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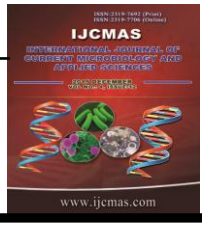

Original Research Article

Utilization of Synthetic Dairy Waste Water and Waste Oil for the Production of Sophorolipid from *Starmerella bombicola* MTCC 1910 and Testing its Antimicrobial Activity

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Keywords

Biosurfactants, Sophorolipid, *Starmerella bombicola*, Dairy waste waters

A B S T R A C T

Surfactants are amphiphilic compounds that reduce surface and interfacial tensions by accumulating at the interface between two immiscible fluids. Biosurfactants have been increasingly attracting the attention of the scientific community as promising candidates for the replacement of a number of synthetic surfactants by way of maintaining the cost of the raw material in its production process at a minimum. Renewable substrates from various sources, particularly from well-known industrial wastes can be utilized for the production of biosurfactants. The wastewaters generated from dairy industries contain large amount of fats and oils that makes such wastewaters not easily biodegradable. Utilization of dairy wastewater by microorganisms for the production of valuable bioproduct can solve both purposes: pretreatment of the wastewater and cost reduction in the bioproduct production process. Towards this goal, synthetic dairy wastewaters (SDWW) were prepared in our laboratory and tested for SLs production by the yeast *Starmerella bombicola*. The extracted sophorolipids were tested for its antimicrobial activity against the bacterial culters.The results states that the zone formation showed that the pathogens like *E.coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* were sensitive to sophorolipid and *Vibrio cholerae* was resistant for both crude and dialysed sophorolipid.

Introduction

Biosurfactants are amphiphilic compounds possessing both hydrophilic and hydrophobic moieties. They can reduce surface and interfacial tensions by accumulating at the interface between two immiscible fluids, thus stabilizing emulsions, or increasing the solubility of hydrophobic on insoluble organic compounds in aqueous media. They can be synthetic or biological origin and the market for these compounds is on expansion (Lin, 1996). Due to their interesting properties such as lower toxicity, higher biodegradability, higher foaming capacity and higher activity at extreme temperatures, pH levels, and salinity, bio surfactants have

been increasingly attracting the attention of the scientific community (Cirgliano, 1984, Zinjarde, 2002, Saruddo, 2007).

The majority of microbial biosurfactant described in literature is of bacterial origin and the genders most reported as biosurfactant producers as *Pseudomonas sp., Acinetobacter sp., Bacillus sp. Arthobacter sp.* However, due to the pathogenic nature of the producing organisms, the applications of these compounds are restricted, not being suitable for use in food industry. The great advantage of using yeasts in biosurfactant production is the GRAS (generally regarded as safe), organisms with GRAS status are not toxic of pathogenic, used in food and pharmaceutical industries (Barth, 1997). The study of biosurfactant production by yeast had reported by the *Candida sp., Pseudozyma sp., Yarrowia sp.*

Biosurfactants significantly reduce the air-water surface tension, Good biosurfactant characteristics such as high emulsification activity, simultaneously presenting low toxicity and good biodegradability (Rosenberg, 1999). Biosurfactants are categorized mainly by their microbial origin and chemical composition. Most extracellular yeast surfactants characterized and reported in literature have been identified as the Sophorolipid, produced by *Candida (Starmerella) bombicola, Torulopsis petrophilum, Torulopsis apicola* and consist of a dimeric carbohydrate sophorose linked to a long chain hydroxy fatty acid (Cooper, 1980).

Starmerella bombicola is a basidiomyceous fungus that reproduces vegetatively by budding, cells are oval to long oval, arranged in single or pair, appear in creamy white colonies in petriplates, surface is shiny, they ferment glucose, galactose, sorbose (Spencer *et al.,* 1970, Rosa and Lanchance, 1998). Biosurfactants can replace synthetic surfactants by way of maintaining the cost of the raw material in its production process at a minimum. In this aspect, renewable substrates from various sources, particularly from industrial waste can be utilized for the production of biosurfactants (Solaiman 2007). Dairy industry is one of the major food industries in India, the wastewater generated from dairy industries contain large amount of fats and oils in these wastewater not easily biodegradable. Further, high levels of fats and oils in these wastewater cause gross pollution of land and water due to their high biochemical oxygen demand (BOD) and chemical oxygen demand (COD), pretreatment of these fats and oil is necessary before subjecting the wastewater to biological treatment operations. Utilization of dairy wastewater by microorganisms for the production of valuable bioproduct solves both purposes: pretreatment of the wastewater and cost reduction in the bioproduct production process. Synthetic dairy wastewater (SDWW) were prepared in our laboratory and tested for SLs production by *Starmerella bombicola* , is known to utilize lipid source for the production of sophorolipid in shake flask experiments (Fleurackers, 2006).

