EFFECTS OF pH ON THE GROWTH, LIPID AND FATTY ACID PRODUCTION OF *Candida utilis* AND *Candida tropicalis* GROWN IN CANE MOLASSES

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Abstract
There was a decrease in viable yeast counts depending on the duration of incubation when Candida utilis was grown in medium at pH 4.0. At pH 5.0 and 6.0, the greatest amounts of viable yeast cells were observed with the overall best growth of $4.1x10^8$ occurring at pH 5.0 after 72 h incubation. The total viable cells of Candida tropicalis grown in media at different pH show that at pH 4.0, 5.0 and 6.0 were the greatest amounts of viable yeast cells. At these pH, the growth of Candida tropicalis was not highly influenced by the incubation time. In contrast, adjusting the initial pH to 7.0 and 8.0 produced low yeast cell yields. At pH 9.0, growth occurred to a much lesser extent than at the other pH values. The best pH for maximum biomass production by Candida utilis was 5.0 while maximum amount of lipid was produced at 9.0. There was a net decrease in the total lipids associated with increases in the yeast biomass. Maximum lipid amount, produced by Candida tropicalis,is 59.4 (mg/L) and this was obtained at pH 9.0. The maximum biomass concentration of 8.1 (g/L) was obtained at pH 6.0. Changes in the fatty acid composition of Candida utilis at different initial media pHs show that at most pH values, the $C_{16:0}$, $C_{18:1,}$ $C_{18:2}$ fatty acids predominated. The fatty acid profiles of Candida tropicalis at different initial medium pH show that linoleic acid ($C_{18:2}$) was the predominant fatty acid at most pH values.

Key Words: *Candida utilis,Candida tropicalis*, yeast cell growth, yeast biomass, fatty acids, lipids.

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1. INTRODUCTION

There is increasing interest in searching new oil feedstock for biodiesel production. Among them are oils and lipids produced from oleaginous microorganisms. They are now considered as promising feedstock because of their similar fatty acid composition to that of vegetable oils. Microorganisms are not affected by seasons nor by climate and can accumulate lipids within a short period of time as well as they grow well on a variety of substrates. Lipids synthesized by microorganisms have been mainly used in pharmaceutical industry, for technical purposes and more rarely as fodder. Microbial lipids have been known as a source of special oils and fats with high industrial potential for application and evaluated as an alternative source of animal and plant oils (D'Amico et al., 2006).

Microbial oils and lipids produced from oleaginous microorganisms including yeasts and moulds, which have the ability to accumulate lipids over 20% of their biomass, are considered as the third generation of biodiesel feedstock due to some advantages such as short production period, higher biomass production and faster growth and easiness to scale up compared to other energy crops, (Meng et al., 2009; Ahmad et al 2011). Microbial biomass production as a source of nutrients in human foods or animal feeds comprises the production of dried cells of different microorganisms (algae, bacteria, molds and yeasts) in large scale culture systems. The production of yeast biomass is advantageous because of its nontoxic nature, low level of nucleic acid content and high productivity. Moreover, yeast biomass is important as a raw material for food, pharmaceutical, cosmetic and other industries, in addition to being an excellent source of nutrients, such as protein and lipids.

Yeasts are unicellular fungi which reproduce by budding or fission. The following yeast genera are capable of producing lipids: *Candida*, *Lipomyces*, *Rhodospiridium*, *Rhodotorula*, *Saccharomyces*, *Torulopsis*,

Trichosporon etc. (Aggelis et al., 1996; Akhtar et al., 1998). The most deeply investigated oleaginous yeasts belong to the genera *Yarrowia*, *Candida*, *Rhodotorula*, *Rhodosporodium*, *Cryptococcus* and *Lypomyces* (Ageitos et al., 2011; Li et al., 2008; Rossi et al., 2009).*Candida* has been utilized commonly as microbial specimen for lipid synthesis (Aggelis et al., 1996). The yeast *Candida* has received much attention due to its biotechnological potential and ability to metabolize several important industrial and agro-industrial residues. Oleaginous yeasts accumulate lipids to levels corresponding to 60% of their dry weight under cultivation conditions (Li et al., 2008).

Microorganisms utilize various carbon sources as energy compounds for the production of lipids. Organisms that utilize cheap and inexpensive carbon substrates and industrial waste products are very attractive for industrial product formation from the economic view point. The use of industrial waste products as substrates for microbial oil production is an interesting area of research. This work aims to evaluate the influence of the initial media pH on the growth, biomass, lipid and fatty acid production of *Candida utilis* and *Candida tropicalis* grown in cane molasses.

