BIOSURFACTANT PRODUCTION POTENTIALS OF SOME BACTERIA RECOVERED FROM AGRO-INDUSTRIAL WASTES

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Abstract: Biosurfactants are amphiphilic molecules that accumulate at interfaces, decrease interfacial tensions and form aggregate structures. Biosurfactants are important alternatives to chemical surfactants due to low toxicity, thermo-tolerant, specificity and ability to produce renewable cheaper substrates. The aim of this research is to isolate some bacteria from Agro-industrial wastes and screen for their ability to produce biosurfactant. Three (3) screening methods; blood haemolysis, emulsification index (EI_{24}) and blue agar hydrolysis were used to confirm biosurfactant production. The most outstanding isolates in order of their potential to produce biosurfactants are: *Enterobacter cloacae*, *Bacillus cereus*, *Bacillus coagulans*, *Acinetobacteria mallei*, *Bacillus megaterium*, *Corynebacterium striatum*, *Escherichia coli*, *Corynebacterium pilosum*, *Bacillus laterosporus*, *Enterobacter intermedius*, *Bacillus brevis*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Bacillus polymyxa*. *Enterobacter cloacae* is isolated as the most outstanding isolated from Palm kernel, other successful isolates are from Cassava flake, followed by isolates from Poultry dung. It is recommended that more research should be focused on *Bacillus* species for optimisation and production of biosurfactant production since despite its different origins, it is always successful, also, more promising isolates should be researched from Palm kernel.

Keywords: Agro-industrial, *Bacillus* species, biosurfactants, *Enterobacter cloacae*, wastes

Introduction

The release of harmful of substances into our environment directly or indirectly will definitely lead to pollution which if care is not taken will subject our natural dwelling place into an inhabitable niche, rendering many natural resources to wastes and some other subsequent damages that may lead to shortage in availability, meanwhile some of these wastes are indirectly useful in balancing the ecosystem as they serve as food for some minor organisms as they largely depend on it as food or as carbon sources or for some other beneficial uses. Hence, since man cannot do without releasing wastes into its environment, then the urgent challenge to turn these wastes to wealth (Reis *et al.*, 2011).

The use of Biosurfactants (BS) for this purpose has been found to be an eco-friendly approach and also an alternative to conventional systems. Sequel to the diversity of biosurfactants, they are considered as great potential solution to environmental clean-up of pollutants. Biosurfactant are surface-active biomolecules produced by microorganisms with wide-range of applications. Recently, BS have gained considerable interest considering their vast application in mining, petrochemical, bioleaching, fertilizers, organic chemicals, food emulsifiers and demulsifiers, beverages, agrochemical, cosmetics, pharmaceutical and also their advantages over their chemical counterparts in terms of specificity, biodegradability, low toxicity, thermo-tolerant, better environmental compatibility and ability to be produced from renewable and cheaper substrates (Vijayakumar and Saravanan, 2015). Basically BS are categorized by their microbial origin and chemical composition viz; glycolipids (rhamnolipids, sophorolipids, trehalolipids), lipopeptides and lipoproteins, phospholipids, lipopolysaccharides, fatty acids (mycolic acids) and the complete cell surface itself.

BS have been tested in environmental and dispersion of oil spills, enhanced oil recovery and transfer of crude oil, and are thought to be potential candidates to replace chemical surfactants in the future, especially in food, cosmetics, health care, industrial cleaning and in agricultural chemicals (Vijayakumar and Saravanan, 2015).

Nevertheless, from economical stand point, BS are not yet competitive with the synthetics. BS can only replace synthetic surfactants if the cost of the raw material in the process is minimal. Furtherance to this, this study is aimed to isolate bacteria from Agro-industrial wastes and screen their ability to produce biosurfactant.

Materials and Methods

Study area

The study area for the research are; Agricultural Farm Settlement of the Olabisi Onabanjo University, Ago-Iwoye, Apoje Farms, Ijebu-Igbo and Ogburo Poultry Dung, Ibadan expressway, Ibadan road, Oyo state are some of the reputable farm settlements within the South-Western Nigeria where samples were collected.

Materials for the study

The materials and samples used for this research include; cassava wastes, poultry wastes, palm kernel wastes, test tubes, petri dishes, media, spatula, hand gloves, ethanol, aluminum foil papers, distilled water, beakers, hot air oven, incubator, measuring cylinders, glass slides, cover slips, weighing balance, cotton wool, microscope, funnel, water bath, MarConkey bottles, pipette, etc.