The present study, evaluates the antibacterial activities of sophorolipids produced by *Starmerella bombicola* MTCC 1910 using low cost substrates, synthetic dairy waste water and restaurant waste oil.

Materials and Methods

The strain *Starmerella bombicola* MTCC 1910 purchased from Chandigarh, India. Loopfull of culture was inoculated into 25 ml media containing 2.5 g glucose, 0.25 g yeast extract, 0.025 g urea, the culture was

incubated at 30º C, 200 rpm for 72 hrs in a rotary shaker (Nagarajan Vedaraman 2010). Comparative study of sophorlipid production was done using Synthetic dairy wastewater, Restaurant waste oil, and oleic acid. (Deverey and Pakshirajan, 2009).

Extraction of Crude Sophorolipids

Sophorolipids were separated by centrifugation at 10,000xg for 10mins at 25ºc then ethyl acetate and hexane was added to remove unutilized oil and any hydrophobic substances such as fatty acids and alcohols, supernatant and pellet were obtained.

Biomass Estimation

The aqueous layer was centrifuged at 5000 x g for 20 mins at 25º C, cell pellets were washed twice with distilled water and dried to weigh for determining the yeast biomass concentration, then the pellet was mixed with 10ml distilled water and kept in incubator shaker for 30 mins at 200 rpm. The biomass concentration can be determined by UV spectrophotometer at a nanometer of 630 nm (Fabio Raphael Accorsini, 2012).

Oil Collapse and Displacement Test

Qualitative drop collapse test was performed by adding 2 µl of oil to 5 µl of culture supernatant to microtitre plate, the shape of drop on the oil surface was observed after 1min. In oil displacement test dispersion of oil can be seen by adding 50ml of distilled water to Petri dish and 20 µl oil was dropped onto the surface of water followed by 10µl of culture supernatant, the clear halo visualized under visible light after 30 sec.

Tilted Glass Slide Test

The colony of *Starmerella bombicola* was mixed with a droplet of 0.9% Nacl at one end of the glass slide, the slide is tilted and droplet was observed. Biosurfactants producers are detected by observation of droplet collapsing down (Perrson and Molin, 1987).

Carbohydrate Estimation by Anthrone Method

Colorimetric detection can be used to determine the presence of glycolipid in the culture medium, anthrone reagent prepared by adding 5ml ethanol to 200 mg anthrone in standard flask and it was made up to 100 ml by adding 75% sulphuric acid. The supernatant was added to the standard flask at different concentration as 2 ml, 4 ml, 6 ml to 10 ml, 20 ml, 40 ml of anthrone for estimation then was heated in boiling water bath for 10 mins, carbohydrates were dehydrated by sulphuric acid to form hexose or 5-hydroxyl methyl furfurol (pentose). Furfurol condenses with anthrone that produce green complex which measured in colorimeter at 620-630 nm (Daverey, 2009).

Sophorolipid Extraction by Dialysis

25 ml of sample was taken in a beaker; 13.8 gms of ammonium sulphate salt was added and centrifuged at 3000 rpm for 15 minutes, refrigerated for overnight at 4º C. Sample solution was filled in dialyzed tube using pipette, the bag was placed in appropriate buffer solution for 3-4 hrs at required temperature. Then the bag was taken and soaked into the jar containing sucrose and refrigerated for overnight at 4º C, bag contains desalted lipids was used for further procedures (Surekha Satpute, 2010).

Seperation of Lipids by Thin Layer Chromatography

5 gms silica gel was added in 10ml distilled water, slurry coated over the glass plate at a

thickness of 0.25 mm. The samples and the respective standard were spotted onto the plate, solvent as chloroform: methanol: acetic acid (6.5:1.5:0.2) was used, Anthrone reagent was sprayed evenly on the glass plate (Subhi. Hamza, 1994).

Antagonism of Sophorolipid

Sophrolipids from *Starmerella bombicola* inhibits or arrest the growth of other microorganisms. Antimicrobial activity was performed by well diffusion method. The pathogenic organisms like *Escherichia coli* MTCC 581, *Staphylococcus aureus* MTCC 3160, *Klebsiella pneumoniae* MTCC 7028, *Vibrio cholerae* MTCC 3904, *Pseudomonas aeruginosa* MTCC 4673 were used for sensitivity test. Sophorolipds were added to the wells with different concentrations 50 µl, 75 µl, 100 µl; the plates are then incubated for 18-24 hrs (Bluth, 2006).

