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Citric Acid Production from Kenana Cane Molasses by *Aspergillus niger* in Submerged Fermentation

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ABSTRACT

Sugar-bearing raw materials are the most suitable resources as feed stocks for biotechnological processes using effective, stable whole organism for biocatalysis. We have selected five *Aspergillus niger* isolates from a group of indigenous *Aspergillus niger* population, prescreened for their potential as citric acid producers. The five isolates abilities for citric acid production was determined at 30°C, pH 3.0 and 100 g/L sugar concentration under submerged fermentation conditions in sucrose salt medium (SSM). Based on the productivity of citric acid under these conditions one *Aspergillus niger* isolate; SUD 27 was selected for optimization of various citric acid production parameters in SSM. Optima obtained for the studied parameters were: Temperature, 30°C; initial pH, 3.5; ethanol, 4%; EDTA; 250 ppm and an initial sucrose concentration 150 g/L. The potential of this isolate for citric acid production from ferrocyanide pretreated Kenana cane molasses was investigated under the predetermined fermentation conditions. SUD 27 demonstrated citric acid yield of up to 68.21 g/L from ferrocynide pretreated (200 ppm) Kenana cane molasses fortified with 3% methanol plus 250 ppm EDTA under submerged fermentation conditions of 30°C, 3.5 pH and 15% Kenana cane molasses initial sugar concentration. Further research is required before this *A. niger* isolate can be recommended for commercial production of citric acid from Kenana cane molasses in Sudan.

Key Words: Kenana molasses, sudan, *aspergillus niger*, citric acid.

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INTRODUCTION

Citric acid is an intermediate in the tricarboxylic acid cycle when carbohydrates are oxidized to CO_2. It is used as an acidifying agent, flavor enhancer, preservative, antioxidant and stabilizer in food, beverages and pharmaceutical industries *(Soccol et al., 2006 and Anwar et al., 2009)*. Conventionally, the acid is produced mainly by submerged or solid state fermentations using *Aspergillus niger* from a variety of substrates such as molasses *(Rajoka et al., 1998 and Saad et al., 2003)*, starchy materials *(Mourya and Jauhri, 2000 and Anwar et al., 2009)*, sugar cane-pressmud *(Shankaranand and Lonsane, 1993)*, sugar cane bagasse *(Soccol et al., 2006)*, cassava bagasse *(Prado et al., 2005)*, date syrup *(Saad, 2006)*, cheese whey *(El-Holi and Al-Delaimy, 2003)* and undersized semolina *(Alben and Erkmen, 2004)*. Although high levels of citric acid are currently achieved but research is in progress in various laboratories on ways of improving the efficiency of the fermentation process. Trials included the use of, different fermentation techniques *(Lu et al., 1995; Mazaheri and Nikkhah, 2002; Kumar and Jain, 2008 and Darouneh et al., 2009)*, different substrates (*Shankaranand and Lonsane, 1993; El-Samragy et al., 1996 and Anwar et al., 2009*), strain improvement by mutation *(Haq et al., 2001 and 2004)*, improvement of pellet morphology (*Gomez et al., 1988 and Couri et al., 2003*), inoculum age and level (*Ganne et al., 2008*), addition of lipoids *(Oderinde et al., 1990)*, metal complexing agents (*Choudhary and Pirt, 1966*), alcohols *(Hang et al., 1987 and Bari et al., 2009)*, EDTA *(Anwar et al., 2009)*, potassium ferrocynide *(Shankaranand and Lonsane, 1993)* nutrient optimization *(Haq et al., 2002 and Bari et al., 2009)* as well as optimization of fermentation conditions and pH modling of fermentation broths *(Ali et al., 2002; Haq et al., 2002; Demirel et al., 2005 and Ganne et al., 2008)* .

In Sudan, thousands of tons of cane molasses are produced by sugar processing industries and there is an urgent need to find suitable applications of this by-product. In addition, the demand for citric acid in Sudan is increasing, due to rapid expansion of food, beverages and pharmaceutical industries. Therefore, the aim of this study was to optimize the conditions for citric acid production from Kenana cane molasses in submerged fermentation using an indigenous isolate of *A. niger.*

MATERIALS AND METHODS

Citric acid production in SSM:

Five *Aspergillus niger* isolates, indigenous to Sudan, were selected from a population of isolates prescreened, according to the method of *Kumar, et al. (2003)*, for their potential as citric acid producers. These five isolates were further screened for their abilities to produce citric acid under submerged fermentation conditions. This was carried out by scraping the spores of each isolate from a five to seven day old PDA slant culture grown at 25°C, then the scraped spores were suspended in sterile distilled water and diluted to obtain a concentration of $1x10^9$ spores ml.