2. MATERIALS AND METHODS

Yeast strains: The two yeast strains *Candida utilis* and *Candida tropicalis* used in this study were isolated from rotten fruit samples. The strains were maintained on Sabouraud Dextrose agar (Oxoid Ltd., UK) slants and stored at 4°C.

Cane molasses: Molasses samples were collected from a sugar factory in Lagos, Nigeria. The total reducing sugar content of the samples was determined by the dinitrosalicylic acid (DNS) method of Miller (1959).

Cultivation conditions: The strains were aerobically cultured at 30°C in Sabouraud Dextrose broth (Oxoid) and maintained at 4°C in slants of Sabouraud Dextrose agar. Lipid production was evaluated in the medium which contained in g/L: total reducing sugar, 120; yeast extract, 3; glycerol, 20; KH_2PO_4, 8; $MgSO_4 \cdot 7H_2O$, 0.5. The pH was adjusted either with sterile lactic acid or sodium hydroxide solution. All flasks were incubated in a rotary shaker at 30^{o}C operated at 50 x g. The numbers of viable *Candida* cells present in all flasks were determined after 24 h over a 96 h by counting the number of colony-forming units present on Sabourand Dextrose agar plates. The biomass was separated by centrifugation at 2515 x g for 15 min. and washed twice with distilled water. Dry weight of the biomass was determined after drying at 105°C until constant weight.

Lipid studies: Lipid determinations were performed on cells harvested after 48 h incubation in media adjusted to initial pH of 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0. The yeast cells were recovered by centrifugation at 6000 x g, washed twice with sterile distilled water, and re-suspended in a minimal amount of distilled water. The cells were frozen in an acetone dry ice bath. Cells were weighed to obtain dry cell weights and then stored at 5^{o}C. Extractions of total lipids were done by the technique of Huston and Albro (1964). Acetone was added to the cells in a ratio of 15ml of acetone per gram of cells. The mixture was then shaken at room temperature for 1 h and filtered through Whatman No 1 filter paper. The residue was then subjected to two successive 2 h chloroform:methanol (2:1 v/v) extractions. To remove non lipid contaminants, the combined extracts were washed with 10 ml of normal saline solution for 12 h. The purified lipid extract was concentrated in an evaporator, resuspended in 10 ml of chloroform : methanol (2:1 v/v) and the volume reduced to 5 ml in an evaporator. The final product was then evaporated to a constant weight at 60^{o}C and designated as extractable lipid.

Analysis of Fatty acids: Fatty acid composition was determined by gas chromatography (GC) according to the method of Christie (2003). The purification of fatty acid methyl esters was done by Silica gel thin layer chromatography on 20 cm x 20 cm plates which was covered with 0.2 mm Silica gel 60 G layer (Merck, Darmstadt, Germany) and the mobile phase was n-hexane:acetone 100:8l. GC was carried

out on HP 5890gas chromatograph which was equipped with a 30 m x 0.25 mm (I.D.) capillary InnoWax column (cross-linked PEG, Hewlett Packard) with flame ionization detector. Temperature of the column was from 165^{o}C to 240^{o}C at 4^{o}C/min and held for 10 min. The injector and detector temperatures were 250^{o}C. Nitrogen was used as carrier gas at a flow rate of 0.8 ml/min; while split was 100:1. Identification of fatty acids was done by the comparison of the retention times with those of standard fatty acids subjected to gas chromatography under similar experimental conditions.

Determination of reducing sugar: The total reducing sugar was determined by a modification of the dinitrosalicylic acid (DNS) method of Miller (1959) as described by Nwokoro *et al*., (2003).

3. RESULTS AND DISCUSSION

Candida utilis grew in the chemically defined media in which the pH was varied (Table 1). The data presented shows that the pH and incubation time had marked effects on yeast cell growth. Growth in medium at pH 4.0 caused a decrease in yeast viable counts throughout the duration of incubation. At pH 5.0 and 6.0, the greatest amounts of yeast viable cells were observed with the overall best growth of 4.1x10^{8} occurring at pH 5.0 after 72 h incubation. Cultivation in media at pH 8.0 and 9.0 was accompanied by decreases in yeast counts. The lowest cell count of 2.1 x10^{3} was observed at pH 9.0 after 96 h incubation (Table 1).

