



Screening for Biosurfactant Production from Lactic Acid Bacteria isolated from African fermented milk 'Wara'

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Abstract

Lactic Acid Bacteria (LAB) from 'wara' an African fermented milk (cheese) was screened for the production of surface-active agent (biosurfactant) from five species of *Lactobacillus*. *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus brevis*, *Lactobacillus casei*, and *Lactococcus lactis* using red blood cell haemolysis test, emulsification index and oil spreading assay. *Lactobacillus fermentum* and *Lactobacilli brevis* showed the largest zone of haemolysis when screened on blood agar. The highest emulsification index of 20% was by *Lactobacillus plantarum* while the least emulsification index of 11% was recorded for *Lactobacillus casei*. All the isolates showed surface activity after being grown in 2%, 4% and 8% glucose and lactose respectively.

KEYWORDS: Biosurfactant, Lactic Acid Bacteria (LAB), African Fermented Milk, Emulsification index. *Lactobacillus* sp.

INTRODUCTION

Biosurfactants are surface-active compounds produced by living cells, especially by microorganisms. Although they are produced by some yeasts and some filamentous fungi, bacteria are the main group of biosurfactant-producing microorganisms (Desai and Banat, 1997). They are surface-active amphiphatic molecules which comprises both hydrophilic and hydrophobic moieties (Thimon *et al.*, 1995). Biosurfactants have a wide range of potential applications in the medical field. They are useful as antibacterial, antifungal, antiviral agents and they also have the potential for use as major immunomodulatory molecules, adhesive agents and in vaccines and gene therapy (Rodrigues *et al.*, 2006). Frequently, biosurfactants have several advantages over synthetic surfactants such as: higher biodegradability, lower toxicity, good compatibility with eukaryotic organisms and effectiveness at a wide range of temperatures, pH values and salinities (Desai and Banat, 1997).

Biosurfactants are mostly produced by microorganisms that are not regarded as safe (Saharan *et al.*, 2011). Hence, the need to explore and screen for non-pathogenic biosurfactant-producing microorganism. Among microorganisms, LABs are found as microbiota in man and are generally found naturally in food especially fermented foods (Korhonen, 2010)). This study looked at biosurfactant-producing potentials of LABs from

'wara' an indigenous fermented milk from southwestern, Nigeria.

MATERIALS AND METHODS

Sample collection

African cheese 'wara', indigenous to southwestern, Nigeria were aseptically collected from Ojoo market in Ibadan, Oyo state, Nigeria and were taken to the laboratory in a cold chest. Samples were stored at 4 °C until analysed

Isolation of Lactic Acid Bacteria from wara

Fresh 'wara' sample was homogenized in sterile distilled water. One gram of the mixture was serially diluted in sterile distilled water under aseptic conditions. One millilitre of appropriate dilutions of the mixture were plated out on De Mann Rogosa and Sharpe (MRS) Agar (De Mann *et al* 1960) and incubated at 37 °C under anaerobic conditions. Colonies were streaked on MRS agar to obtain pure cultures of LAB. Slants of pure LAB cultures were stored at 4 °C.

Characterization and Identification of Lactic Acid Bacteria

Isolated LAB were characterized and identified using various morphological and biochemical tests: Gram's reaction, cell morphology, catalase, spore forming, nitrate reduction, indole test, gas production, Methyl Red Vogues Proskauer and oxidase. Fermentation of sugars such as sucrose, Galactose, Sorbitol, Mannitol, Raffinose, Arabinose, Lactose, Sorbose, Xylose and Fructose. All the above-mentioned tests were carried out according to the Bergey's Manual of Systematic Bacteriology.

Screening for Biosurfactant Production from Lactic Acid Bacteria

Screening for biosurfactant production was carried out by red blood cell haemolysis, emulsification index test and oil spreading test.

Haemolytic Activity Test: Three percent of defibrinated rabbit blood was aseptically added to 100mL of sterilized nutrient agar that had been slightly cooled and poured into plates and then incubated anaerobically at 37 °C for 48 h. Pure culture of Lactic Acid Bacteria (LAB) isolates were streaked on the agar medium. Haemolysis of red blood cells was then observed as described by Bodour *et al.*, (2003); Carillo *et al.*, (1996) and Rodrigues *et al.*, (2006).