One ml of the prepared suspension was used, in each case, to aseptically inoculate 50ml of the sucrose salt medium (SSM) *(Wang and Liu, 1996)* in a 250 ml flask. The inoculated medium was kept at 200 rpm for 168 hrs. At the end of the fermentation period samples of broth were filtered and citric acid concentration, residual sugars and mycelial dry weight were determined according to the methods of *Marier and Boulet (1958), Dubois, et al. (1956) and Esuoso (1994)*, respectively.

According to the results obtained, the isolate SUD27 was selected for its merit as the highest citric acid producer among the five isolates for further studies.

Optimization of fermentation conditions for enhanced citric acid production in SSM:

Citric acid production, using isolate SUD27, was studied at various incubation temperatures (25, 30, 35, 40 °C), pH (2.0, 2.5, 3.0, 3.5, 4.0 4.5) and sugar concentration (12.5, 15, 17.5, 20, 22.5 %). Optimum values obtained were used to investigate enhanced citric acid production at various methanol (0.0, 2, 3, 4%), ethanol (0.0, 1, 2, 3, 4%) and EDTA (50, 100, 150, 200, 250, 300 ppm) concentrations. In all cases the citric acid concentration, amount of consumed sugar and dry mycelium weight were determined in g/L.

Effect of pretreatment of cane molasses with potassium ferrocyanide on citric acid production parameters:

Cane molasses obtained from Kenana Sugar Mill, Sudan were used as a substrate for citric acid production by the *Aspergillus niger* isolate SUD27. The molasses medium was diluted with sterilized distilled water to maintain a sugar concentration of 15% and the pH was adjusted to 3.5 using 1 N HCl. The effect of pretreatment of cane molasses with potassium ferrocyanide on citric acid production by *Aspergillus niger* isolate SUD27 was studied. Potassium ferrocyanide was added 24 hours after inoculation at concentrations of 0.0, 50, 80, 100, 200 and 250 ppm. Fermentation was performed in 250 ml shake flasks (200 rpm) containing 50 ml molasses for 168 hrs, 30°C and pH 3.5.

Effect of Alcohols and EDTA on citric acid production from Kenana cane molasses:

The metal constituents and the total reducing sugars of the Kenana molasses were determined by atomic absorption spectroscopy *(Perkin Elmer Manual, 1993)* and spectrophotometrically *(Dubois et al., 1956)*, respectively. The effect of each of methanol (3%), ethanol (4%), EDTA (250 ppm) and a combination of methanol (3%) and EDTA (250 ppm) on the kinetic parameters of citric acid production (Pirt, 1975) by *Aspergillus niger* isolate SUD27 was investigated in 250 ml shake flasks containing 50 ml cane molasses at 200 rpm for 168 hrs at 30°C and pH 3.5.

RESULTS AND DISCUSSION

Citric acid production in SSM:

Table 1 shows the production of citric acid by five, locally isolated, *A. niger* isolates in SSM. Citric acid production (g/L) was in the range of 11.65 - 29.28 with a percentage yield (based on sugar consumed) ranging between 13.57 - 41.57%.

A relatively higher citric acid concentration (29.28 g/L) was produced by the isolate SUD27. Dry cell mass and sugar consumed by this isolate at the end of the incubation period were 21.72 and 70.43 g/L, respectively. Hence, SUD27 was selected for further tests. In order to optimize conditions for citric acid production by SUD27, the effect of different incubation temperatures and pH values each-at-a-time were studied. Determined optima for temperature and pH were then used in a series of experiments to optimize the effect of initial sugar concentration and three different citric acid enhancers (methanol, ethanol, EDTA) on the production of citric acid by *A. niger* using SSM as a basic substrate.

Table 1: Screening of five *A. niger* isolates for Citric acid production in SSM.