Collection of samples

Soils contaminated with agro-industrial wastes were collected inside sterile polythene bags and appropriately labeled from three (3) different sites within Ogun State, Nigeria for laboratory analysis at the Federal Research Institute, Oshodi, Lagos State.

Isolation and enumeration method

Each sample collected were serially diluted, sample mixtures were prepared by 10 fold serial dilutions using 1 gram of grounded sample with peptone water as diluents. 0.1 ml aliquots of ideal dilutions were spread on triplicates of aseptic nutrient agar. The plates were incubated for 24 h in the incubator at 28^{o}C and total heterotrophic bacterial count present in each sample incubated was determined by plate method on nutrient agar.

Values were expressed as Cfu/g. Enumeration of total heterotrophic bacteria was carried out using stated procedures according to Chikere *et al.* (2009) and Nwachukwu *et al.* (2010).

After incubation, morphologically different colonies were observed on the plates and were sub-cultured on a nutrient agar to recover pure cultures and were subsequently transferred into nutrient agar slants. The slants were kept in the refrigerator as stock culture at 4^{o}C.

Total heterotrophic bacteria count

The total heterotrophic bacterial count (THBC) was carried out using the method of Rahman *et al*. (2002). One gram of each of the samples was serially diluted nine-fold in sterile distilled water and 1 ml of the diluents was aseptically dispensed into sterile Petri-dishes. The pour plate method and plate count agar was poured aseptically on the sterile plates. The plates were incubated at 28°C for 24 h after which the colonies were counted. This was done in replicates. The various colonies were then sub-cultured to obtain pure colonies (Rahman *et al.*, 2002).

Total hydrocarbon degrading bacteria count

Hydrocarbon utilizing bacterial count was carried out on Mineral Salt Medium (MSM) agar on which Dual Purpose Kerosene (DPK) was used as major carbon source, before this, the DPK was filtered using a Whatman filter paper No 1. Two percent (2%) agar was added to enable solidification the medium.

Surface active bacterial count

The screening for surface-active bacteria was done on blood agar through blood heamolytic activity. The blood agar composes of Nutrient Agar containing 5% (v/v) defibrinated rabbit blood. The method was carried out according to Tabatabaee *et al.*, (2005).

Screening methods

Three different screening methods were used to screen possible isolates:

Blood heamolysis: Blood heamolytic activity was done as complimentary test for biosurfactant production. It is a qualitative screening test for the detection of biosurfactant producers. Bacteria cultures were streaked on nutrient agar supplemented with 5% fresh human blood and incubated at 37^{o}C for 48-72 h. Visual inspection for haemolysis was an indication of red blood lysis. The blood agar method was used for a preliminary screening of microorganisms for the ability to produce biosurfactants on hydrophilic media according to Vijayakumar and Saravanan (2015).

Results were recorded based on the type of clear zone observed i.e. α-heamolysis when the colony was surrounded by greenish zone, β-heamolysis when the colony was surrounded by a clear white zone and γ-heamolysis when there was no change in the medium surrounding the colony. Observation was made for α, β and γ heamolysis according to Vijayakumar and Saravanan (2015).

Blue agar hydrolysis: Mineral Salts Agar (MSA) supplemented with carbon source (2%) and cetyltrimethylammonium bromide (CTAB: 0.5 mg/ml-methylene blue (MB: 0.2 mg/ml) were prepared as reported by Nordiyana *et al.* (2013). Carbon sources tested were mannitol, glycerol, sodium citrate, sodium acetate, peptone and glucose. A dark blue halo around the culture was considered as positive for biosurfactant production.

Emulsification index (EI24): Bacterial isolates that showed positive result from the above complimentary screening tests were grown on MSM (Mannitol Salt Media), supplemented with 1% Kerosene for 7 days in an orbital incubator at 180 revolutions per minute (rpm) at 28ºC. Cell free supernatant was obtained by centrifuging the broth culture at 15,000 rpm for 15 min and was used for the experiment as previously done by some researchers. The emulsification index for surface active agents producing bacteria was carried out using the method of Ellaiah *et al.* (2000). Two millilitres of the supernatant of each organism was put in reaction tube and 2 ml of kerosene added as hydrocarbon substrate tested. The mixture was vortexed at high speed for 2 minutes and observed for percentage emulsification at intervals 4 h through 24 h. Emulsification index (EI24) was calculated by measurement of the height of the emulsion layer (a) divided by the total height (b), multiplied by 100 (EI = a/b x100). The emulsification activity is one of the most important factors of a surfactant. This assay was performed in same size glass test tubes according to (Ellaiah *et al.*, 2000).

