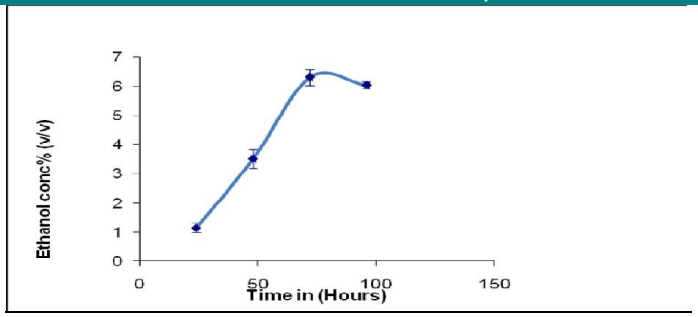

Figure7. Daily ethanol production of *M4* yeast isolate at optima pH, temperature and soy flour supplement
(Values are means of three different measurements; Mean ± SD, n=3)

Conclusion

Coffee pulp waste from Coffee processing industries in Tanzania contained 7-9% sugar content which makes it a potential raw material for the production of bioethanol. The optimum temperature and pH of the CPW for ethanol production by the yeast strain studied, *Pichia anomala* M4 correspond to the natural conditions of the CPW, thus making it a user friendly material at the industrial processing levels. However, supplementation of CPW with soy flour increased significantly the production of ethanol from 4.05% (v/v) to 6.04% (v/v). More studies are needed on the use of other supplements for production of higher levels of ethanol from CPW.

Acknowledgments

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