Emulsification Index Test: Eighteen hours old colonies were grown in MRS broth at 37 °C for 24 hours. Broth culture was centrifuged at 3,000 rpm for 15 minutes and the supernatant was decanted. Equal volumes (2 mL) of cell-free supernatant and sterilized refined groundnut oil were vigorously mixed with a vortex mixer in a glass test tube for 2 minutes and left standing undisturbed for 24 h. The height of oil that separated after 24hour of standing was measured using a metre rule. The emulsification index was taken as a percentage of the height of emulsified layer divided by the total height of the liquid column (Balogun, 2009).

Oil Spreading Assay: To determine the surface activity, 1mL of the fermented broth was centrifuged at 8,000g for 10 minutes after 24 and 48 hours to obtain the supernatant. Twenty microlitres (20µL) of motor oil 10 W-40 (Selenia, Italy) was deposited onto the surface of 20 mL of distilled water in a Petri dish (90 mm in diameter) to form a thin membrane. Twenty microlitres of each bacterial suspension was gently put onto the centre of the oil. Diameters of cleared zone of oil displacement were then measured using a metre ruler (Morikawa *et al.*, 2000).

Production of Biosurfactant by isolates when grown on Lactose and glucose

The LAB isolates were tested for ability to produce biosurfactant when grown on 2, 4 and 8% glucose and Lactose broth respectively.

RESULTS AND DISCUSSION

Five Lactic Acid Bacteria isolates were obtained from 'wara'(Table 1). Olatunji *et al.*, (2006) reported that wara has been found to harbour Lactic acid bacteria such as *Lactobacillus*. Presence of *L. brevis*, *L. fermentum*, *L. casei*, *L. plantarum* (Guetouache and Guessas (2015)) and *Lactococcus lactis* (Oyeleke *et al.*, 2006; Olukoya, 1993) in wara and other african fermented milk have been reported by many workers.

All isolates were able to utilize the sugars; Maltose, Sucrose, Galactose, Arabinose, Lactose, xylose and fructose as their source of carbon. *L. brevis* and *L. plantarum* were able to utilize sorbitol, *L. plantarum* was the only isolate that can utilize manitol, *L. fermentum* and *L. plantarum* were also able to utilize raffinose whereas *L. casei* was the only bacterium isolate that is able to utilize sorbose as carbon source (Table 2).

From this study, *Lactobacillus fermentum* (Plate 1) and *Lactobacillus brevis* (Plate 2) were screened as strong biosurfactant producing micro-organisms due to their high haemolytic activity on rabbit red blood cells. Ability to haemolyse red blood cells have been reported by many workers as one of the major characteristics for identifying biosurfactant producing microorganisms (Carillo *et al.* (1994); Lin (1996); Banat (1995), Velraed *et al.*, 1998 Moran *et al.* 2002).

Biosurfactants production by isolates was determined by emulsification index. All the isolates were able to produce emulsion. *L. plantarum* had the highest emulsification index of 20% followed by *L. brevis* with emulsification index of 17%. *L. lactis* produced the emulsification index of 16% followed by *L. fermentum* with emulsification index of 14% and *L. casei* with emulsification index of 11% (Figure 1). Organisms with high emulsifying activity are promising microbial candidates for biosurfactant production. In line with this study, the highest value of emulsification index was found in *L. plantarum* compared to other isolates. This shows that it is an emulsifier and has the ability to reduce surface tension (Bento *et al.*, 2006; Balogun and Fagade 2010).

The lactic acid bacteria *L. fermentum*, *L. plantarum*, *L. brevis*, *L. casei* and *L. lactis* were found to be biosurfactant producing organism since they are able to reduce the surface activity at both 24 and 48 hours. In this study, biosurfactant production continued for all strains up to 48 hours of fermentation but at a very slow production rate. Depending upon the nature of the biosurfactant and the producing microorganisms, several patterns of biosurfactants production by fermentation are possible as reported by Desai and Desai, (1993).