Statistical analysis

Data obtained were subjected to analysis of variance and mean were separated with Duncan Multiple Range Test using Statistical Package for Social Sciences (SPSS) version 20.0, ($P<0.05$).

Results and Discussion

The resultant results from the screening of bacterial isolates from the Agro-industrial wastes showed that Cassava flake has four out of five of the isolates are from genus *Bacillus* while the last isolate is a *Corynebacterium*. *Escherichia coli* was only isolated from fermented cassava shaft as been successful based on the screening activity result.

Poultry dropping has three successful isolates. Poultry waste recorded no successful organism has organisms isolated from it showed poor result from the preliminary screening test for BS production. Palm kernel has four successful isolates been dominated with genus *Corynebacterium* having two out of the four isolates.

Bacillus genus also dominated isolates from poultry sewage with two out of three with only one genus *Acinetobacteria* and thus presented in Table 1.

Table 1: Successful isolates and their corresponding screening pattern

S/N	Name	Isolate Code	Blood Haemolysis (mm)	EI_{24} (%)	Blue Agar Hydrolysis
1.	*Acinetobacteria mallei*	PS 104	19	1.61	++
2.	*Acinetobacteria mallei*	PD 131	0	0	++
3.	*Bacillus brevis*	PK 351	0	0	++
4.	*Bacillus cereus*	CF 121	15	42.67	++
5.	*Bacillus coagulans*	CF 122	11	5.48	++
6.	*Bacillus intermedius*	PS 101	8	0	-
7.	*Bacillus megaterium*	PS 103	10	2.70	++
8.	*Bacillus laterosporus*	CF 321	0	2.86	+
9.	*Bacillus subtilis*	CF 322	0	0	-
10.	*Bacillus polymyxa*	PD 112	0	0	-
11.	*Corynebacterium pilosum*	PK 353	0	0	-
12.	*Corynebacterium pilosum*	PK 104	6	1.54	-
13.	*Corynebacterium pilosum*	PK 106	0	1.33	+++
14.	*Corynebacterium striatum*	PK 107	0	0	-
15.	*Corynebacterium striatum*	CF 124	0	7.35	++
16.	*Enterobacter cloacae*	PK 105	20	5.56	+++
17.	*Escherichia coli*	FCS 111	10	3.41	-
18.	*Pseudomonas aeruginosa*	PD 133	0	0	++

PK-Palm kernel, CF- Cassava flake, PS- Poultry sewage, FCS- Fermented Cassava Shaft, PD- Poultry dropping, PS-Poultry waste, - = No activity, ++ = moderate activity, +++= highest activity

As shown in Table 1 above, PK 105 has the highest blood heamolytic activity; isolate CF121 has the highest emulsification index, and both PK 105 and PK 106 have the highest blue agar heamolysis. Thus, it can be inferred that from cumulative comparison, PK 105 (*Enterobacter cloacae*) was outstanding from all the isolates by having the highest potential from two out of three of the screening result.

Biosurfactants have been reported severally in different literatures as suitable alternatives to conventional surfactants due to their properties like biodegradability, eco-friendly, high specificity, selectivity at temperature, salinity, pH, less/no toxicity and synthesis from cheaper renewable substrates. The functional properties such as wetting, emulsification, forming, surface activity, cleansing, phase separation and reduction in viscosity of crude oil for transportation are fascinating.

