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A New Method for Estimation of Surfactin

MUHAMMAD USMAN MUBARAK[1,2], AHMAD ADNAN[2], MUHAMMAD IMTIAZ SHAFIQ[1,3,*], ABDUL REHMAN[4] and JAVEED A. AWAN[4]

[1]Institute of Chemistry, University of the Punjab, Lahore-54000, Pakistan
[2]Government College University, Lahore-54000, Pakistan
[3]Institute of Biochemistry and Biotechnology, University of the Punjab, Lahore-54000, Pakistan
[4]Institute of Chemical Engineering and Technology University of the Punjab, Lahore-54000, Pakistan

*Corresponding author: Fax: +92 42 99230355; Tel: +92 99230355; E-mail: imtiazshafiq@gmail.com

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Surfactin is a lipopeptide biosurfactant produced by some strains of *Bacillus subtilis*. Due to its surface tension reducing ability it has a potential application as a drug in certain types of cancer diseases. It has a strong ability to cause hemolysis. In this work the hemolytic activity of surfactin was used for its estimation by measuring released haemoglobin spectrophotometrically. Herein a new method has been developed involving microscopic counting of red blood cells for surfactin estimation. There has been a good agreement between the newly developed microscopic counting of red blood cells and already reported spectrophotometric method.

Keywords: Surfactin, *Bacillus subtilis*, Red blood cells, Haemoglobin, Hemolytic activity.

INTRODUCTION

Surfactin is a lipopeptide biosurfactant synthesized by many stains of *Bacillus subtilis* [1]. Surfactin shows different characteristics like antiviral, antimycoplasma, antifungal, antibacterial, haemolytic activities [2-4]. Surfactin made of 3-hydroxy fatty acid which is linked to lipopeptide containing seven amino acids. Its structure has heptapeptide group with sequence Glu-Leu-D-Leu-Val-Asp-D-Leu-Leu, which is closed to lactone ring by fatty acid chain of 13 to 15 carbon atoms. In the solution, peptide ring adopts structure like 'horse saddle' that explains its wide spectrum of biological activity. Glycine residue at position 1 and asparagine residue at position 6 constituting a polar domain and at position 4, a valine residue extend down to fatty acid chain [5].

Most frequently used methods for the estimation of surfactin is the measurement of the surface tension in series of diluted samples to determine the critical micelle concentration (CMC) [6], HPLC [7] or dry weight method [8]. Surfactin shows an outstanding amphiphilic property which induces hemolysis at some specific concentration [9]. This hemolytic property uses to screen biosurfactant producers by using blood agar plate technique [10] as well as minimal haemolytic concentration (MHC) for surfactin was also reported [11,12].

By following spectrophotometric method [13], a quantitative method for estimation of surfactin was developed in which red blood cells are counted after treatment with surfactin present in supernatant.

EXPERIMENTAL

Culture development: Nutrient broth 1.5 % was prepared and inoculated with *B. subtilis* DSM 3258. This inoculated medium was incubated for 48 h in rotary flask shaker at 37 °C with agitation of 120 rpm. After 48 h incubation period, fermented broth was centrifuged at 12000 g for 20 min. The supernatent was used in haemolytic reaction.

Preparation of red blood cell suspension: Fresh human blood was obtained and anti-coagulated with 20 µM EDTA. Red blood cells were washed twice with isotonic solution of NaCl. After centrifugation at 340 g for 3 min, hematocryt obtained after draining supernatant. Hematocryt was diluted in 0.9 % NaCl solution to make 1 % cell suspension.

Haemolysis reaction: Surfactin and red blood cell reaction mixture have following composition: culture supernatant 1.5 mL (0, 0.25 mL, 0.5 mL, 0.75 mL, 1 mL, 1.25 mL, 1.5 mL to make total volume 1.5 mL, dilution was done with freshly prepared 1.5 % nutrient broth), buffer saline solution 4.5 mL (20 mM sodium phosphate buffer (pH 7.2), NaCl 40 mM and 145 mL/L ethanol, diluted with freshly prepared medium) and 3 mL of prepared suspension of red blood cells.

The mixtures were gently mixed in vial and incubated in flask shaker at 37 °C. After 1 h, reaction mixture was taken

out from the incubator and the numbers of cells were counted in haemocytometer microscopically. Spectrophotometric measurements (405 nm) were also done by centrifuging (790 g) the same sample after cell counting. Freshly prepared 1.5 % nutrient broth was used as control. Haemolysis was calculated as:

$$\text{Haemolysis}\,(\%) = \frac{N_R}{N_T} \times 100 \qquad \text{(I)}$$

where, N_R is numbers of reduced cells which is difference of total number of cells taken (N_T) and number of cells remained after incubation. One haemolytic unit (%) was converted into concentration unit (g/L), considering that 0.02 g/L was equivalent to one haemolytic unit [13]. Furthermore, from a separate vial both measurements were noted after each 30 min to assess the haemolytic activity comparison in both types of measurements.

RESULTS AND DISCUSSION

Reduction of red blood cells with surfactin concentrations: When red blood cells interact with surfactin, they undergo breakdown. As shown (Fig. 1), with increasing concentration of surfactin, there was decrease in cell numbers (surfactin concentration was increased as the volume of culture supernatant increases). Thus, there was a same positive linear relationship between surfactin concentration and red blood cells number. Similar trend is shown with spectrophotometric method (Fig. 2), which inferred that with increase of surfactin concentration, there was increase in absorbance.

