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Full Length Research Paper

Production of bioemulsifier by an unusual isolate of salmon/red melanin containing *Rhodotorula glutinis*

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An unusual isolate of *Rhodotorula glutinis* was obtained. The yeast produces a spore and a crystalliferous protein per cell. Sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed that the parasporal crystals contained polypeptides with a molecular mass of approximately 55, 47, 40, 36 32.5, 30 and 25 kD. The yeast also produces a salmon/red pigment at late stage of exponential growth, which has physical and chemical properties that are consistent with it being a form of melanin. The organism has been used to produce bioemulsifier with emulsion index of 80% when tested against kerosene and crude oil. The bioemulsifier has been shown to be capable of eliminating about 76% of crude oil pollutant. The colonial and mycelia forms of *Rhodosporidium toruloides* and *Rhodosporidium sphaerocarpum* have been successfully isolated from the unusual organism.

Key words: Bioemulsifier, rhodotorula glutinis, teleomorph, *Rhodosporidium toruloides*.

INTRODUCTION

Strains of a red yeast isolated by Ruinen (1956) from leaf surface of citrus plants in Indonesia were classified by Deinema (1961) as *Rhodotorula graminis di Menna* CBs 3053. Since this earliest work on the genus other strains of *Rhodotorula* such as *R. glutinis* have been isolated (Ammers et al., 1964).

From the works of Marchant and smith (1968) and Strock et al. (1969) it has been shown that there are basic differences between budding in *R. glutinis* and *Saccharomyces cerevisiae*. Budding in *R. glutinis* has been shown to be similar to that of the heterobasidiomycetous species, *Sporobolomyces roseus* (Marchant and Smith, 1968). *Rhodotorula* genus, thus regarded as heterobasidiomycetous yeast have striking similarities to the basidiomycetous fungi. The heterobasidiomycetous *yeasts* has deoxyribonucleic acid base composition (guanine plus cytosine contents) well within the range of basidiomycetous fungi, whereas the *ascomycetous* yeast, in general, exhibit distinctly lower guanine – cytosine contents. Cazin et al. (1969) found that yeast suspected of being heterobasidiomycetes differed from their ascomycete counterparts in that they were capable of producing extracellular deoxyribonuclease and urease. This work is a report of some unusual features of a local isolate of *R. glutinis* and its used in the production of bioemulsifier.

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MATERIALS AND METHODS

Isolation procedure

The unusual red yeast is routinely isolated from soil samples collected in different locations in Ladoke Akintola University of Technology, Ogbomoso Nigeria. A suspension of the soil sample in sterile distilled water is plated onto potato dextrose agar (PDA). Following several days of incubation at room temperature the

salmon/red pigmented organism was subcultured on fresh PDA plates.

Preparation of coconut water

R. glutinis has been found to grow well on a medium containing coconut water. Coconut water is prepared as follows: an average size coconut is broken carefully and the liquid inside is collected in a beaker. Small pieces of coconut are blended with water, filtered with cheese cloth and then clarified by centrifugation in a sorval centrifuge using an SS–34 rotor at 8000 x g at 4°C for 20 min. The clear filtrate is then mixed with the previously set aside coconut water. The mixture is autoclaved and stored at 4°C until needed. The final product is referred to in this paper as coconut water. About 200 ml of coconut water can be obtained from a medium size coconut.

Isolation of teleomorph

R. glutinis was streaked on PDA slant and incubated in refrigerated incubator for 30 days. Cell suspensions from such aged slant were harvested using sterile distilled water. The suspension was refrigerated at 4° C for 2 to 3 days to obtain the teleospores of *Rhodosporidium toruloides* and *Rhodosporidium sphaerocarpum*, which are the two teleomorphs of *R. glutinis* obtained. Crystals, were separated from spores as previously described (Aronson et al., 1991). The identities of the teleomorphs were confirmed by the spore shape as described by Newell and Hunter (1970).