The total viable cells of *Candida tropicalis* grown in media at different pH values is shown in Table 2. At pH 4.0, 5.0 and 6.0 there were the greatest amounts of yeast viable cells. At these pH levels, the growth of *Candida tropicalis* was not influenced greatly by the incubation time. In contrast, adjusting the initial pH to 7.0 and 8.0 produced low yeast cell yields. At the extreme high pH level of 9.0, yeast growth occurred to a much lesser extent than at the other pH values. This observed decrease of viable cell counts at high alkalinity is certainly not unexpected, since it is well known that unfavorable hydrogen ion concentrations can adversely affect the cellular enzymatic activities of yeasts. Ratledge (1994) has pointed out that as pH rises above 6.2, the rate of uptake of alkali metal cations by yeasts declines, resulting in a general reduction of inward transport. Therefore, any consideration of growth inhibition at pH 9.0 may also take into account possible alterations in yeast cell permeability.

Results in Table 3 indicate that the best pH that lead to the maximum biomass production of *Candida utilis* was 5.0, while maximal amount of total lipids was obtained at pH 9.0. There was a net decrease in the total lipid content with increases in yeast biomass indicating that the tested oleaginous yeast preferred an alkaline pH to accumulate maximum amount of lipid.

Table 4 reveals that the maximal lipid concentration produced by *Candida tropicalis* is 59.4 (mg/L) and this was obtained at pH 9.0. The maximum biomass concentration of 8.1 (g/L) was obtained at pH 6.0. Lipid and biomass concentration at each pH showed a parallel pattern. Rattray et al, (1975) reported that slight changes in the medium pH influenced the general composition of the cellular lipids of yeasts rather than the total amount produced. Zalashko et al, (1972) reported that *Lipomyces lipoferus* growing at non optimal pH values produced fewer lipids. In the present study maximum lipid production by *Candida tropicalis* was observed under conditions that produced an inhibition of growth.

Lipid contents of 22.3, 39 and 53% were reported for *Rhodotorula glacialis*, *Aspergillus niger* and *Cunninghamella echinulata* respectively by Makri *et al.* (2010), André *et al.* (2010) and Fakas *et al.* (2009) respectively. Production of lipids by *Trichosporon fermentans* with lipid content of 63, 58, and 37% after cultivation on sucrose, xylose and molasses respectively was reported by Zhu et al., (2008). Papanikolaou *et al*. (2002) produced biomass at the concentration of 6-7.5 g/ L, containing 5-10% lipids in shaken flask

culture with *Y. lipolytica* LGAM SC711. *Candida curvata* (Holdsworth and Ratledge, 1991) and *Candida freyschussii* (Amaretti et al., 2011) synthesized and stored significant amounts of lipids. *Microsphaeropsis, Phomopsis, Cephalosporium, Sclerocystis* and *Nigrospora* simultaneously accumulated lipids (21.3 to 35.0% of dry weight) (Peng and Chen, 2007). Pure glycerol supported growth and lipid accumulation of *Rhodotorula glutinis* and *Candida freyschussii* (Easterling et al., 2009; Amaretti et al., 2011), when used as sole carbon and energy source. Liang et al., (2010) made successfully converted crude glycerol into lipids by exploiting the oleaginous yeast *Cryptococcus curvatus* and in a 12-day two-stage fed-batch where raw glycerol was fed, the biomass density and the lipid content reached 32.9 g/L and 52%, respectively.

Changes in the fatty acid composition of *Candida utilis* at different initial media pH are shown in Table 5. At most pH values, the $C_{16:0}$ and $C_{18:1}$ and $C_{18:2}$ fatty acids predominated. Unsaturated fatty acids predominated over saturated types at pH 4.0, 6.0 and 9.0 while the saturated types predominated at pH 5.0, 7.0 and 8.0. The fatty acidscontained high fractions of saturated and monounsaturated C_{16} and C_{18} types (Table 5).

Table 1. Effects of initial medium pH on the growth of *Candida utilis*

	Initial pH of the growth medium					
	4.0	5.0	6.0	7.0	8.0	9.0
Incubation Time (h)	Viable cells/ml	Viable cells/ml	Viable cells/ml	Viable cells/ml	Viable cells/ml	Viable cells/ml
24	2.8×10^6	2.7×10^7	3.6×10^8	2.5×10^6	1.4×10^5	1.2×10^4
48	3.1×10^6	3.5×10^8	3.9×10^8	3.1×10^7	2.0×10^5	2.5×10^5
72	3.9×10^5	4.1×10^8	4.2×10^7	4.8×10^7	3.8×10^4	2.8×10^4
96	2.6×10^4	3.6×10^7	3.4×10^7	2.2×10^5	3.3×10^4	2.1×10^3

