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Evaluation of fungus from Traditionally Fermented Cow Milk and Their Application in the Production of Biosurfactant

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Abstract

Yeast are chemoorganotrophs that obtain carbon mostly from hexose sugars, such as glucose and fructose or disaccharides such as sucrose and maltose and these carbon sources are frequently present in yoghurt, cow milk and other fermentable products. Three fungal isolates were isolated from traditionally fermented cow milk which are *saccharomyces cerevisiae, Saccaharomyces pombae* and Pichia anomala. Isolation was carried out by serially diluting the samples in the laboratory which were fermented at room temperature for 24 hours.

All of the yeast isolated are glucose and sucrose fermentors. Biosurfactant was also produced after growing the yeast in a prepared Sabouraud Dextrose Agar (SDA) containing 1.0ml of crude oil at the temperature range between 27-30°C for 72 hours. After incubation oil displacement test was carried out to determine if biosurfactant can be produced from the yeast, from the result Saccharomyces cerevisiae and saccohromyes pombae showed clear zones with diameter 96.0 mm/cm^3 and 40.4mm/cm^3 respectively.

Conclusively, biosurfactant was extracted from *Saccharomyces cerevisiae* which was measured to be 220.mg/ml while *Saccharomyces pombae* did not produced biosurfactant when grown in Sabouraud Dextrose agar (SDA) containing 1.0 ml of crude oil at a room temperature for 24 hours. Based on the above result it was recommended that, yeast can be isolated from fermented cow milk and it is able to utilized sugars present in milk.

Yeast isolated from fermented cow milk can be used to produce biosurfactants industrially. There is need to increase interest in the use of yeast to produced biosurfactant so as to increase the amount of biosurfactants produced

Keywords: *Biosurfactant; Cow milk; Chemoorganotrophs; Fungal; Yeast*

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Introduction

Yeast are unicellular fungus that has a single nucleus and reproduces either asexually by and transverse division or sexually through bud formation. Each bud that separate can grow in to new cell and some group together to form colonies. Generally yeast cells are larger than bacteria, varies considerably in size, and is commonly spherical to egg shape. Yeast lack flagella and cilia but posses most other eukaryotic organells (Presscott., *et al.* 2011).

Yeasts are chemoorganotrophs that obtain carbon mostly from hexose sugars, such as glucose and fructose or disaccharides such as sucrose and maltose (Barnett, 1975). Some species can also metabolize pentose sugars like xylose (Chaudhary and Qazi 2006), alcohols and other organic acids. Yeast species require either oxygen for aerobic cellular respiration (obligate aerobes), or are anaerobic but also have aerobic methods of energy production (facultative anaerobes).

The useful physiological properties of yeast have led to their use in the field of biotechnology. Fermentation of sugar by yeast is the oldest and largest application of this technology. Some isolates have the ability to carry out an alcoholic fermentation while others lack this property. Thus, many types of yeasts are used for making many foods; yeast in wine fermentation (Chaudhary and Qazi 2006) and for xylitol production (Sreenivas Rao., *et al*. 2004).

Yeast cells, especially *Candida species*, are common in oral cavities and in immunocompromised and immunocompetent individuals (Chaudhary and Qazi, 2006), with a predominace of *Candida albicans*. Their isolation from the mouth can be used to investigate reduced salivary flow rate (Parvinen and Larmas 1981), excessive consumption of fermentable carbohydrates (Samaranayake., *et al*. 1986). Their isolation can also be used in dealing with infectious diseases, surgeries, antibiotics administration and medical immunosuppression (Chaudhary and Qazi 2006), which can trigger the development of candidosis caused by pathogenic *Candida species*.

These are therefore, good reasons to evaluate and improve the characterization methods currently used for these microorganisms (Rodrigues, 2000). Microbial biosurfactants are amphiphilic metabolites with a pronounced surface activity with a broad range of chemical structures (such as glycolipids, lipopeptides, polysaccharide-protein complexes, phospholipids, fatty acids, and neutral lipids) with several advantages over chemical surfactants (Chanchaichaovivat., *et al*. 2007). That is, low toxicity, biodegradable, and effective at different ranges of temperature and pH (Oyeleke and Jibrin, 2009). They are being used for industrial applications in the pharmaceuticals, biomedical, and food processing industries (Saharan., *et al.* 2014).