Pigment isolation and purification

Pigmented yeast cell were scraped from the surface of PDA cultures, suspended in sterile distilled water and sonicated (Braun-Sonic 2000) for 5 min on ice. The suspension was pelleted by centrifugation in a sorval centrifuge using an SS-34 rotor at 8000 rpm (4°C) for 10 min and the pellet was discarded. The pH of the supernant was adjusted to 3 before it was centrifuged for additional 10 min. The pigmented pellet was purified and characterized as described (Fuqua et al., 1991; Liu et al., 1993).

Purification and solubilization of crystaliferous protein

The yeast cell was grown with vigorous shaking for six days in potato dextrose broth. The harvested cultures were pelleted by centrifugation and then washed once with 10 mL of 1 M KCl, 5 mM EDTA, once with 10 mL of deionized water containing 5 mM phenylmethlsulfonyl fluoride (PMSF) and twice with 10 mL of deionized water aqueous solution of 0.2% Triton X-100 (Aronson et al., 1991). 6 mL of the cell suspension was layered over 18 mL of a discontinuous gradient of equal volumes of 50, 63, and 73% (w/v) sucrose (Ingle et al., 1993). The tubes were centrifuged at 16,000 X g in a sorval HB4 rotor for 60 min at 4°C (Meenakshi and Jayaraman, 1979). The inclusion band, which contains the crystal protein, was removed with a pasteur pipette, and its purity was ascertained with 1% CarbolFuchsin. When this band was contaminated with spores and/or cellular debris, it was repurified through a second discontinuous sucrose density gradient. The final band was diluted approximately five-fold with water, pelleted at 8000 x g for 20 min in a sorval ss-34 rotor at 4°C and washed twice with deionized water before being dried in a Savant speed-Vac (Aronson et al., 1991).

For the solubilization assay, 75 µL of an intact crystal suspension (2 mg/mL in distilled water) was mixed with 75 µL of different buffers (Aronson et al., 1991; Du et al., 1994). The initial buffer, 0.3 M Na_2CO_3, β-mercaptoethanol (pH 9.2) was selected based on conditions reported by others for the solubilization of crystal protein found in *Bacillus thurugiensis* strains. More reducing conditions at a higher pH were then used; and finally buffers consisting of 6 M urea-1% SDS, 0.5 M dithiothreitol, 2 mM PMSF (UDS-PMSF) at pH 9.5, 10.5, 11.5 and 12.5 were used. Protein levels were determined by the method of Bradford (1976) using bovine serum albumin as standard.

Insecticidal activity

Aedes aegypti eggs were kindly supplied by Dr. B. Diel-Jones of the Department of Biology, University of Waterloo. In order to measure the insecticidal activity against *A. aegypti,* protoxins solubilized in 0.3 M Na_2CO_3, 2% β-mercaptoethanol (pH 9.7) were dialyzed at 4°C against several changes of 0.03 M $NaHCO_3$ (pH 8.5) (Aronson et al., 1991). Ten fourth instars larvae were incubated at 28°C (Khawalled et al., 1990) with the suspension of the inclusion in the latter butter. The assays were performed in duplicate and mortality was scored after 48 h (Ingle et al., 1993). In addition, intact cells harvested from PDA slant after four days of incubation and other cells harvested from slant after 30 days of incubation were separately assayed for insecticidal activities.

SDS-PAGE

Sodium dodecyl sulfate (SDS-PAGE) was performed according to Laemmli (1970) with 12% separating and 4% stacking gels. Following electrophoresis the gels were stained with 0.4% Coomassie blue R-250. The molecular masses of the solubilized proteins were determined in comparison with known protein standard (Bio-Rad).

Electron microscopy

Scanning electron microscopy was carried out as described by Calabrese and Nikerson (1980) with the technical assistance of Mr. Dale Weber.

Production of bioemulsifier

The local isolate of *R. glutinis* has been found to produce both amylase and lipase. Its ability to utilize starch and soybean oil in the production of bioemulsifier was therefore investigated as follows. A 4% cassava starch broth (CSB) medium of the following composition in g/liter is used: Cassava starch, 40; yeast extract 5, $MgSO_4$, 2; kH_2PO_4, 5. The medium was thoroughly boiled to homogenization before sterilized. A ten-liter medium was inoculated with yeast cell suspension obtained from growth from 35 yeast-extract Petri-dish culture. 10, 45 and 75 mL of soybean and palm oil were separately added to the CSB medium to initiate lipase formation. Fermentation was allowed to take place inside a home-made twenty liter bioreactor equipped with propeller for agitation and source of aeration. After three days of fermentation 100 mL of oil was added and fermentor allowed to run for another 72 h.