A. niger isolates	mycelial dry weight (g/L)	Citric acid (g/L)	Sugar consumed (g/L)	Yield %*
SUD1	18.14	19.62	88.03	22.29
SUD3	17.63	16.41	76.33	21.50
SUD5	16.46	15.67	77.41	20.24
SUD13	13.37	11.65	85.82	13.57
SUD27	21.72	29.28	70.43	41.57

*Based on sugar consumed.

Effect of incubation temperature:

Figure 1 shows the effect of temperature on citric acid production. Maximum citric acid production (34.52 g/L) was achieved at 30°C. Sugar consumption at this temperature was 84.31 g/L with 40.94 % yield while dry mycelial weight was 20.91 g/L. When the incubation temperature was increased to 35 and 40°C, the percentage yield decreased to 34% and 20.04%, respectively. At 40°C, decrease in percent yield was accompanied by a noticeable increase in sugar consumption and, to some extent, in dry mycelial weight. The optimum citric acid production temperature determined in this study (30°C) is similar to the findings of *Ali, et al. (2002)* and is also similar to the findings of *Haq, et al. (2002)* who reported that increment of incubation temperature above 35°C was inhibitory to citric acid production due to the increased production of by-product acids and inhibition of culture growth.

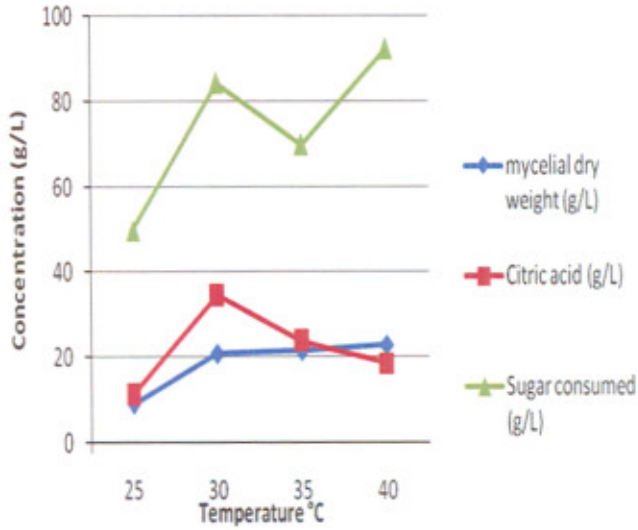

Figure 1: Effect of incubation temperature on citric acid production by SUD27. pH, 3.5; sucrose concentration, 100 g/L.

Effect of initial pH:

Figure 2 shows the effect of level of initial pH on citric acid production, mycelial dry weight and sugar consumption by *A. niger* isolate SUD27 at 30°C. Maximum citric acid concentration (48.93 g/L), with a percentage yield of 58.28 based on sugar consumption was obtained at pH 3.5. A very sharp decrease in citric acid yield was observed when the pH was increase0d to 4.0 and to 4.5. However, this reduction was not accompanied by a similar reduction in mycelial dry weight or sugar consumption. The gram per liter mycelial dry weight and sugar consumption were very low at 2 and 2.5 pH values, but sharply increased when the pH was increased to 3.0 and 3.5. No increase in mycelial dry mass or sugar consumption was further observed with pH increase. Apparently the maintenance of a favourable pH is essential for successful production of citric acid. A lower initial pH of less than 2.5 was reported to inhibit the growth of *A. niger (Haq et al., 2002)* and is thus expected to negatively affect citric acid production. *Shadafza, et al. (1976)* reported that a pH higher than 3.5 leads to accumulation of oxalic acid and gluconic acid at the expense of citric acid production. On the other hand, *Pessoa, et al. (1984)* reported that decrease of pH to a value lower than 3.5 results in decreased citric acid production. Oxalic acid is an undesirable by-product of citric acid fermentation and is considered to be a main impurity in the commercial citric acid process from *A. niger* (*Paul et al., 1999*).