Results and Discussion

Cost effective substrates were considered highly rich in biodegradable carbon and nitrogen source and hence utilization as a low cost fermentative media for sophorolipid production was tested with the aim to reduce the biological load in environment. It was observed that maximum sophorolipid production of 31 ml/l was obtained by supplemented with oleic acid, 28 ml/l was obtained with synthetic dairy waste, 26 ml/l obtained with waste restaurant oil (Table :5,6,7). The comparison of the sophorolipid production was done by using different substrates were given as (Graph: 1).

The yeast biomass produced was examined on 8th day of fermentation, production was more in oleic acid containing medium of about 0.34g/10ml, synthetic dairy waste produced 0.31g/ml of biomass, and 0.29 g/10ml biomass by restaurant waste oil. The weight of biomass from various substrates was obtained by drying it under vacuum at 65^{o} C (Table: 4). The concentration of the biomass yield was determined by using UV visible spectrophotometer at a wave length of 630 nm and the results were obtained (Graph: 2).

The oil collapse and displacement test showed the positive result, that were observed in figures showing the collapsing oil and clear halo zone by *Starmerella bombicola* that produced sophorolipid (Figure: 1). The halo zone formation and dispersion of oil was given along with control and test sample that shows the positive result (Figure: 2).

The comparison of carbohydrate production was given in (Graph: 3), that shows more production in oleic acid, best production by synthetic dairy waste water and better production by restaurant waste oil.

Thin layer chromatography technique was used for analyzing lipid by visualizing its absorption by using dyes. The samples contain lipids and their Rf values were calculated using formula. The movement of the solvent and solutes with different substrate produced different levels of sophorolipid was given in Figure: 3.

Antimicrobial activity of sophorolipid was done by well diffusion method, the test showed the pathogens was more sensitive to biosurfactant produced by Starmerella bombicola and it showed that organisms were not sensitive to pellet but the organism showed sensitivity to crude supernatant and dialysed supernatant showed at concentrations of 50 µl, 75 µl, 100 µl. The results states that the zone formation showed that the pathogens like *E.coli* (Figure: 4), *Staphylococcus aureus* (Figure: 5),

Klebsiella pneumoniae (Figure: 6), *Pseudomonas aeruginosa* (Figure: 7) were sensitive to sophorolipid and *Vibrio cholerae* was resistant for both crude and dialysed sophorolipid.

The wastewaters generated from dairy industries contain large amount of fats and oils that makes such wastewaters not easily biodegradable these wastewaters cause gross pollution of land and water. Therefore, utilization of dairy wastewater by microorganisms for the production of valuable bioproduct can solve both purposes: pretreatment of the wastewater and cost reduction in the bioproduct production process. Towards this goal, synthetic dairy wastewaters (SDWW) were prepared in our laboratory and tested for SLs production by the yeast *Candida bombicola*. *S. bombicola* has the ability to utilize restaurant oil waste as the lipid source for the production of sophorolipids so there is a degradation of oil in shake flask experiment.

Table.1 Sophorolipid Production by using Oleic Acid as a Substrate

S.No	Incubated days	Sophorolipid Production
1.	3	1.4
2.	4	1.7
3.	5	1.9
4.	6	2.5
5.	7	2.8
6.	8	3.1

Table.2 Sophorolipid Productions by using Synthetic Dairy Waste as a Substrate

S.No	Incubated days	Sophorolipid Production
1.	3	1.0
2.	4	1.3
3.	5	1.6
4.	6	2.0
5.	7	2.4
6.	8	2.8

Table.3 Sophorolipid Productions by using Restaurant Waste Oil as a Substrate

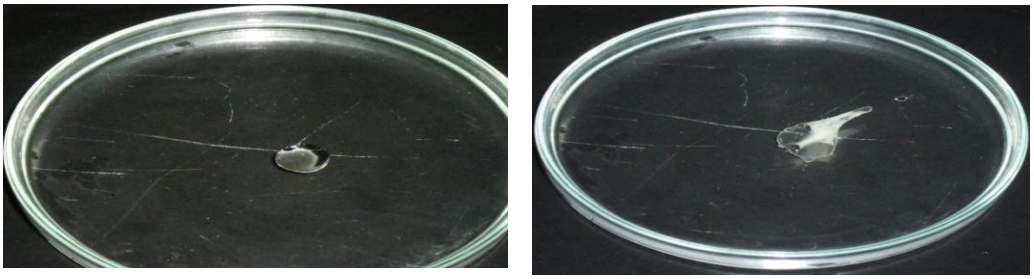
S.No	Incubated days	Sophorolipid Production
1.	3	0.7
2.	4	0.9
3.	5	1.3
4.	6	1.9
5.	7	2.2
6.	8	2.6