Biosurfactants are amphipathic molecules mostly excreted by microorganisms outside the cells, and in some cases attached to the cells, predominantly during growth on water-immiscible substrates. They contain both hydrophilic and hydrophobic parts. Hydrophilic parts can consist of amino acids or peptides, phosphate, alcohol and mono- di- or poly-saccharides. Hydrophobic parts consist of unsaturated or saturated fatty acids (Desai and Banat, 1997).

Several properties and physiological functions in the producer organisms have been described for different groups of biosurfactant, that include solubility of hydrophobic compounds, heavy metal binding, bacterial pathogenesis, cell adhesion and aggregation, quorum sensing, and biofilm formation (Van Hamme., *et al.* 2006). Microbial biosurfactants are amphiphilic metabolites with a pronounced surface activity with a broad range of chemical structures. In terms of their structure, biosurfactants are; Hydroxylated and cross-linked fatty acids, Polysaccharide-lipid complexes, Glycolipids, Lipoprotein-lipopeptides, Phospholipids and Complete cell surfaces (Boulton, 1987).

Surfactants are chemically synthetic substance that are produced industrially which are employed in different fields. Nowadays, microbiologist introduced a technology that is used to produce biosurfactants from microorganisms which are used instead of synthetic surfactants (Cooper and Paddock, 1984). The study of biosurfactant production by yeast has been growing in importance, with production being reported mainly by the genders *Candida species, Pseudozyma species* and *Yarrowia species*.

The great advantage of using yeast in biosurfactants production is the generally regarded as safe (GRAS) status that most of these species present, for example *Yarrowia lipolytica, Saccharomyces cerevisiae* and *Kluyveromyces lactis.* Organisms with GRAS status are not toxic or pathogenic, allowing the application of their products in the food and pharmaceutical industries (Barth and Gailard, 1997). The aim of this research is to isolate and characterize yeast and to determine its application in biosurfactant production. The objectives for the study are to isolate and characterize yeast from traditionally fermented Cow milk, and to demonstrate the application of the isolates in biosurfactants production.

Materials and Methods

Sample collection

The sample was collected from five (5) different Cows at Sidi Mamman Asarakkawa farms in Sokoto metropolis by using sterilized containers. The collected sample was kept at room temperature for about 2 days to ferment. The fermented samples were taken to mycology laboratory of Usmanu Danfodiyo University, Sokoto for further analysis.

Isolation of Yeast

The samples were aseptically picked using a sterilized syringe and homogenized into 10 ml of distilled water. From each sample, 1:6 dilutions was subsequently made using distilled water followed by making a 6 fold serial dilution. 0.1 ml of each diluents was inoculated in duplicate into the prepared medium (MOelofse., *et al.* 2008).

Preparation of Media

The media to be used in this work was Sabouraud Dextrose agar (SDA). The media was prepared according to the manufacturer's instructions. The powder (5.9g) was dissolved in 180 ml of distilled water. The mixture was heated until it dissolved and autoclave at 121°C for about 15 minutes (Cheesbrough, 2006).

Inoculation of Samples

The prepared media was allowed to dry in the Hot air oven at a temperature of about 121°C for about 15 minutes. A small quantity of 0.1mililitre of the diluents from the third test tube was taking using a sterilized syringe. Two (2) drops was inoculated on the surface of solidified agar, and then spread on the surface of the agar using a sterilized bend glass rod quickly and carefully. The same procedures were carried out on the rest of the samples. All the plates were then incubate for 3 days at 37°C (MOelofse., *et al*. 2008).

Characterization of Yeast

Gram's staining

Gram staining was performed to distinguish whether the isolates are yeast or not and to examine their different morphologies. A drop of normal saline will be placed on a slide using a heated wire loop (after allowing to cool) a colony will picked and emulsified on the slide and fix the smear so as to allowed cells to retain on the slide. Crystal violet stains will be applied on the fixed smear which was served as a primary stain and leave for 30–60 seconds. Rapidly wash off the stain with clean water. Tip off all the water, and apply iodine on the smear to serve as a mordant and leaved for 30–60 seconds. Washed off the iodine with clean water.