Level of Lipid Inhibition to *R. glutinis*

The level and nature of lipid inhibition to *R. glutinis* was determined by inoculating the 4% cassava starch broth (CSB) as shown in Table below: .

Fermentation was allowed to go on for about four days. Rate of fermentation was measured by change in medium color from dirty

Table 1. Method of inoculum.

Medium code	Inoculum description
A	Cell suspension mixed with same volume of soybean oil and allowed to stand for one hour.
B	As in A above but palm oil was used instead of soybean oil
C	Cell suspension added separately to CSB medium after addition of same volume of soybean oil.
D	Same as in C above except that palm oil was used

CSB = Cassava starch broth.

gray to white. This is based on the previous work of the author that establishes the fact that the degree of fermentation of CSB is dependent on how whitish the medium becomes.

Determination of emulsion index

Emulsification index was determined as described (Cooper and Goldenberg, 1987) in which 6 mL of kerosene or crude petroleum were separately added to 4 mL of the bioemulsifier and vortexed at high speed for 2 min. The emulsion index is the height of the emulsion layer, divided by the total height, multiplied by 100.

Bioremediation experiment

Modified method described (Nwachukwu and Ugoji, 1995) in which garden soil (200 g) was contaminated by mixing with 500 g Nigerian crude oil was used. This level of contamination has been known to make the soil sterile (Nwachukwu et. Al., 2001). 500 mL of the bioemulsifier was then added and this set-up was designated "experiment (E)". A control consisted of the same materials present in E but without the addition of biemulsifier. Three replicates of E and the control were kept at room temperature throughout the six weeks for oil degradation. They were watered at weekly intervals.

Determination of residual oil concentration (ROC)

The mean changes in ROC over six weeks' period were determined by a gravimetric method. Three samples of 10 g each were randomly and aseptically taken from each of the replicates of the set-up at weekly intervals for six weeks and analyzed. The samples were collected to include surface, middle and bottom samples and mixed thoroughly before analyzing. Oil was extracted from each sample by mixing 5 g soil with 30 mL volume of n-hexane; dichloromethane solvent system (1:1) and stirred for 5 min in a beaker at room temperature (Yveline et al., 1997). The mixture was filtered through a glass wool in a funnel. The extraction procedure was repeated three times and combined.

The solvent system was evaporated by placing the extract in an oven at 80°C for 5 min. The beaker containing the oil extract was allowed to cool in a desiccator to room temperature and then weighed. The weight of the ROC was obtained by difference (Yveline et al., 1997).

RESULTS

Morphology

The yeast is capable of forming a spore and a crystalliferous protein per cell usually in 24-48 h following growth on PDA at 26°C. The spores are oval shaped with an average size of approximately 1 x 1.9 µM (Figure 1). The spores and the crystal protein are found at the polar ends of the cell (Figure 2). On agar plates the colonies are medium sized (approximately 2-3 mm in diameter), slightly shiny, with flat smooth boarders and have a salmon/reddish colour (Figure 3). A 24 h culture of vegetative cells consists of cocobacilli cells approximately 1.5-2.5 x 4.1 – 6.0 µM (Figure 4).

