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Review Article

# The Intriguing Extrapolations of Haemolysis Assay as Screening Criterion for Selecting Biosurfactant-Producing Microorganisms in Petroleum Industries Process-Conditions

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## ABSTRACT

Commonly-used screening techniques for determination of biosurfactants production by microorganisms include haemolysis assay, generally depicted to confirm the ability of microorganisms in production of biosurfactants. Diameters of zones of haemolysis surrounding microbial colonies are considered as quantitative indication of biosurfactant production whereas; haemolytic reactions on blood agar plates are specifically associated with pathologic types of erythrocyte lysis by microorganisms, due to haemolysins production. Haemolytic microorganisms can destroy erythrocyte membranes, by compromise in integrity of cytoplasmic membranes, through pore-forming mechanisms, multiple-hit mechanism, formation of sphaerocytes, derangement of membrane integrity, detergent-like action, or lipase activity. Relative levels of acute toxicity, cell invasiveness and virulence factors, which can make biosurfactants become opportunistic pathogens that use haemin or haemoglobin as a source of iron, have also been reported. Haemolysins are further classically defined as exotoxins that can be thermostable, and can cross membranes of microorganisms. Haemolysis assay thus, identifies haemolytic microbial strains with lytic, pathogenic, toxigenic, and/or virulent potentials, rather than biosurfactant-producing potential, as the assay does not correlate particularly with specific characteristics of biosurfactants' production. However, based on new insights and perspectives appropriately extrapolated for the first time in this report, microbial haemolysis assay is considered, the easiest, most-economical, non-animal-based, highly-determinative, reliable and sensitive biosafety selection criterion protocol, for selection of safe and environmental-friendly biosurfactant candidates, for the petroleum industries' process conditions.

**Keywords:** Biosurfactants; Blood agar; Microbial haemolysis assay; Microbial toxicity; Petroleum industries

## INTRODUCTION

Microbial surfactants (biosurfactants) are one of the wide ranges of extracellular compounds that are produced by microorganisms, particularly, bacteria and fungi (yeasts, moulds and mushroom), more especially when grown on hydrophobic substrates. They are surface-active microbial amphiphilic compounds, which are produced on living surfaces, mostly, on microbial cell surfaces or excreted as extracellular hydrophobic and hydrophilic moieties. These characteristics thus, confer on the biosurfactants-producing microorganisms, the ability to accumulate between fluid phases, and also possess the characteristic property for reducing surface and interfacial tension, at surfaces and interfaces respectively. By accumulating at the interfaces of immiscible fluids, biosurfactants have been reported to be able to increase the solubility, bioavailability and subsequent biodegradation of hydrophobic or insoluble organic compounds [1-10]. However, biosurfactants use similar mechanisms to the chemical surfactants but mostly with certain more established advantages [11-13].

In addition to having many unique properties and applications, the ability to exhibit biosensoactives (surface- active) properties, which lower the surface tension and the interfacial tension of their growth media, allow biosurfactants to play diverse key beneficial roles [13-28]. Furthermore, the vast structural diversities that characterise

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biosurfactants, may also explain the reason for their continual intrigue to scientific interests [16,29,30]. Increased environmental awareness has been the main driver for the search of biosurfactants, as replacement for chemical surfactants [2,13,24,29-34].

## LITERATURE REVIEW

### Benefits and applications of biosurfactants in petroleum industries

Biosurfactant production is considered one of the key technologies for development in the 21st century, and biosurfactants are widely applicable in almost every area of human endeavours, especially in the field of petroleum technology and processes. Specifically, due to their efficacy as dispersion and remediation agents, biosurfactants have several potential applications across the oil-processing chains, and in the formulations of petrochemical products, microbial enhanced oil recovery, anti-corrosives; biocides for sulphate-reducing bacteria; emulsification and emulsified fuels; de-emulsification; oil waste treatment; enhancement of crude oil transportation through pipelines; crude oil spill clean-ups/ bioremediation of crude-oil polluted soils; including the removal of crude oil from contaminated soils and water bodies by indigenous microbes, biodegradation. Some other benefits of biosurfactants are environmental remediation processes like- oil storage bottom sludge tank cleaning; sediment remediation, soil washing and soil flushing, extraction of bitumen from tar sands, and extraction of hydrocarbon compounds from oil shales, in order to utilise them as a substitute for petroleum energy fuel [10,12,16,17,22,24,35-86].