Therefore, the search for newer biosurfactant producing microorganisms becomes very essential as a crucial area in environmental microbiology. In this research, three screening methods were adopted for selecting biosurfactant producing bacteria from six agro-industrial wastes. Through confirmation of heamolytic activity which is a commonly preferred method to screen biosurfactant producing culture, it was inferred from the present study that it is not very useful for hydrocarbon utilizing bacteria. Further same reference cultures negative for heamolytic activity did show biosurfactant production in heterotrophic isolates. As also noticed by some other researchers, confirmation of biosurfactant production through other screening methods becomes essential to select potent biosurfactant producers as proven in this research. However, none from the hydrocarbon utilizing bacteria showed positive result from blood heamolysis as reported by Batista *et al.* (2006). Emulsification activity is one of the most significant techniques in the screening for potential biosurfactant producers. Emulsifying activities (EI24) determine productivity of bioemulsifier. Ellaiah *et al.* (2000) in their study screened 68 bacterial isolates from soil sample and found that only 6% of isolates recorded emulsification activity of up to 61%. In this research, emulsification of kerosene by *Enterobacter cloacae* and *Bacillus cerues* was high. This observation is important to suggest that potent biosurfactant producing cultures can be detected through such assays. The cultures showing above 1.0 emulsification activity were also positive for biosurfactant production in one or two other methods. It is also possible to detect biosurfactant producing and hydrocarbon degrading activity simultaneously on agar plate by overlaying with hydrocarbon (Kokare *et al.* 2007).

Maximum number of isolates positive for kerosene, hexadecane, benzene, toluene and diesel utilization. Measurement of emulsification units help to choose the carbon and energy source for biosurfactant production. It was sited from Satpute *et al.* (2008) that it is important to note that most of the researchers have used maximum two or three screening methods for selection of biosurfatcant producers, they suggested that a single method is not suitable to identify all type of biosurfactants. Therefore, a combination of various methods is required for effective screening. This research agreed with the work of Fox and Bala (2000) and also isolated *Bacillus subtilis*. They used Potato substrate as carbon source for biosurfactant production. Nitschke and Pastore (2004) used a cassava flour processing effluent as a substrate surfactants produced by *B. subtilis* which agreed with this work. *Acinetobacter* spp was isolated from Soapstock, a gummy amber coloured byproduct of oil seal processing from the work of Maneerat, (2005) while this research isolated it from poultry dropping. Hence, from the results obtained in this study, effluents of cassava wastes have the largest number of colony counts, as heterotrophic bacteria has more colony counts over the hydrocarbon utilizers because they are fastidious. Also, most of the heterotrophic isolates were gram positive rods same with the hydrocarbon utilizers which are also gram positive rods (mucoidal). Meanwhile, *Bacillus* species were isolated from all the wastes, even though more are from cassava flake. *Corynebacterium pilosum* was only isolated from Palm kernel, while *Corynebacterium striatum* was isolated from Palm kernel and Cassava flake. *Enterobacter cloacae* were isolated from Palm kernel. *Escherichia coli* were isolated from fermented cassava shaft. *Pseudomonas aeruginosa* was isolated from Poultry dung.

The most outstanding isolates in order of their potential to produce biosurfactants are: *Enterobacter cloacae, Bacillus cereus, Bacillus coagulans, Acinetobacteria mallei, Bacillus megaterium, Corynebacterium striatum, Escherichia coli, Corynebacterium pilosum, Bacillus laterosporus, Enterobacter intermedius, Bacillus brevis, Pseudomonas aeruginosa, Bacillus subtilis, and Bacillus polymyxa*. Also it is of note that none of the most outstanding biosurfactant producing bacteria came from Poultry waste but poultry dropping and poultry sewage. More of the promising bacteria were from Cassava flake. The most outstanding isolate *Enterobacter cloacae* were isolated from Palm kernel followed by isolates from Cassava flake.

The search for the discovery of more promising organisms is very essential. *Bacillus* species must be continuously optimized and given a deep attention as from most literature it always promising to produce biosurfactant, of which it's same with this research has it has the highest number of successful isolates. More promising isolates should be researched from Palm kernel because only limited attention has be given to it, meanwhile from this research, it has the most outstanding isolate *Enterobacter cloacae* which has not be detected from literature. Cassava flake are very promising, thus more of wastes of cassava should be worked on.

Likewise, poultry dropping and sewage had successful isolates which calls for further research. It is of great importance to note that most research report have concentration on oil, marine and soil contaminated sites. However, this research show wastes from Agro-industrial having promising bacteria that can produce biosurfactant. Hence, more research should be carried out on wastes from other prominent areas excluding these two above. Pharmaceutical wastes, Mill effluents, Distiller waste, Renewable sources, Industrial and municipal waste, Chemical wastes should be considered.

In conclusion, there should be further studies on novel methods which will be capable of increasing the yield of production and making isolation of potent organisms easier.

Conflict of Interest

Authors declare that there is no conflict of interest.

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