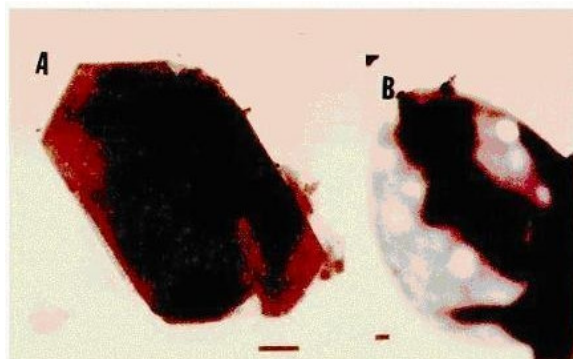

Figure 1. Reversed image shadow cast transmission electron micrograph of crystal (A) and spore (B) from *R. glutinis.* Bar=0.1µm

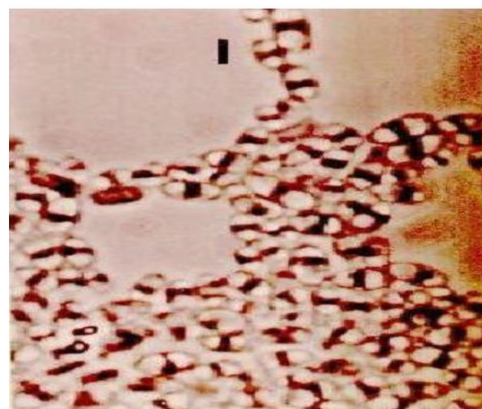

Figure 2. Light microscope image *of R. glutinis.* Bar=2.15 µm.

Figure 3. Salmon/red coloureed colonies of *Rhodotorula glutinis.*

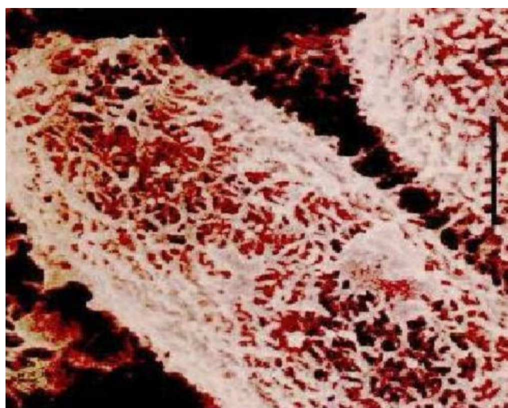

Figure 4. Scanning electron micrograph of Rhodotorula glutinis.

Table 2. Effect of medium composition on the formation of insecticidal crystal and salmoned/red pigment by *R. glutinis*.

Medium	Growth Pigmentation	Crystal formation, % of cells
LB agar	++ light red	2
LB agar + 5mg/mL tyrosine	++ light red	2
NA	+ light red	<1
PDA	+++ salmon/red	3
PDW agar	+++ salmon/red	>95
CWA	+++ salmon/red	>99.5

Growth Conditions

R. glutinis grew poorly and produced crystals and pigment poorly on both nutrient agar and Luria broth agar compared with on potato dextrose agar supplemented with coconut water (PDW) and 100% coconut water (WW) (Table 2). The organism produced the salmon/red pigment when it was grown on PDA but with poor crystal formation. When the organism was cultured on PDA containing 10% coconut water, it not only produced the pigment but also more than 95% of the cells formed crystal inclusions. When the growth medium consisted entirely of coconut water and agar, more than 99% of the cells form crystal inclusions as well as pigment.

Isolated teliomorphs

When *R. glutinis* is streaked on PDA slant and incubated in refrigerated incubator for 30 days, the suspension obtained in sterile distilled water is found to be made up of lysed cell debris with free cell inclusions. The cell inclusions are made up of crystal proteins and teliospores. If the suspension is stored at 4°C for 2 to 3 days the cell debris the pigment and some teliospores settled while the crystals and many of the teliospores remain in the suspension. Crystals are separated from the spores by sucrose gradient centrifugation. To confirm purity, when safranin is added and observed under x 40 objective lens, crystals were seen to be colourless as against spores which take up safranin.

The teliospores were identified as described by Newell and Hunter (1970). *Rhodosporidium toruloides* has teliospores which are highly angular whereas the teliospores of *Rhodosporidium sphaerocarpum* are spheroidal (Figures 5, 6).

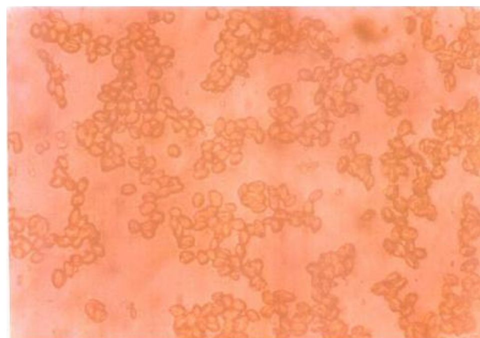

Figure 5. Teliospore of *R. toruloides* x 100.