Biosurfactants being diverse amphiphilic molecules, with wide structural and functional diversities, and because great diversity also exists among biosurfactant roducing microorganisms, there is adoption of different screening techniques for their determination; although, almost all the screening methods can give qualitative and/or quantitative results [87-93].

Some screening methods for biosurfactant production, which are basically automated and/or miniaturised rapid-screening assays, are available in present times. However, the major regular direct and indirect biosurfactants screening assays are presented in Figure 1 [19,88,58,90,93,94-122].

#### Screening assays for selecting biosurfactant-producing microorganisms

Each biosurfactant screening method, as presented in Figure 1, has its advantages and disadvantages; so, a combination of different methods has been suggested as appropriate for successful screening of biosurfactants [15,87,88,121,123]. In addition to the physiological nature of the biosurfactant-producing microorganisms, screening for biosurfactant producers somehow depends, both on the type of carbon source(s) present, and also the types and amounts of other nutrients in the screening media [92,124-130]. The screening medium used will therefore, tremendously influence production or non-production of biosurfactants; and also influence the type and amount of biosurfactants produced [19].

### Haemolysis assay in biosurfactant determination

It was reported that haemolytic activity of biosurfactants was first discovered when Bernheimer and Avigad [131] recorded that surfactin, the biosurfactant produced by *B. subtilis*, lysed erythrocytes.

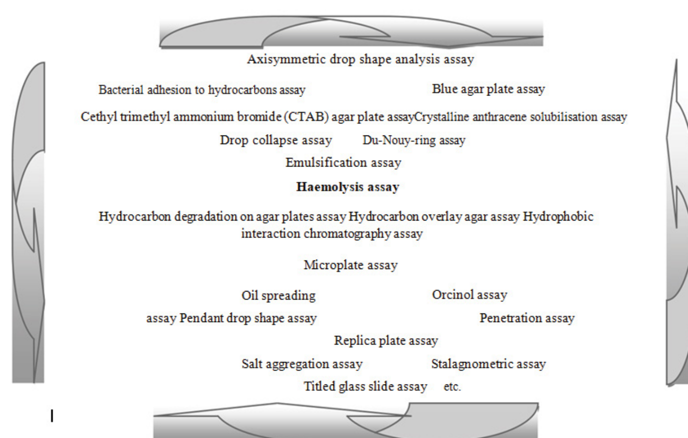


**Figure 1:** Common direct and indirect non-automated biosurfactants screening assays.

Afterwards, haemolysis assay for biosurfactant determination was also reportedly developed by Mulligan et al. [96]. Following the development of haemolysis assay for biosurfactant determination, Carrillo et al. [106] also claimed to have discovered an association between haemolytic activity and surfactant production. Several studies similarly reported the impossibility of biosurfactant production without haemolytic activity, as haemolysis has been referred to as a determination of biosurfactant [87,106,113,121,131-134]. Whereas, as summed up by Kabir et al. [25], very few bacteria would have the selective advantage of lysing erythrocytes, yet haemolysis test has always been considered an ideal assay for determining surfactant production, as it is commonly claimed that biosurfactants cause lysis of erythrocytes, and this is usually the principle adopted in the haemolysis assay for biosurfacts determination.

In several studies on biosurfactants, haemolysis assay on blood agar plates has always been an exclusive experimental screening method to monitor biosurfactant production [19]. Based on the reference that biosurfactant-producing capacity in liquid medium was found to be associated with haemolytic activity, the use of blood agar lysis (haemolysis assay) was considered and recommended as appearing to be a good primary (and in few cases, secondary) screening criterion/method for biosurfactant production, by surfactant-producing microbial strains, and regarded as indicative of biosurfactant production [16,19,25,87,88,96,102,106,113,117,135-145].