Decolorize rapidly (few seconds) with alcohol. Wash immediately with clean water. Safaranin was then applied which served as a counter stain and leave for 2 minutes. Washed off the safaranin with clean water. Wipe the back of the slide clean, and placed it in a draining rack for the smear to air-dry. Examine the smear microscopically with the X40 objectives magnification to examine the morphology of the organisms by applying oil immersion on the smear. Yeast was seeing as dark pale purple (Cheesbrough, 2006).

Biochemical Characteristics of Isolated Yeast

The ability of yeast to utilize different sugars (Glucose, Maltose, Lactose and Sucrose) were tested for by weighing 6g of peptone and dispensing in 100 ml of distilled water and adding 0.1 of yeast extract and 6g of the sugar (this was done for each sugar). 5 ml of the solution from the initial prepation was then dispensed into different test tube respectively. The test tubes were then covered with cotton wool and aluminum foil and sterilized by autoclaving at 121°C for 15 minutes. The set up was then cooled to about 45°C and samples from petri dishes 01-10 were inoculated into the different test tube. The inoculated tubes were benched at room temperature for 24-48 hours (Cheesbrough, 2006).

The same procedure was carried out for each of the sugars. After incubation, change in coloration and turbidity showed that the yeast had fermented the sugars and absence of turbidity detected otherwise. The final results obtained were compared to a standard chart and the yeast identified (Cheesbrough, 2006).

Test for Biosurfactants Production

In order to test the ability of the isolates to produce biosurfactants, carbon substrate will be used. This is crude oil (Bony light- a Nigerian type of crude oil). Using a sterilize conical flask, 3.36g of Sabouraud Dextrose agar (SDA) will be prepared according to manufacturer's instructions in form of broth medium and dispensed into test tubes. To each of the test tube, 1ml of crude oil will be added (SeghalKiran., *et al*. 2010). The set up was replicated into 2 times and incubated at room temperature for ten (10) days. The amount of biosurfactant produced will be monitored after 2 days interval during the period of incubation. To test whether the isolates produce Biosurfactant molecules, the supernatant will be test by the oil spreading test (Rodrigues., *et al.* 2006).

Oil spreading Test

A Petri dish (9 cm diameter) was filled with tap water and a droplet of motor oil will be added. The oil formed a hydrophobic film on the surface. Twenty (20) microliter of supernatant will be also deposited on this surface. The diameter of the appearing clear zones were then measured (Rodrigues., *et al.* 2006).

Extraction and Purification of Biosurfactants

To extract the biosurfactants produced by the yeast organism, the yeast cells were removed by centrifugation (12000 rpm for 10 minutes) using centrifugation machine, and culture supernatants were acidified with HCL (0.1M) to obtain the pH of 2. The extraction of the biosurfactants was performed with a mixture of methanol (4v/v) which was added into the supernatant, after being vigorously shaken, and allowed to stand until phase separation. Extract were concentrated by rotary evaporation and then anhydrous sodium sulfate was added to remove water (Thampaayak., *et al*. 2008). The biosurfactant was appeared white crystals.

S/n.	Yeast isolates	Frequency of occurence.	Percentage Frequency (%)
1	*Saccharomyces cerevisiae*	3	15
2	*Saccharomyces pombae*	1	5
3	*Pichia anomala*	8	40
4	*Aspergillus niger*	2	10
5	*Aspergillus fumigatus*	6	30
	Total	20	100

Table 1: *Organisms Isolated from Fermented Cow Milk.*

Results

The organisms that were isolated from this research were: *Saccharomyces cerivisiae, Saccharomyces pombae and Pichia anomala*. Table 1 shows organisms isolated from traditionally fermented cow milk. Table 2 shows fungal species isolated from cow milk and their biochemical characteristics. After incubation at temperature ranging from 27-30°C for one (1) day.