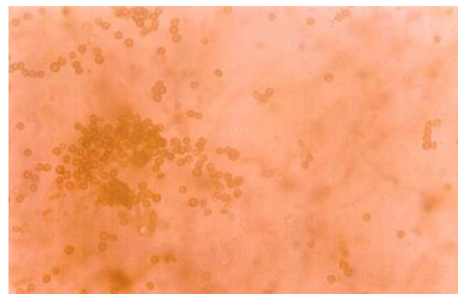

Figure 6. Teliospore of *R. sphaerocarpum* X 100.

Characterization of the salmon/red pigment

The pigment had characteristics consistent with melanin including solubility in 1 M NaOH; precipitation in 1 M HCl or 5 mM $FeCl_3$; and bleaching with 20% H_2O_2. Furthermore, the purified pigment shows a steady increasing absorption with no peak at wavelengths of UV high ranging from 300 to 210 nm (Figure 7).

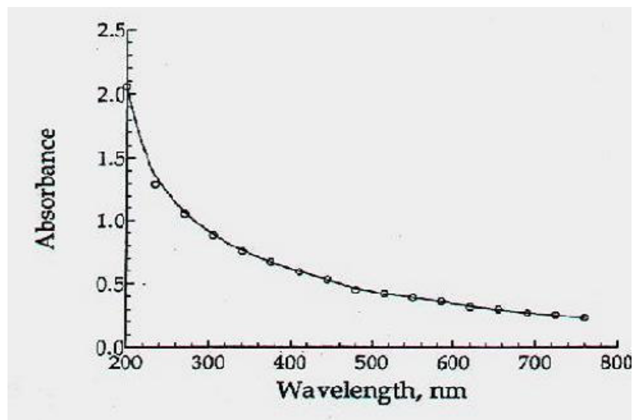

Figure 7. Ultraviolet and visible light absorption of the salmon/red melanin isolated from a culture of *R. glutinis*.

Table 3. Solubilization of inclusion protein from *Rhodotorula glutinis*.

Buffer	Amount of solubilized protein, µg/mL
0.03 M Na_2CO_3, 0.1% MBE,pH 9.2	29.1
0.03 M Na_2CO_3, 2% BME, pH 10.0	59.4
0.80 M Na_2CO_3, 5% BME, pH 12.5	41.5

Crystal solubilization

The behaviour of crystalliferous proteins in solubilization buffers at different pH values can serve as an indication of the insecticidal activity of the protein in the crystal (Aronson et al., 1991; Du et al., 1994). Therefore, the solubility of the crystaliferous protein in two types of buffers viz. β-mercaptoethanol pH 9.2, 10.0 and 12.5 and UDS-PMSF at pH 9.5, 10.5. 11.5 and 12.5 were investigated. For the first set of buffers, the highest amount of solubilized protein was obtained when moderate reducing conditions (i.e. 0.3 M Na_2CO_2, 2% β-mercaptoethanol pH 10) (Aronson et al., 1991) were used to dissolve the crystal. Using a buffer that was more reducing and provided a higher pH (i.e. 0.8 M Na_2CO_3, 5% BME pH 12.5), a lower amount of protein was solubilized. With the second set of solubilization buffers maximal solubilization occurred at pH 11.5 (Table 3; Figure 8). The solubilized protein obtained following solubilization of the crystal with UDS-PMSF PH 11.5 buffer was examined by SDS-PAGE. Seven major bands with the following molecular weights were observed: 55, 4 7, 40, 36, 32.5, 30 and 25 kDa (Figure 9).