### Preparation of blood agar for haemolysis assay

Blood agar is an enriched and differential solid growth medium with general composition of-blood agar peptone: 10 g/l; yeast extract: 3 g/l; NaCl: 5 g/l; blood: 100 ml/l (as the basal medium), of which specified mls. of human or animal (rabbit, sheep, horse or cattle) blood is added, for the growth of many microorganisms, especially, the fastidious microorganisms (i.e., microbial species that do not grow easily on general purpose culture media, which are microbial culture media that lack special nutrients). Firstly, for haemolysis assay, the isolated microbial colonies may be sub-cultured from primary plates, by four-corner streaking or repeated microbial colony transfers (for mostly fungal isolates) on appropriate sterile culture media, in order to obtain pure microbial cultures. The pure microbial cultures can then be inoculated on any of the various modified blood agar plates, such as, Zobell marine medium supplemented with 5% fresh human blood [121]. Other basal

culture media to which human or animal blood can be added, in order to prepare blood agar include, blood agar base, tryptone soy agar, nutrient agar, plate count agar, Mueller-Hinton agar, potato dextrose agar, Sabouraud dextrose agar, etc.

Inoculation of blood agar plates is usually followed by incubation at 25-37°C for 24-72°C or 96 hrs, depending on the bacterial or fungal species. The blood agar plates are then visually inspected for haemolysis (clear zone) around the haemolytic microbial colonies. Secondly, the initial isolation of suspected biosurfactant-producers may also primarily be done on blood agar plates, based on the acclaimed ability of many biosurfactants to lyse erythrocytes, which then results in haemolysis around suspected biosurfactant-producing microbial colonies, on the blood agar plates [25,87,131,135,146-148].

## Blood agar and haemolysis

Haemolytic microbial strains cause lysis of erythrocytes, and exhibit haemolytic zones, which can be complete or partial haemolysis around the haemolytic microbial colonies. As introduced by Brown, the three basic types of haemolysis (haemolytic reactions) that can be observed on blood agar plates are designated, alpha (∝), beta (β) and gamma (γ) haemolysis [148,149], as denoted in Figure 2.

Alpha-haemolysis (α-haemolysis) is a greenish discoloration that surrounds a haemolytic microbial colony, growing on blood agar plate. This type of haemolysis represents a partial (greenish) lysis or incomplete decomposition (reduction) of the haemoglobin of the erythrocytes (red blood cells). Alpha haemolysis is caused by hydrogen-peroxide produced by alpha-haemolytic bacteria or fungi, which oxidise haemoglobin to green methaemoglobin, in the medium surrounding the colony. Thus, alpha-haemolytic microbes thus, produce greenish diffusible appearance on blood agar plates [149-152].

Beta-haemolysis (β-haemolysis) represents a complete haemolysis (complete breakdown) of the haemoglobin of the red blood cells surrounding a microbial colony, on blood agar plate, giving a transparent or translucent clearing of the blood agar around the microbial colony. Beta haemolysis is more pronounced when the blood agar plate is incubated anaerobically, although some microorganisms are weakly beta-haemolytic species [149].

Gamma-haemolysis (γ-haemolysis/non-haemolysis) is the third type of haemolytic reaction, in which there is actually no haemolysis at all, as there is lack of haemolysis in the area surrounding the microbial colony on blood agar plates. Gamma-haemolysis show neither typical alpha nor beta haemolysis, due to no haemolytic change around the microbial growth on blood agar plates (http://www.encyclopedia.com/science/encyclopedias-almanacs-transcripts-and-maps/blood-agar-hemolysis-and-hemolytic-reactions). There may however, be, slight brownish discolouration (not haemolysis) on the blood agar plates [149]. Zonee of alpha and beta-haemolysis surrounding microbial colonies on blood-agar plates are however, designated as hallmark phenotypic features of various pathogenic microbes.

## Haemolysins as microbial toxins and virulence factors

Haemolysins, sometimes classified as enzymes, are lipids and proteins that have been extensively reported and studied in bacteria, fungi, various species of plants, invertebrates, mammals, and also denoted as perforins, in fungi, plants, invertebrates, and mammals [153-168]. Haemolysins cause lysis (destruction) of erythrocytes (red blood cells), by destroying their cell membrane (Figure 3), with release of their haemoglobins; thereby, providing iron, for bacterial growth. Through haemolysins enzymatic attack on phospholipids, the cell membranes are subsequently destabilised [169], as shown in Figure 3.

Pore formations in microbial cell membranes, derangement of microbial cell membrane integrity, detergent action, or lipase activity are the major mechanisms by which microbial haemolysisns cause haemolysis [69-173]. It was further proposed that the hydrophilic part (the cationic part) of biosurfactants initiate electrostatic interaction with the negatively charged components of the bacterial cell membranes; while the hydrophobic portion was supposed to permit the peptides to insert into, and permeate the bacterial cell membranes [174]. Some haemolysins however, attack the phospholipid of the host cytoplasmic membrane, by using phospholipases lecithinases, and the phospholipids, lecithin (phosphatidylcholine), often used as substrate; although, some haemolysins affect the sterols of the host cytoplasmic membrane [87].