The organisms isolated from sample number three (3) is more significant than other samples with 40% of occurrence, followed by sample 5 having 30% of occurrence, while sample two and four (2 and 4) showed low growth having 5 and 10% of occurrence respectively. 15% of occurrence was observed from sample number 1. The biochemical test showed that all the isolates are glucose and sucrose fermentors, two of the isolates are lactose fermentors, while two of the isolates ferment xylose negatively.

S/N	SAMPLE	GLU	SUC	LAC	FRU	XYL	ORGANISMS
1	C_1	+	+	-	+	-	*Saccharomyces cerevisiae*
2	C_1	+	+	+	+	+	*Pichia anomala*
3	C_3	+	+	-	+	+	*Saccharomyces pombae*
4	C_3	+	+	+	+	+	*Pichia anomala*
5	C_4	+	+	-	+	-	*Saccharomyces cerevisiae*
6	C_4	+	+	-	+	-	*Saccharomyces cerevisiae*

Key: *C_1 = Cow number 1, C_2 = Cow number 2, C_3 = Cow number 3, C_4 = Cow number 4 and C_5 = Cow Number 5, Glu = Glucose, Suc = Sucrose, Lac = Lactose, Fruc = Fructose, Xyl = Xylose.*

Table 2: *Fungal Specie Isolated from Cow milk.*

The results obtain from oil spreading test show that Biosurfactants are highly significant produced by *Saccharomyces cerevisiae* while *Pichia anomala* do not form any clear zone of inhibition after inoculating the isolates in conical flask containing SDA broth with 1 ml of crude oil and incubated at room temperature for 2 weeks. After incubation period the media broth was centrifuge so as to separate cells and supernatant, the supernatant was used to carried out oil spreading test and diameter of clear zone formed after dispersing 1 drop of supernatant on the the surface of the plate containing 40 ml of distilled water and 10 um of motor oil (petrol) was measured.

S/N	Yeast	Diameter of clear zone of Inhibition (mm)
1	*Saccharomyces cerevisiae*	96.0
2	*Pichia anomala*	No zone
3	*Saccharomyces pombae*	40.4
4	*Pichia anomala*	No zone
5	*Saccharomyces cerevisiae*	31.6
6	*Saccharomyces cerevisiae*	67.0

Table 3: *Oil Spreading Test Applied for screening for Isolate Produced biosurfactants.*

Petri dish was filled with distilled water and a droplet of motor oil was added. The oil forms a hydrophobic film on the surface. 10 microliters of the suspension of each isolate was dispensed on the surface. The diameter of the appearing clear zones was measured.

The result from the extraction of biosurfactant was measured after extraction of the supernatants which was acidified with 10 ml of Hydrogen chloride. High amount of biosurfactant was extracted from Saccharomyces cerevisiae isolated from fermented cow milk

and no biosufactant extracted from *Saccharomyces pombae*. It can be concluded that *Saccharomyces cerevisiae* was able to produce biosurfactant when grown in SDA with crude oil serving as a source of energy for the yeast. Table 4 shows the amount of biosurfactant produced by *Saccharomyces cerivisiae*

S/N	YestIsolates	Amount of extracted biosurfactant (mg)
1	*Saccharomyces cerevisiae*	220.0
2	*Saccharomyces pombae*	00.0

Table 4: *Biosurfactants extracted from the Isolate.*

Yeast cells were removed by centrifugation (12000 rpm for 10 minutes) using centrifugation machine, and culture supernatants were acidified with HCL (0.1M) to obtain the pH of 2. The extraction of the biosurfactants was performed with a mixture of methanol (4v/v) which was added into the supernatant, after being vigorously shaken, and allowed to stand until phase separation. Extract were concentrated by rotary evaporation and then anhydrous sodium sulfate was added to remove water. The biosurfactant appeared as white crystals (Thampaayak., *et al*. 2008).

Discussion

The yeast were isolated from different fermented cow milks of different cow under study, they includes *Saccharomyces cerevisiae*, Pichia anomala and *Saccharomyces pombae* as showed in Table 1. The yeasts were found to be fermentative in the breakdown of glucose and fructose sugars. Several workers such as Oyeleke and Jibrin (2009), Mohd., *et al.* (2011) have reported the activities of some of the yeast strains in the fermentation processes. Species of yeast like *Candida* have not been extensively reported as fermentative yeast for industrial utilization such as the production of biosurfactants nor in the production of other useful organic compounds except as causal agents of human diseases.