Table 2. Effect of initial medium pH on the growth of *Candida tropicalis*

	Initial pH of the growth medium					
	4.0	5.0	6.0	7.0	8.0	9.0
Time (h)	Viable cells/ml	Viable cells/ml	Viable cells/ml	Viable cells/ml	Viable cells/ml	Viable cells/ml
24	4.9×10^7	3.6×10^7	5.2×10^9	3.1×10^7	4.8×10^5	1.6×10^3
48	3.1×10^8	4.5×10^9	5.0×10^9	4.2×10^7	2.6×10^6	2.5×10^5
72	2.1×10^8	2.3×10^8	2.5×10^7	5.1×10^5	3.9×10^5	3.1×10^4
96	3.6×10^6	2.1×10^6	2.0×10^7	2.9×10^5	3.2×10^3	2.0×10^3

Table 3. Effects of initial medium pH on cell weight and lipid content of *Candida utilis*

Initial pH of medium	Dry cell weight (g/L)	Total lipids (mg/L)
4.0	6.16	36.0
5.0	8.21	30.3
6.0	7.81	25.5
7.0	5.12	43.1
8.0	4.06	60.5
9.0	3.57	68.9

Table 4. Effects of initial medium pH on the cell weight and lipid content of *Candida tropicalis*.

Initial pH of medium	Dry cell weight (g/L)	Total lipids (mg/L)
4.0	5.4	28.9
5.0	7.9	32.6
6.0	8.1	29.5
7.0	4.2	35.3
8.0	4.9	50.7
9.0	4.6	59.4

Table 5. Effects of initial medium pH on the fatty acid composition of *Candida utilis* after 48 h incubation

	Initial pH of the growth medium					
	4.0	5.0	6.0	7.0	8.0	9.0
*Fatty acids	%	%	%	%	%	%
$C_{14:0}$	1.63	1.49	10.91	17.23	9.00	12.05
$C_{16:0}$	28.90	40.10	25.22	32.83	22.41	16.24
$C_{16:1}$	8.86	3.14	8.43	10.11	18.28	6.83
$C_{17:0}$	3.34	9.21	2.86	3.56	12.96	7.32
$C_{18:0}$	2.98	8.09	3.48	5.60	11.83	14.27
$C_{18:1}$	18.20	20.92	26.47	21.25	13.23	15.18
$C_{18:2}$	27.65	12.44	21.82	8.44	10.86	27.46
$C_{18:3}$	8.44	4.61	0.81	1.16	1.43	0.65
% saturated	36.85	58.89	42.47	59.04	56.20	49.88
% Unsaturated	63.15	41.11	57.53	40.96	43.80	50.12

*$C_{14:0}$- Myristic acid; $C_{16:0}$- Palmitic acid; $C_{16:1}$- Palmitoleic acid; $C_{17:0}$- Margarinic acid; $C_{18:0}$- Stearic acid; $C_{18:1}$- Oleic acid; $C_{18:2}$- Linoleic acid; $C_{18:3}$- Linolenic acid.

Table 6. Effects of initial medium pH on the fatty acid composition of *Candida tropicalis* after 48 h incubation

	Initial pH of growth medium					
	4.0	5.0	6.0	7.0	8.0	9.0
*Fatty acids	%	%	%	%	%	%
$C_{12:0}$	0.54	2.93	1.56	1.28	0.47	0.93
$C_{14:0}$	8.75	4.11	13.08	10.90	9.72	17.28
$C_{16:0}$	25.22	18.78	20.61	26.42	23.16	18.82
$C_{16:1}$	3.10	4.81	2.33	9.62	2.38	5.46
$C_{17:0}$	10.34	8.54	0.86	4.77	17.95	9.26
$C_{18:0}$	0.92	2.61	9.42	0.18	3.86	3.33
$C_{18:1}$	14.49	15.63	16.85	16.46	12.14	12.09
$C_{18:2}$	28.25	31.94	20.96	29.54	28.11	16.48
$C_{18:3}$	8.39	10.65	14.33	0.83	2.21	16.35
% saturated	45.77	36.97	45.53	43.55	55.16	49.62
% unsaturated	54.23	63.03	54.47	56.45	44.84	50.38

*$C_{12:0}$- Lauric acid; $C_{14:0}$- Myristic acid; $C_{16:0}$- Palmitic acid; $C_{16:1}$- Palmitoleic acid; $C_{17:0}$- Margarinic acid; $_{18:0}$- Stearic acid; $C_{18:1}$- Oleic acid; $C_{18:2}$- Linoleic acid; $C_{18:3}$- Linolenic acid.