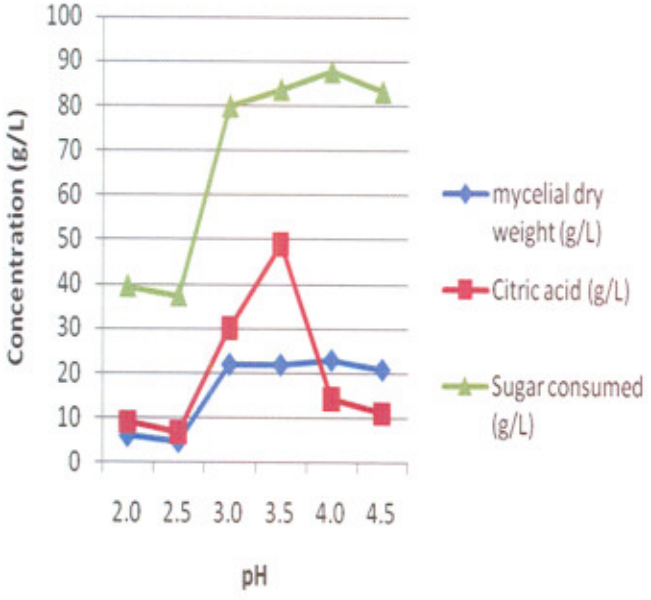

Figure 2: Effect of medium initial pH on citric acid production by SUD27. Temperature, 30°C; sucrose concentration, 100 g/L.

Effect of initial sugar concentration:

Figure 3 shows the effect of initial sugar concentration on mycelial dry weight, sugar consumption and citric acid production by isolate SUD27 under the previously determined optimum conditions of temperature (30°C) and pH (3.5). The highest citric acid production (58.81 g/L) was obtained at 150 g/L sucrose with a percentage yield of 49.84. At 175 g/L sucrose, citric acid concentration was 56.84 g/L, but with a lower percentage yield of 41.94 based on the sugar consumed. Mycelial dry weight demonstrated a steady increase with the increase of sugar concentration beyond 150g/L. *Demirel, et al. (2005)* reported maximum citric acid production at sucrose concentration of 140 g/L. The optimum sugar concentration determined in this study is 150 g/L. This result is comparable to the results reported by *Pazouki, et al. (2000)* who reported that a sugar concentration higher than 1618%- leads to accumulation of greater amounts of residual sugars making the process uneconomical while a lower sugar concentration leads to lower yields due to accumulation of oxalic acid in the culture broth. Similar conclusions were also reached by *Haq, et al. (2002) and Anwar, et al. (2009)* who observed significant reductions in citric acid yield when sugar concentration was increased beyond 150g/L. The latter authors explained this reduction by overgrowth of the mycelia resulting in increased viscosity of the medium. These findings are in line with the steady increase in mycelial dry weight reported in this study beyond 150g/L sugar concentration. Addition of 15% sucrose was reported to enhance citric acid production from cheese whey while significantly lower yields were obtained when other sugars were added *(El-Holi and Al-Delaimy, 2003).*

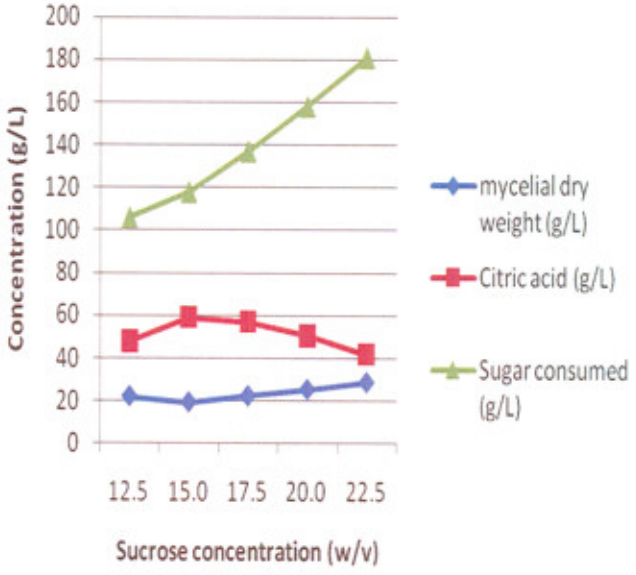

Figure 3: Effect of initial sucrose concentration on citric acid production by SUD27. Temperature, 30°C; pH, 3.5 g/L.

Effect of enhancers:

Table 2 shows the effect of adding different concentrations of methanol, ethanol and EDTA on mycelial dry mass, sugar consumption and citric acid production by SUD27. Both methanol and EDTA had significant favorable effects on citric acid yield. At 3 % methanol, citric acid production and sugar consumption were 76.81 and 122.32 g/L, respectively with a conversion rate of 62.96% which is significantly better, by 37%, than the control ($p \leq 0.05$). Addition of EDTA at a concentration in the range of 150250- ppm stimulated citric acid production with a maximum concentration of 79.28 g/L obtained at 250 ppm.