Ellis., *et al.* (2007) reported Candida tropicalis as the causal agent of candidiasis in man; they are opportunistic fungi which live in most human organs. However, recent reports by Kathiresan and Saravanakumar (2011) and Senthilraja., *et al.* (2011) have shown that species of Candida are not just pathogens but can be useful tools for bioethanol production, as they were able to use Candida tropicalis and Candida albicans isolated from marine environment to produce biosurfactants.

In yeast (fungi) taxonomy, conventional methods such as physiological and morphological analysis are not enough to adequately identify yeast especially with the emergence of new strains. Molecular identification is known to provide a more objective separation of genera and species than phenotypic analysis. They showed interesting features such as extra cellular enzyme and fermentation capability which facilitate the opportunity for identification of the yeasts (Rajoka*., et al*. 2003).

The result of this study indicated that these indigenous yeasts, isolated from cow milk showed good fermentation attributes, which could enhance biosurfactants yield that would contribute to the cost effective role in the production of biosurfactants and enzymes of industrial importance, hence, increasing the varieties of yeast and decreasing its importation.

In accordance with previous works, there had been reports on the isolation of yeast from dairy products such as yoghurt (Rohm., *et al*. 1992) and milk (Gadaga., *et al.* 2000). *P. caribbica* and *C. tropicalis* were isolated from *Z. mays* and Cola acuminate, respectively. Contrary to literature reports that species of Saccharomyces cannot ferment lactose as they lack the enzyme lactase, the *S. cerevisiae* strain (from fermented cow milk) isolated in the course of this study was able to ferment the lactose used in the fermentation test (Rajoka., *et al.* 2003).

This strain could be used for the purpose of fermentation to produced biosurfactants and other derivatives. This is probably the first report of isolation and characterization of yeasts from these substrates that can be used in fermentation for the production of bioethanol in Nigeria. Most workers had reported the use of *S. cerevisiae* for fermentation in the production of bioethanol (Abouzied and Reddy, 1986).

Yeasts isolated from fermented cow milk were able to grow in Mineral Salt broth containing 1 ml of crude oil at 28-300C for 72 hours to give biosurfactants as they by-products. The result indicate that isolated yeast (*Saccharomyces cerevisiae and Saccharomyces pombae*) they all form clear zone of diameter after carried oil spreading test for each isolate having 31.6-96.0mm and 40.4mm diameter of clear zone respectively as presented in Table 3 in accordance with others research, It is agreed that yeasts isolated from fermented cow milk are not only to cause diseases to human it can be also used to produce biosurfactant industrially after growing in a medium supplemented with adequate carbon sources (crude oil, glucose, etc.) (Abouzied and Reddy, 1986). Organism like K. marxianus was also able to ferment galactose which is an indication of the presence of β-galactosidase to produced biosurfactant (Abouzied and Reddy, 1986), while Rajoka., *et al*. (2003) had earlier extracted the biosurfactants from K. marxianus.

This report therefore, gives an array of prospective fermentative species of yeast isolated from fermented cow milk which can be of industrial benefits. The organisms were able to degrade the carbon sources because they contain the enzymes necessary for the conversion of sugars to other products such as biosurfactants. It can be concluded that yeast isolated from fermented cow milk can be used to produce biosurfactants industrially.

Conclusion

Base on the above result, it is concluded that yeasts are found in traditionally fermented cow where it utilized the sugar present in milk to obtained energy. Yeasts are also used to produce biosurfactants that play a vital role in industries at a huge amount when grown in Media supplemented with carbon sources.

It is recommended that, yeast can be isolated from fermented cow milk and it is able to utilized sugars present in milk. Yeast isolated from fermented cow milk can be used to produce biosurfactants industrially. There is need to increase interest in the use of yeast to produced biosurfactant so as to increase the amount of biosurfactants produced

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