Table 1: Biochemical characterization of biosurfactant producing LAB from wara

S/N	GR	CM	Os	Cs	SF	MR	VP	Ar	G.PG	G.15°C	G25°C	G45°C	GpH3.9	GpH9.6	NR	CU	G1%N	G2%N	G4%N	Probable Organism
1	+	R	-	-	-	-	-	+	+	+	+	+	+	+	-	+	+	+	+	<i>L. fermentum</i>
2	+	R	-	-	-	-	-	+	+	+	+	-	+	+	+	-	+	+	+	<i>L. brevis</i>
3	+	C	-	-	-	-	-	+	+	+	+	-	+	-	+	+	+	+	+	<i>Lactococcus. lactis</i>
4	+	R	-	-	-	-	-	+	+	+	+	-	+	+	+	-	+	+	+	<i>L. casei</i>
5	+	Cb	-	-	-	-	-	+	+	+	+	+	+	+	-	+	+	+	+	<i>L. plantarum</i>

KEYS:

GR - Gram's reaction, CM - Cell morphology, R - Rod, C - Coccus, Cb - Coccobacillus, Os - Oxidase, Cs - Catalase, Sf - Spore forming, MR - Methyl Red, VPt - Voges P test, AR - Arginine test, GPG - Gas production from Glucose, G.15°C - Growth @ 15°C, G.25°C - Growth @25°C, G45°C - Growth @ 45°C, GpH3.9 - Growth @ pH 3.9, GpH9.6 - Growth @ pH 9.6, NR - Nitrate Reduction, Cu - Citrate utilization, G1%N - Growth @ 1% NaCl, G2%N - Growth @ 2% NaCl, G4%N - Growth @ 4% NaCl, PO - Probable Organism, *L. f* - *Lactobacillus fermentum*, *L. b* - *Lactobacillus brevis*, *L. l* - *Lactococcus lactis*, *Lactobacillus casei*, *L. p* - *Lactobacillus plantarum*.

Table 2: Sugar fermentation pattern of biosurfactant producing LAB isolates from wara

S/N	Maltose	Sucrose	Galactose	Sorbitol	Manitol	Raffinose	Arabinose	Lactose	Sorbitose	Xylose	Fructose	Probable organism
1	+	+	+	-	-	+	+	+	-	+	+	<i>L. fermentum</i>
2	+	+	+	+	-	-	+	+	-	+	+	<i>L. brevis</i>
3	+	+	+	-	-	-	+	+	-	+	+	<i>L. lactis</i>
4	+	+	+	-	-	-	+	+	+	+	+	<i>L. casei</i>
5	+	+	+	+	+	+	+	+	-	+	+	<i>L. plantarum</i>

KEYS:

L. f - *Lactobacillus fermentum*, *L. b* - *Lactobacillus brevis*, *L. l* - *Lactococcus lactis*, *Lactobacillus casei*, *L. p* - *Lactobacillus plantarum*, + indicates Positive, - indicates Negative.

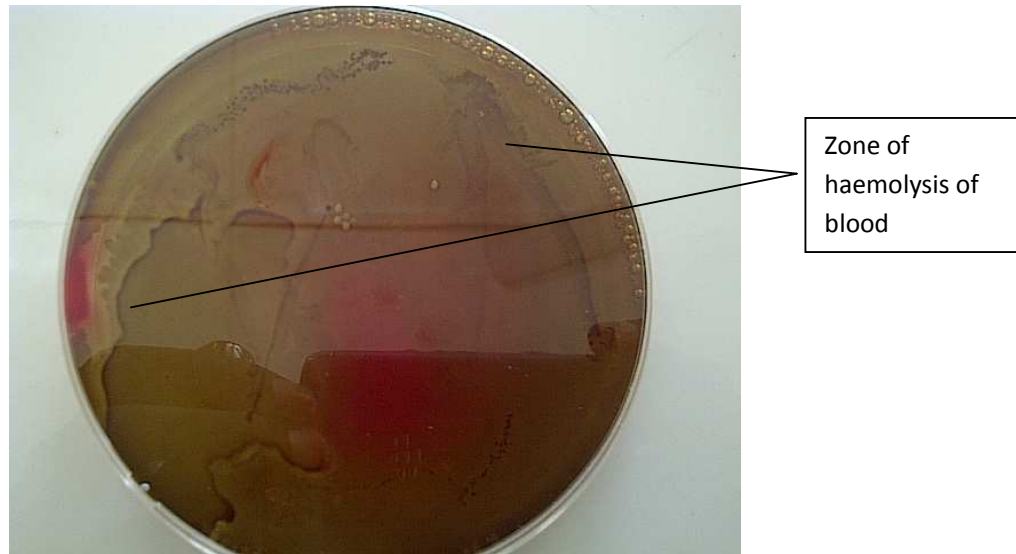


Plate1: Haemolysis of rabbit red blood cells on blood agar by *Lactobacillus brevis*.