Addition of 1% ethanol resulted in a slight increase of citric acid production and had a slightly better effect than the addition of methanol at the same concentration. However, no significant differences were observed between the two treatments with respect to percentage yield.

Table 2: Effect of different concentrations of alcohols and EDTA on citric acid production by SUD27 using sucrose salt medium.

Parameters studied		mycelium dry weight (g/L)	Citric acid (g/L)	Sugar consumed (g/L)	Yield (%)*
Methanol (%)	1	24.51	59.39	112.35	52.86
	2	24.82	73.14	123.43	59.26
	3	20.04	76.81	122.32	62.96
	4	21.63	58.52	108.61	53.88
	control	22.28	56.12	141.50	39.66
Ethanol (%)	1	22.16	61.31	116.91	52.44
	2	24.56	59.80	124.45	48.05
	3	22.55	62.43	120.00	52.03
	4	26.67	64.92	121.24	53.55
	control	23.13	59.11	122.37	48.30
EDTA (ppm)	50	22.23	56.12	112.82	49.74
	100	25.14	57.80	117.73	49.10
	150	25.83	61.83	116.17	53.22
	200	29.12	63.32	122.33	51.76
	250	28.98	79.28	136.53	58.06
	300	31.41	62.84	128.42	48.93
	control	21.94	58.19	119.63	48.64

* Based on sugar consumed.

Citric acid production from Kenana molasses by *A. niger* SUD27:

Effect of pretreatment of Kenana molasses with ferrocyanide on citric acid production:

The metal composition (mg/L) of the molasses used in this study was Cu, 0.14; Zn, 0.27; Mn, 0.15; Fe, 0.66; Na, 4.0; K, 36.0; Ca, 16.0 and Mg, 16.5. The effect of adding different concentrations of ferrocyanide to Kenana molasses on citric acid production by SUD27 was studied and the results are shown in (Figure 4). Both citric acid production (g/L) and yield (%) increased with the increase of ferrocyanide concentration upto 200 ppm. Further increase in ferrocyanide concentration resulted in the decrease of citric acid production, mycelial dry weight and sugar consumption. Addition of potassium ferrocyanide in the range 0.0 - 0.1 ppm in solid state fermentation was reported to have no effect or it slightly increased citric acid productivity from date pulp *(Mazaheri and Nikkhah, 2002)*. A slight difference was also observed in the production of citric acid by *A. niger* when potassium ferrocyanide was added in the range 50300- ppm to hydrolyzed raw starch *(Anwar et al., 2009)*. However, *Ali (2004)* reported that addition of ferrocyanide to molasses resulted in a highly significant increment of citric acid production, 2.38 folds higher, when compared to control.

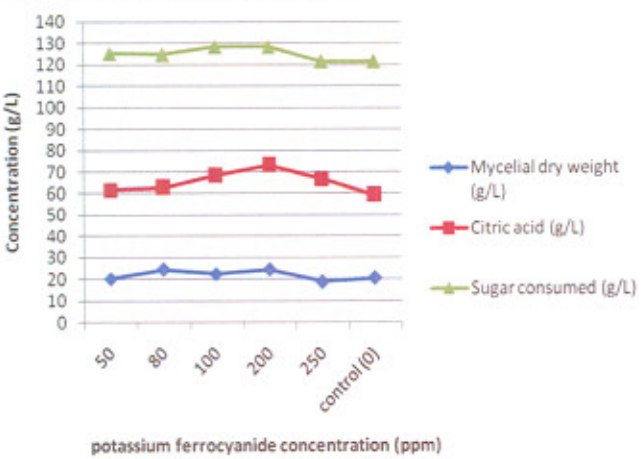

Figure 4: Effect of pretreatment of cane molasses with different concentrations of potassium ferrocyanide on citric acid production by *A. niger* isolate SUD27.