Table.4 Biomass Estimation Based on Weight on 8th day

S.No	Substrates	Weight g/10ml
1.	Oleic acid	0.34
2.	Synthetic dairy waste water	0.30
3.	Restaurant waste oil	0.29

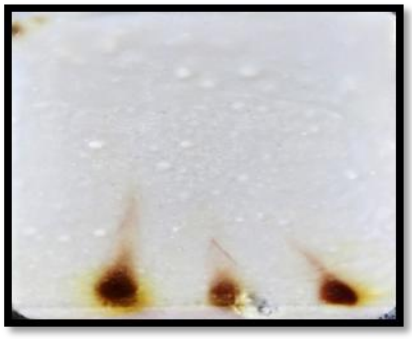
Figure.1 Drop Collapse Test

Figure.2 Oil Displacement Test



Control Test sample

Figure.3 Thin Layer Chromatography



Antimicrobial Activity of Sophorolipid

Figure.4 Activity on *Escherichia coli*

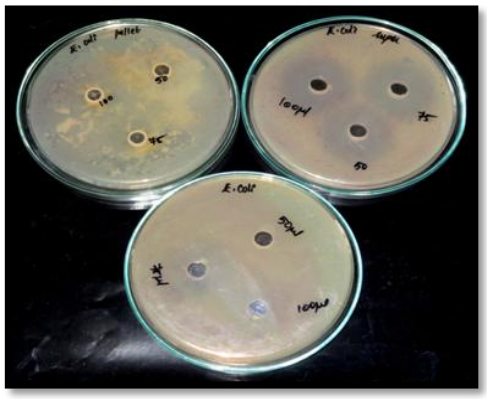


Figure.5 Activity on *Pseudomonas Aeruginosa*

Figure.6 Activity on *Staphylococcus aureus*

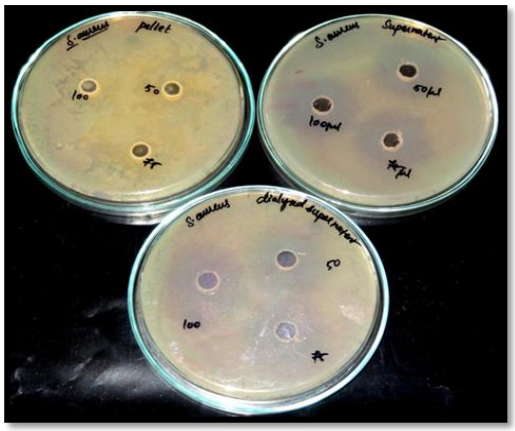
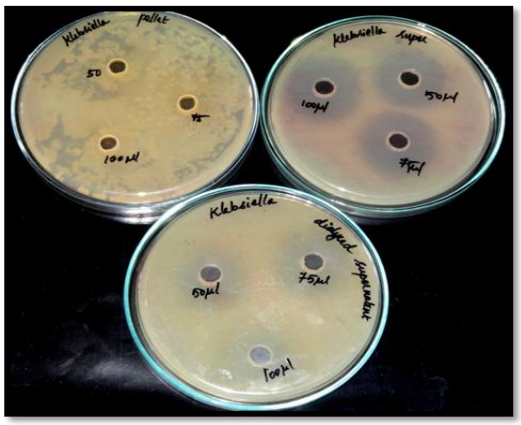
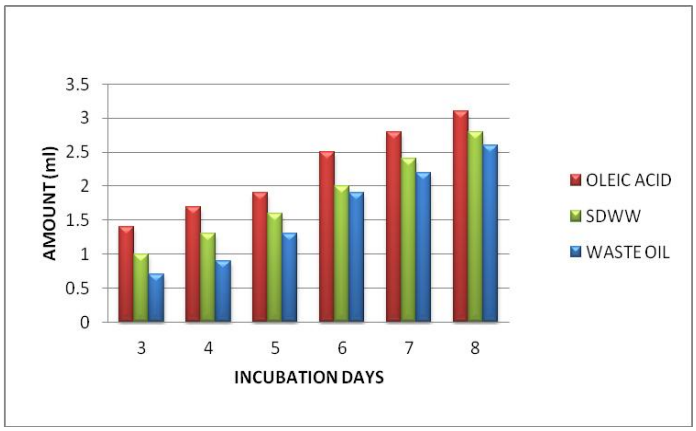