Palmitic acid ($C_{16:0}$) was present with values ranging between 16.24% at pH 9.0 and 40.1% at pH 5.0. Low amounts of Myristic acid ($C_{14:0}$) and Linolenic acid ($C_{18:3}$) were found in lipid of the yeast at almost all pH values.

Fatty acid profiles of *Candida tropicalis* at different initial medium pH are shown in Table 6. Nine fatty acids were detected and linoleic acid ($C_{18:2}$) was the predominant fatty acid at most pH values. The values of unsaturated fatty acids were 54.23, 63.03, 54.47, 56.45, 44.84 and 50.38% at pH 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 respectively. Values of saturated fatty acids were much lower than the unsaturated types at all pH values, except at pH 8.0. Lauric acid ($C_{12:0}$) was present in negligible amounts at all pH values.

High contents of stearic acid ($C_{18:0}$)(48-57%) produced by *Aspergillus* sp. was reported by Venkata and Venkata (2011) while low contents of stearic acid were produced by *Mucor circinelloides* (7%) (Vicente *et al.* 2010) and (1%) by M*ucor isabellina* (Liu and Zhao, 2007). Large amounts of palmitic acid were reported for all tested fungi ranging from 18.09 (*Aspergillus niger*) to 22.75 (*Mucor hiemalis*); while oleic acid contents were 0.1-1.6, 28 and 55.5% in lipids of *Aspergillus* sp., *Mucor circinelloides* and *M. isabellina*, respectively (Venkata and Venkata, 2011; Vicente *et al.*,2010; Liu and Zhao, 2007). The yeast strain reported by Towijit *et al*., (2014) produced the fatty acids profiles similar to butanoic acid ($C_{4:0}$) and capric acid ($C_{10:0}$), with palmitic acid

($C_{16:0}$), oleic acid ($C_{18:1}$) and linoleic acid ($C_{18:2}$) reported as the minor components. Leesing and Karraphan (2011) showed that the lipid extracted from *Torulaspora maleeae* Y30 mainly contained 25.69% palmitic acid ($C_{16:0}$), 23.39% stearic acid ($C_{18:0}$), 45.41% oleic acid ($C_{18:1}$), 3.41% linoleic acid ($C_{18:2}$) and 1.83% linolenic acid ($C_{18:3}$). The unsaturated fatty acids and saturated fatty acid amounted to about 49.31 and 50.69% of the total fatty acids, respectively. Li *et al*., (2010) reported that the fatty acids from *Rhodotorula mucilaginosa* TJY15a were mainly composed of palmitic acid, palmitoleic acid, stearic acid, oleic acid and linolenic acid. C_{16} and C_{18}fatty acids were most found in the crude lipid compounds of *Rhodotorula glutinis* cultivated on monosodium glutamate wastewater (Xue et al., 2008). Zhu et al. (2008) reported that the lipid of the yeast,*Trichosporon fermentans* mainly contained palmitic acid, stearic acid, oleic acid and linoleic acid and the unsaturated fatty acids amounted to about 64% of the total fatty acids produced by the yeast.

4. CONCLUSION

Media pH was varied to test its effect on the production of yeast viable cells, biomass, lipid and fatty acid content in *Candida utilis* and *Candida tropicalis*. The results showed a decrease in yeast viable counts when *Candida utilis* was grown in medium at pH 4.0. At pH 5.0 and 6.0, the greatest amounts of yeast viable cells were produced. Total viable cells of *Candida tropicalis* grown in media at different pH show that at pH 4.0, 5.0 and 6.0 were produced the highest amounts of yeast viable cells. At these pH values, the growth of *Candida tropicalis* was not largely influenced by the incubation time. Adjusting the initial pH to 7.0 and 8.0 produced low yeast cell yields with the lowest growth at pH 9.0.Best pH for maximum biomass production by *Candida utilis* was 5.0 while maximum amount of lipids was produced at 9.0. Maximum lipid production by *Candida tropicalis* was obtained at pH 9.0, while maximum biomass was obtained at pH 6.0. Fatty acid composition of *Candida utilis* at different initial media pH shows that at most pH values, the $C_{16:0}$, $C_{18:1}$, $C_{18:2}$ fatty acids predominated. Unsaturated fatty acids predominated over saturated types at pH 4.0, 6.0 and 9.0, while the saturated types predominated at pH 5.0, 7.0 and 8.0. Fatty acid profiles of *Candida tropicalis* at different initial medium pH show that $C_{18:2}$ was the most predominant type at various pH values. Levels of unsaturated fatty acids produced by *Candida tropicalis* were much higher than the saturated types.

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