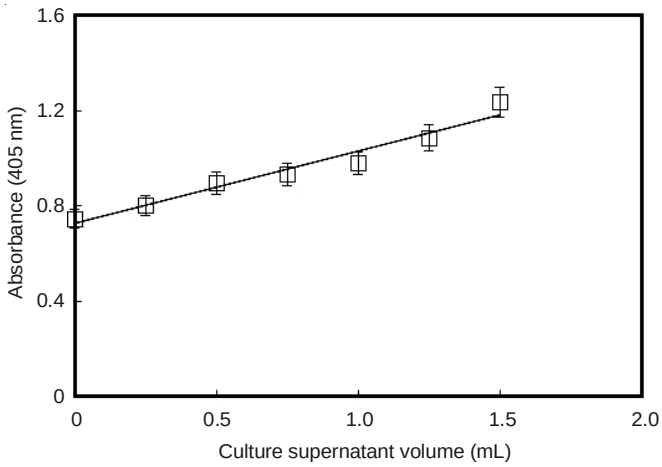

Fig. 1. A linear trend of absorbance as a function of supernatant volume (mL), increasing volume elaborate increasing surfactin produced by *B. subtilis* DSM 3258, where (□); experimental data measured, solid line; tendency curves, error band; experimental uncertainty ± 5 %

Optimization of red blood cells counting method: Organic solvents which are water miscible such as ethanol cause dissociation of micelles into isolated molecules resulting the decrease of affinity of surface active agents for the interface thus surfactants exist in monomer form [14]. By the addition of alcohols surfactin exists in monomer form hence interact efficiently with red blood cells, which decrease the minimal haemolytic concentration value and increase the sensitivity for estimation of surfactin through haemolytic activity. Excess

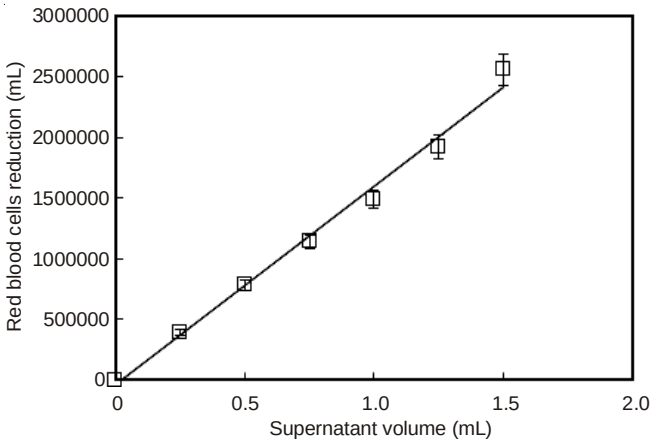

Fig. 2. A linear trend of red blood cells reduced per mL as a function of supernatant volume (mL), increasing volume elaborate increasing surfactin produced by *B. subtilis* DSM 3258, where (□); experimental data measured, solid line; tendency curves, error band; experimental uncertainty ± 5 %

of alcohol can cause the unspecific haemolysis, because ethanol causes destabilization of cells [15]. Thus, ethanol attributed to effects, increasing fragility of cells as well as providing maximum free monomer concentration. As shown (Fig. 3), at ethanol concentration of 145 mL/L for red blood cells counting assay, haemolytic curves were almost superimposed and showing that at this concentration of ethanol, there was a fine parallelism between two methods.

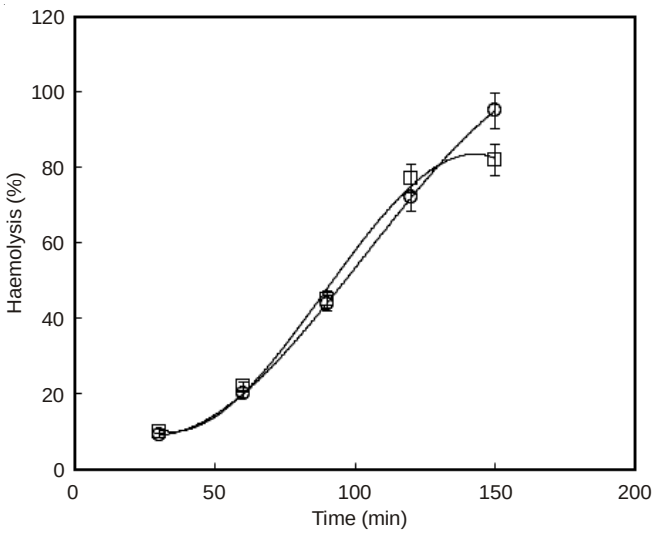

Fig. 3. Haemolysis (%) as function of time (min), where (o); experimental data measured with spectrophotometer to derive haemolysis (%), (□); experimental data measured with cell counting, solid line; tendency curves, error band; experimental uncertainty ± 5 %

Minimal haemolytic concentration value is equivalent to one haemolytic unit so it can be converted into surfactin concentration (g/L). Minimal haemolytic concentration value of surfactin is 0.02 g/L at the ethanol concentration of 145 mL/L [13]. In red blood cells counting method, product of minimal haemolytic concentration value and haemolysis (%) gave concentration of surfactin. The difference in haemolysis percentage of both methods was calculated as average of values of haemolysis (%) at different times then converted into surfactin

concentration (g/L). Thus, result difference of cell counting method compared to spectrophotometric method was ± 0.016 g/L at ethanol concentrations of 145 mL/L.

Conclusion

An easy and new approach for the measurement of surfactin produced by *B. subtilis* during fermentation process has been reported in this work. This involves the counting of red blood cells reduced by surfactin present in culture supernatant, numbers of cells reduced were used to calculate the percentage of haemolysis at any time during the biosurfactant production. Similar to spectrophotometric method the new approach is simple and required only the microscopic counting of red blood cells on haemocytometer. Unlike dry weight estimation method it was carried out without any further treatment of supernatant, which prevent loss of surfactin as well as no specific instruments and expensive reagents are required.

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