Plate 2: Haemolysis of rabbit red blood cells on blood agar by *Lactobacillus fermentum*

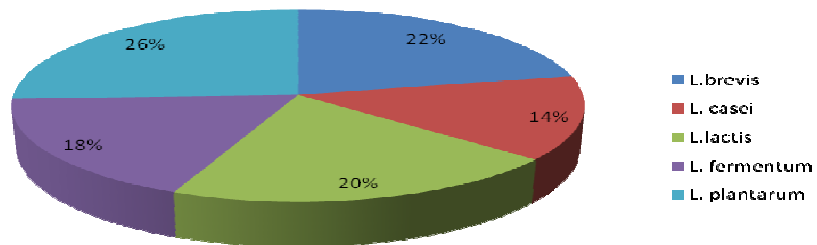


Figure 1: Emulsification indices of LAB isolates from wara

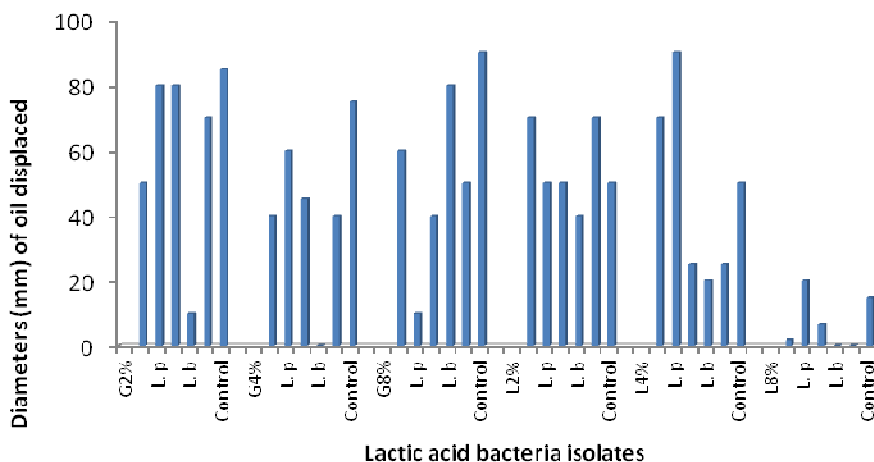


Figure 2: Surface activity of isolates when grown on different concentration of glucose and lactose for 24 hours.

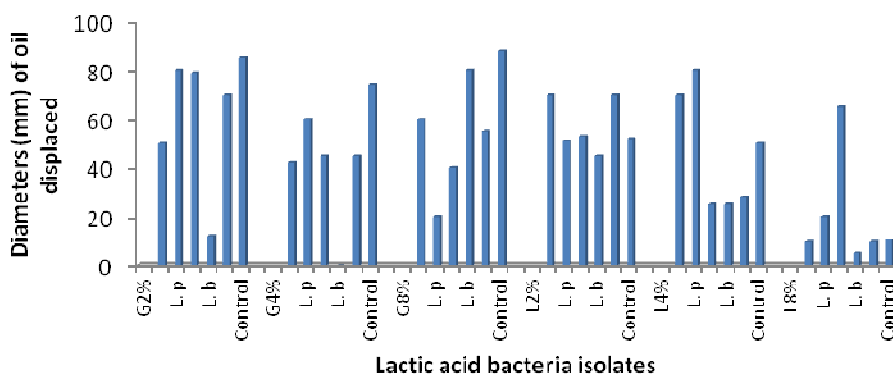


Figure 3: Surface activity of isolates when grown on different concentration of glucose and lactose at 48 hours.