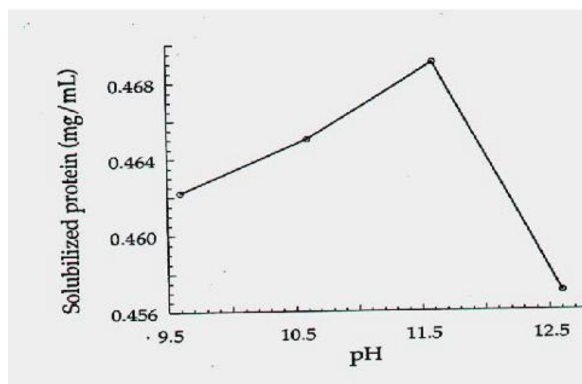

Figure 8. Effect of pH on the solubility of crystals from *R. glutinis***.**

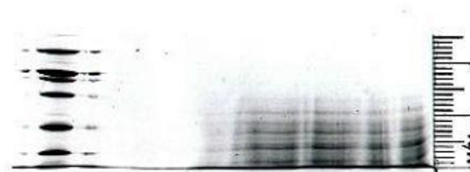

Figure 9. Protein composition of *R. glutinis* crystals determined by SDS-PAGE (15%) (The bands between 1.3 and zero mm have molecular weights of between 66 and 31 KDa).

Insecticidal activity

When the protoxin and intact crystaliferous protein of the yeast were tested against *A. aegypti* larvae and *Trichoplusia ni (*Cabbage lopper) larvae no appreciable insecticidal activity was observed. In addition, the intact cells harvested from four days old PDA slant showed no insecticidal properties. However, the suspension obtained from 30 days old slant killed all the ten larval of *A. aegypti* for each of the replicate within 48 h. Suspension from the 30 days old slant has been shown under light microscope to contain lysed cells with freed teliospores and crystals. Suspension from four-day-old slant is made up of intact cells.

Table 4. Fermentation rate of Rhodotorula glutinis.

Medium code	*Fermentation rate			
	24 h	48 h	72 h	7 days
A	-	-	-	-
B	-	-	-	-
C	+	++	+++	++++
D	-	-		

*Rate determined by degree of color change of CSB medium
- = No fermentation
+ = Low rate of fermentation
++ = Fair fermentation rate
+++ = Good fermentation rate
++++ = Very good fermentation rate
Media A, B, C, and D already defined in Table 1.

Table 5. Final product formation from *Rhodotorula glutinis.*

Medium code	Product formed		
	1	2	3
A	+	-	-
B	+	-	-
C	-	+	-
D	-	+	-

+ = product obtained
- = product not obtained
1 = uncoupled oligosaccahride
2 = oligosaccahride-structured triacylglcerol ligand
3 = uncoupled lipid
Media A, B, C, & already defined in Table 1.

Table 6. Effect of amount of lipids addition on product formation.

Type of Lipid	Amount added (ml/L)	Ligand Formation
a. Palm oil	10	-
	45	-
	75	-
b. Soybean oil	10	+
	45	-
	75	-

+ = ligand formed
- = ligand not formed

Bioemulsifier of *R. glutinis*

The yeast grows readily and forms its crystals in coconut water and PDA supplemented with coconut water. The organism has been found to posses both lipase which hydrolyses soybean oil to triacylglycerol and amylase which hydrolyses cassava starch to oligosacharide (result not shown). The ability of the yeast to couple these hydrolyzed products to bioemulsifier was therefore investigated.

When the yeast cell used as inoculum is suspended in same volume of either soybean oil or palm oil for one hour before its addition to broth (media codes A and B, Tables 4 and 5) there was no fermentation, as the medium colour did not turn from brown to white. However, when cell suspension was added separately to CSB medium after the addition of the same volume of soybean oil, (medium C; Tables 4 and 5) the medium colour changes from brown to whitish colour after about six to seven days of incubation in the fermentator to initiate lipase formation (Table 6). After six days of fermentation in a 20-liter bioreactor, the amylase of the organism hydrolyses cassava starch to oligosaccharide which was coupled with structured triacylglycerol produced from soyabean oil by the lipase of *R. glutinis* to form a ligand whitish, slimy and milky in appearance. The ligand produced has emulsification index of 80% when tested separately against kerosene and crude petroleum (Table 7). The ligand is only formed when 10 mL of soybean oil is added (Table 6). The residual oil concentration was reduced by 76.9% by the ligand (Table 8).