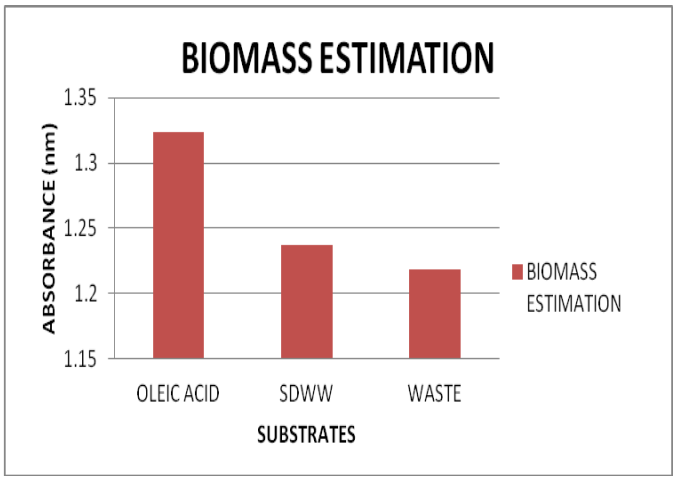
Figure.7 Activity on *Klebsiella pneumoniae*



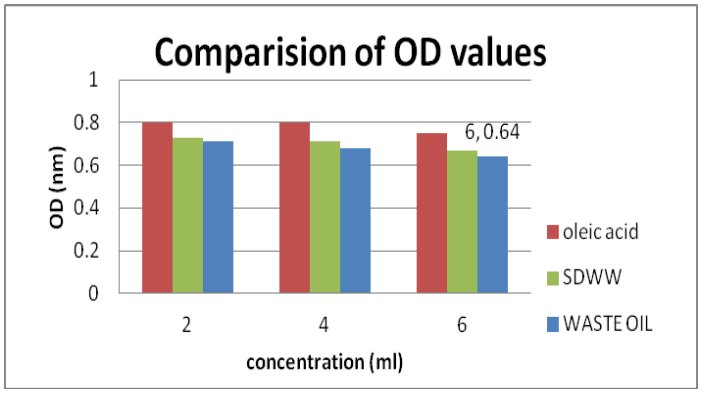
Graph.1 Comparative study of Substrates used for Sophorolipid Production



Graph.2



Graph.3 Comparison of Substrates by OD Values



Starmerella bombicola exhibits good sophorolipid (SL) production from synthetic dairy waste water (SDWW) medium which indicates a good substrate for sophorolipid, it is cost effective (Daverey, 2009). Substrate as restaurant waste oil also produces SL in considerable amount, this shows use of SDWW as a substrate is effective (Sushant Wadekar, 2010) and which are equal in production of sophorolipid that are produced by the chemical substrate, oleic acid which is very costly.

Sophorolipid production was determined by the biomass production, estimation of carbohydrate production from glycolipid using anthrone method. The confirmation for production of sophorolipid by oil collapse test shows the oil getting collapsed when sophorolipid added onto oil surface. Oil displacement is another test shows the oil gets displaced on acting with sophorolipid on forming zone.

Thin layer chromatography was done for analyzing lipid production by visualizing the spots, and reported with its Rf values.

The highest sophorolipid production was on SDWW with 2.8 g/100ml, waste restaurant oil produces 2.6 g/100ml. The oleic acid produces 3.1 g/100ml of sophorolipid.

The use of bio waste in production of sophorolipid will ultimately bring down their production cost and at the same time reduce the environmental pollution due to waste , on production of SL was tested for its antimicrobial activity against pathogens like *E.coli, Pseudomonas aeuroginosa, Klebsiella pneumoniae, Vibrio cholerae, Staphylococcus aureus* , where *V.cholerae* shows resistant to sophorolipid . Other pathogens are sensitive (Dilsad Onbasli, 2008).

In conclusion, the present study showed that the yeast *Starmerella bombicola* can utilize SDWW, with or without other nutrients, for SLs production. The production was increased when SDWW was supplemented with cheap carbon sources of milk powder, ghee and soybean oil. These show a chance of utilizing dairy industry wastewater and restaurant oil waste for the production of SLs. This method of waste oil disposal has the advantage of producing a value-added commercially viable byproduct. Providing the incentive of generating revenue for through waste treatments has been shown to be an attractive way to decrease the disposal of untreated wastes in the environment. Any

type of oils used in restaurants including canola oil and sunflower oil can be used as feed-stock during fermentation.SL is a diacetate ethyl ester derivative and so they are most potent in acting against the pathogenic microbes.

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