Kinetic parameters of citric acid production from Kenana cane molasses:

Results obtained for studies of the effect of methanol, 3%; ethanol, 4%; EDTA, 250 ppm and 3% methanol plus 250 ppm EDTA on the kinetic parameters of citric acid production from Kenana cane molasses pretreated with 200 ppm ferrocyanide are shown in (Table 3). All treatments gave a remarkable increase in citric acid production (g/L) over the control. The highest value (68.21 g/L) of citric acid was obtained in cultures supplemented with 3% methanol plus 250 ppm EDTA demonstrating an increase of 97% over the control. Supplementation of 3% methanol alone came second with a production of 61.62 g/L citric acid demonstrating a comparatively lower increase of 78%. *Hamissa and Radwan (1977)* reported that both ethanol and methanol had a significant favourable effect on citric acid yield and conversion coefficient from cane molasses on a semi-pilot scale, with a more pronounced effective role of methanol than ethanol. Furthermore, the authors attributed the stimulatory effect of alcohols to their inhibition of spore formation and increased tolerance of the microorganisms to the high levels of minerals present in the molasses. There were slight differences in citric acid production when ethanol was added in the range of 14%- however; maximum production (64.92 g/L) was obtained at 4% ethanol. This result is comparable to the findings reported by *Demirel, et al. (2005). Mazaheri and Nikkhah (2002) and Roukas and Kotzekidou (1997)* reported that the highest values for citric acid concentration, sugar utilization and percentage citric acid yield were obtained from date syrup in presence of methanol at a concentration of 4%. The authors attributed the stimulatory effect of methanol to its inhibitory effect on spore formation and increase of microorganism's tolerance to high levels of minerals contained in date syrup. The enhancing effect of moderate methanol concentration on citric acid production was also explained by its reduction of iron and manganese uptake by *A. niger (Dasgupta et al., 1994). Maddox, et al. (1986)* reported that methanol affects cell permeability in a way that allows citrate excretion from the cell. In response, the cell increases its citrate production by repressing 2-oxoglutarate dehydrogenase to maintain an adequate intracellular level of the metabolite.

It is also evident (Table 3) that addition of EDTA to the molasses has increased citric acid production by 58% over the control. However, in solid state fermentation of hydrolyzed raw starch, *Anwar, et al. (2009)* reported a little enhancement of citric acid production over the control due to the addition of EDTA. This contradiction could be attributed to the isolate as well as the substrate used.

Addition of EDTA has also resulted in a remarkable change in the growth pattern of the fungus. In media supplemented with EDTA, the organism grew in the form of separate, round and smooth pellets whereas in the control the fungus had a filamentous mat-like appearance. The enhanced citric acid yield due to the addition of EDTA is attributed to the presence of the roundish pellets in the culture and consequently efficient aeration of the medium *(Chung and Chang, 1990)*. Earlier studies demonstrated that EDTA as well as other chelating agents stimulated citric acid

production with the maximum stimulation reached with the chelating agent in the range 13-mM *(Choudhary and Pirt, 1966)*.

Table 3 also displays comparisons of some kinetic parameters for citric acid production under different treatments. Values of gram cells per gram sugar utilized (YX/S) and gram sugars consumed per liter per hour (QS) for the different treatments were only marginally different from the control. However, when the cultures under different treatments were monitored for gram citric acid produced per gram sugar utilized (YP/S) and gram citric acid produced per liter per hour (QP) there was a significant enhancement, in the case of 3% methanol plus 250 ppm EDTA, 3% methanol and 250 ppm EDTA ($p \leq 0.05$) over the control.

Table 3: Effect of different concentrations of alcohols and EDTA on kinetic parameters during citric acid fermentation by SUD27 using cane molasses.

Treatments	3% methanol	4% ethanol	250 ppm EDTA	3% methanol + 250 ppm EDTA	Control
Citric acid (g/L)	61.62	48.43	59.74	68.21	34.65
g citric acid produced/g sugar consumed (YP/S)	0.52	0.37	0.46	0.51	0.29
g cells/g sugar utilized (YX/S)	0.24	0.21	0.26	0.24	0.20
g citric acid produced/ liter/hour (QP)	0.37	0.29	0.36	0.41	0.21
g sugar consumed/ liter/hour (QS)	0.71	0.78	0.77	0.80	0.72

CONCLUSION

The results obtained showed some important features of citric acid from Kenana cane molasses by *A. niger*. On the basis of the results obtained, it can be concluded that simultaneous addition of 3% methanol and 250 ppm EDTA significantly improved citric acid production compared to addition of each of these factors alone. The optimum temperature and pH for citric acid production from molasses was 30°C and 3.5, respectively. Due to its good keeping quality, low cost and abundance, molasses is considered as an economically attractive substrate for citric acid production especially for a major cane sugar producing country like Sudan.

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