Table 7. Emulsification index.

Hydrocarbon	Emulsion index
R. glutinis bioemusifier	
Kerosene	80
Crude petroleum	80

Table 8. Residual oil concentration (g/5 g soil).

Week	Experimental	Control
1	3.9	4
2	3.1	3.9
3	2.2	3.8
4	1.5	3.7
5	1.2	3.5
6	0.9	3.4

DISCUSSION

It is possible to confuse the strange isolate of *R. glutinis* discussed herein with *B. thuringiensis* as it contains one spore and a crystal per cell (Figures 1,2). The following features however confirms that it is a yeast and not

bacterium: it grows very readily on potato dextrose agar with very poor or no growth on nutrient agar. Isolation of two teleomorphs viz *Rhodosporidium toruloides* and *Rhodospordiium sphaerocapum* is the conclusive evidence that the organism is *R. glutinis* (Figures 5,6). As the organism produces its crystals and grows more readily in fatty acid medium like coconut water, it belongs to the group of yeast referred to as oleaginous yeast. Oleaginous organisms are those organisms that accumulate lipid in cell.

The crystal of the yeast has been found to contain seven major polypeptides of 55, 47, 40, 36, 32.5, 30 and 25KD (Fig. 9), which is completely different from all other reported *B. thuringiensis* strains. For example, the molecular weights of the proteins from solubilized crystals inclusions from sixteen different *B. thuringiensis* were reported to be between 160 and 40 Kda (Calabrese et al., 1980), which is significantly different from what is observed.

The crystal from *R. glutinis* was not solubilized by any of the buffers used for this purpose with different strains of *B. thuringiensis.* Thus, 10 mM EDTA, 50 mM NaOH used to solubilize the *B. thuringiensis* israelensis crystal protein (Ingle et al., 1993); 0.05 N NaOH used to solubilize the crystal from *B. thuringiensis subsp. thuringiensis* (Meenakshi and Jayaraman, 1979) and the solubilization buffer referred to as 'cracking buffers' by Calabrese et al. (1980) used for the solubilization of the crystals of sixteen strains of *B. thuringiensis* were all ineffective. All these further show that the organism though related to *B. thuringiensis* but it is yeast.

Candida bogoriensis seems to behave very closely to this unusual *R. glutinis* strain. When *C. bogoriensis* cells are grown on a glucose yeast extract medium, the organism produced two types of extracellular lipids (Deinema, 1961). One was obtained as a liquid by solvent extraction of the cell-free culture left after centrifugation. The other product remained as crystals with the yeast cells. The crystallline lipid, separated from the yeast cells by acetone or alcohol extraction, melted at 74 to 76°C after recrystalization from aqueous acetone. Apart from the fact that the unusual strain of *R. glutinis* did not produce the lipid of *C. bogoriensis* in glucose-yeast extract medium the crystals of *R. glutinis* cannot be extracted with acetone and alcohol, as they are not soluble in those solvents.

From this work an attractive bioemulsifier has been produced by manipulating a culture containing carbohydrate and lipid to produce a consistent single homogeneous layer. To form a single homogeneous layer with water and lipid is a difficult task since water is not miscible with lipid. The unusual isolate of *R. glutinis*, which is not only oleaginous but also produces an amylase, has been shown to solve this problem. The lipid and carbohydrate content of the medium is hydrolyzed simultaneously by lipase and amylase enzyme system of the organism.

Lipid hydrolysis by lipase producing organisms is a slow process. As a result, lipid hydrolysis at a very large scale is very problematic. Although, the hydrolytic enzyme reaction of lipid can be rapid under the right condition, it is difficult to obtain high conversion; so the reaction is very slow compared with steam hydrolysis, and several days may be required for good conversion in a stirred tank reactor even if calcium is added to counteract the inhibitory action of the fatty acids.

Several expensive techniques have to be designed to bypass lipase inhibition by lipid during hydrolysis. For example, hydrolysis may have to be at very high temperature or in expensive specially constructed vessels.