KEYS:

G2% - 2% of Glucose concentration, G4% - 4% of Glucose concentration, G8% - 8% of Glucose concentration
 L2% - 2% of Lactose concentration, L4% - 4% of Lactose concentration, L8% - 8% of Lactose concentration, L. f - *Lactobacillus fermentum*, L. b - *Lactobacillus brevis*, L. p - *Lactobacillus plantarum*, L. c - *Lactobacillus casei*, L. l - *Lactococcus lactis*

Conclusion

Results from this work showed that LAB isolated from African cheese “wara” can produce biosurfactants. Prominent among which are *Lactobacillus brevis* and *L. fermentum*. These isolates are very promising and important in food fermentation. Since

they are generally regarded as safe, hence these isolates have potential for producing safe and non-toxic biosurfactants that can be used for cosmetics, household and food grade biosurfactants that can be added to food and other daily need products

REFERENCES

Balogun, S. A. (2009). Characteristics and use in crude oil recovery of biosurfactant - producing bacteria from water and soil samples. Unpublished Ph.D Thesis . University of Ibadan, Nigeria. pp 29 - 30.

Balogun, S. A. and Fagade, O. E. (2010) Emulsifying bacteria in produce water from Niger-Delta, Nigeria. *African Journal of Microbiology Research* Vol. 4(9), pp. 730-734,

- Banat I.M. (1995). Characterization of Biosurfactants and their use in pollution removal. State of the art Review. *Acta Biotechnol* 3: 251-267.
- Bodour AA, Dress KP, Maier RM (2003). Distribution of biosurfactant - producing bacteria in undisturbed and contaminated arid southwestern soils. *Appl. Environ. Microbiol.* 69(6): 3280-3287.
- Carrillo, P., Mardaraz, C., Pitta-Alvarez, S. and Giuliotti, A. (1994). Isolation and selection of biosurfactant-producing bacteria, *World Journal Microbiology Biotechnology*, 12:82-84.
- Desai, J. and Desai, A. (1993). Biosurfactants: production, in: Properties and Applications, *Marcel Dekker, New York*, page number 65-97.
- Desai, J. and Banat, I. (1997). Microbial production of surfactants and their commercial potential, *Microbiology Molecular Biology*, page number 47-64.
- Guetouache, M. And Guessas B. (2015) Characterization and iden Guetouache, M. And Guessas B. (2015) Characterization and identification of lactic acid bacteria isolated from traditional cheese (Klila) prepared from cow's milk Vol. 9(2), pp. 71-77,
- Korhonen, J. (2010) Antibiotic Resistance Of Lactic Acid Bacteria. Dissertations in Forestry and Natural Sciences, University of Eastern Finland
- Lin S (1996). Biosurfactants: Recent Reviews *J. Chem. Tech. Biotechnol.* 66: 109-120
- Morikawa, M., Hirata, Y. & Imanaka, T. 2000. A Study on the structure-function relationship of lipopeptides biosurfactants. *Biochimica et Biophysica Acta* 1488: 211-218
- Olatunji, E.A., Shiawoya, E.L. and Ijah, U.J.J. (2006) Bacterial assessment and keeping quality of local cheese (wara) in whey and salt solution. *Journal of Sustainable Tropical Agricultural Research*, 17:125-130
- Olukoya, D.K. (1993). Lactobacillus in human health with reference to locally fermented foods. *Nigerian Medical Journal* 24 (1):28-30.
- Oyeleke, S.B., Faruk, A.K., Oyewole, O.A. and Nabara H.Y. (2006) Occurrence of lactic acid bacteria in some locally fermented food products sold in Minna markets. *Nigerian Journal of Microbiology* 20(2):927-930.
- Rodrigues, L. R., Moldes, A., Teixeira, J. and Oliveira, R. (2006). Kinetic study of fermentative biosurfactant production by *Lactobacillus* strains. *Biochemical Engineering Journal*, 28:109-116.
- Saharan BS, Sahu RK, Sharma D (2011) A review on biosurfactants: fermentation, current developments and perspectives. *Genet Eng Biotechnol J* (1):1-14
- Thimon, L., Peypoux, F., Wallach, J. (1995). Effect of lipopeptide antibiotic, iturin A, on morphology and membrane ultrastructure of yeast cells. *FEMS Microbiol Lett.*, 128: 101 -106.
- Velraeds, M., Van de Belt, B. and Van der Mei, H. C. (1996). Interference in initial adhesion of uropathogenic bacteria and yeasts to silicone rubber by a *Lactobacillus acidophilus* biosurfactant. *Journal of Medical Microbiology*, 47: 1081- 1085