It seems there is a critical concentration in a broth culture at which lipid hydrolysis cannot take place. Above such concentration, oleaginous and lipidophilic organism will adsorb lipid in excessive amount. This has been clearly demonstrated several times in the unusual strain of *R. glutinis*. When the yeast is placed either in a broth containing high amount of lipid or in 100% concentration of lipid; the bacterium lipid granules and other cell organelles adsorb excessive amount of lipid and become swollen. Such cellular abnormalities have been observed several times in the yeast by simply staining such cells with lactophenol.

When attempt was made to use such abnormal cells for lipid hydrolysis it was observed that either the hydrolysis does not take place or it goes on very slowly (Tables 4,5). The excessive lipid in cells may either cause a change in cell physiology making the cells to produce little or minute amount of lipase and/or the lipid may block the cell wall and membrane pores making it impossible for extracellular lipase(s) to diffuse into the medium. There has always been a fermentation delay or no fermentation at all in the cultures that contained abnormal cells.

This has led the author to propose an hypothesis as follows: to produce an attractive bioemulsifier there is a need to couple an oligosaccahride with structured triacylglycerol to produce a ligand. Therefore an oleaginous and/or lipidophilic organism capable of producing amylase will be a very important tool. However great care should be taken so that the organism does not adsorb excessive lipid. This is because if an oleaginous and/or lipidophilic organism is allowed to adsorb excessive lipid, the accumulated lipid induces physiological abnormalities, which has negative effects on the total turnover of lipase. Even the little lipase that may be produced may be expressed with great difficulty into the medium as a result of the cell wall and membrane pore blockade by the adsorbed lipid.

This hypothesis is not only limited in the production of attractive bioemulsifier. In antifoam addition to cultures, a great care must be exercised so that the antifoam does not induce abnormal cells. This will be best done by making sure that the cell does not have direct contact with the antifoam, which is the larger broth.

To prevent lipidolethal effects of lipid on lipase about 0.1 per cent broth volume of the lipid to be hydrolyzed should be used to induce lipase production in a fermentor over a period of time. When enough lipase has been formed, the whole lipid to be hydrolyzed should be added. Such precautions have been tried several times using the yeast and have been found to be effective. Specifically, to obtain high yield of the oligosaccahride-structured triacylglycerol ligand, 10 ml of soybean oil is added separately to 10 L CSB medium in a 20-L bioreactor and stirred for about ten min. Cell suspension obtained from surface growth of 35 yeast-extract agar plates were then added. The bioreactor is allowed to run for three days.

The 10 ml of the soybean oil added is not only serving as antifoam but also as lipase inducers. On the third day, 100 ml of soybean oil is then added to the 10-liter culture (10 ml/liter) and fermentor allowed to run for another three days. If this protocol is followed, at the end of fermentation the culture appears whitish and milky (Tables 4, 5, 6). The coupled oligosaccahride-structured triacylglycerol ligand makes the whole culture to become a consistent single homogeneous layer as opposed to ordinary lipid, which floats on the culture, and uncoupled oligosaccahride, which settles at the bottom of the culture. If the above protocol is not followed, for example, if the cell suspension is mixed with the 10 ml of oil before added to CSB broth or if more oil is used in place of 100 ml of oil, the fermentation will not proceed (Tables 4, 5, 6). Such unfermented culture separates unto three layers as agitation and aeration cease. The top layer is made up of ordinary lipid. The middle layer is always made up of dirty brown unfermented liquid while the bottom layer is made up of unfermented starch and/or uncoupled oligosaccharide.

It was observed in this work that although a four day old cell suspension of *R. glutinis* from a PDA slant does not kill mosquito larvae, the suspension obtained from a 30 day old slant killed all the mosquito larvae within 48 h. While the 4-day old cell suspension appear as intact cells under the microscope the 30 day old cell suspension are made up of disintegrated mass with freed crystals and spores. Maybe the mosquitocidal actity is due to the free teliospores of *Rhodosporidium spaerocarpum* which is already known to possess insecticidal property. The unusual *R. glutinis* species will be further characterized using ribosomal RNA typing.

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