In addition to bacterial growth, due to the release of haemoglobins after red blood lysis; thereby, providing iron, pathogenicity; are also reported, and the responsible haemolysisns considered as toxins. So, being identified as extracellular toxic proteins that are produced by several microbial species, all of which possess a certain pathogenic potential; haemolysins have usually been further considered as virulence factors [175], and sublytic effects of haemolysin can alter host cell regulation and lead to cell death [176,177]. Due to production of cytolytic toxins, haemolysins from several bacterial and fungal strains have been confirmed to possess lytic activities that correlate with severity of haemolysin–induced infections, sometimes, with high mortality rates [168,177-180]. Haemolysis is also considered a pathogenicity indicator tool [180-182], and haemolysins have similarly been linked to increased severity of infections, and concretely associated with virulence, in addition to pathogenesis or pathogenicity [152,183-188].

**Figure 2:** Haemolytic reactions on blood agar plates.

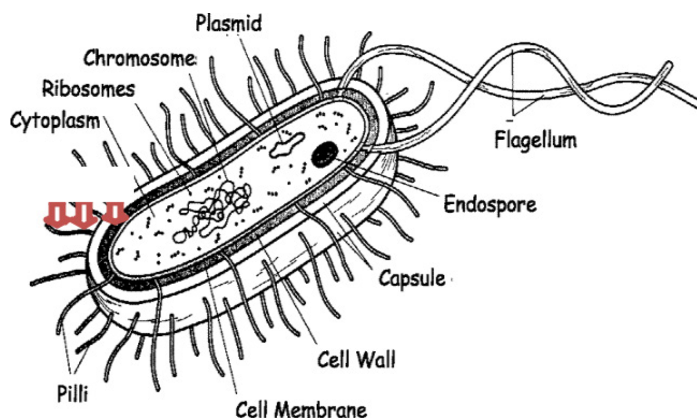


**Figure 3:** Permeability of bacterial cell membrane by haemolysin (biosurfactant).

Among the well-known diverse toxigenic microbial haemolysins are, small β-pore-forming toxins, alpha-haemolysin monomers secreted by *Staphylococcus aureus*, aerolysin, secreted by *Aeromonas hydrophila*; α-toxins, secreted by *Staphylococcus aureus* and *Clostridium septicum*; cholesterol-dependent cytolysins (CDCs), like streptolysin O, secreted by *Streptococcus pyogenes*, and listeriolysin O, secreted by *Listeria monocytogenes* or AB toxins, like the diphtheria toxin, secreted by *Corynebacterium diphtheriae*, as well as the toxic fungal haemolysins like, nigerlysin, aerolysin, ostreolysin, pleurotolysin A and B etc. [166,169,189-193].

Mechanisms of pore-forming toxins are depicted in Figure 4; thereby, it is designated that pore-forming toxins like thermostable direct haemolysin (TDH) are also known to induce haemolysis, by incorporating into cell membranes to form pores [194]. Pore-forming toxins are secreted by microbial pathogens in a water-soluble form that binds to the target cell, then generally multimerises into an amphipathic structure that finally inserts into the target cell membrane, and then forms a pore [169]. The native thermostable direct haemolysin (TDHn) is transformed into non-toxic fibrils, rich in beta-strands, by incubation at 600°C, to form the incubated thermostable direct haemolysin (TDHi). The TDHi fibrils are dissociated into unfolded states by further heating above 800°C (TDHu) but the protein is trapped in the TDHi structure by slow cooling of TDHu, while rapid cooling of TDHu results in refolding of the protein into toxic TDHn [195].

Haemolysins lyse erythrocytes, which results in the release of iron, an important growth factor for microorganisms, especially in pathogenicity, and during infections [196,197], as it is certain that numerous pathogenic microorganisms grow in the host by using haemin or haemoglobin as a source of iron [198-201]. Several fungal haemolysins have also thus, been proposed as virulence factors [202-204]. In addition to cell adherence, cytotoxicity and cell invasiveness, haemolysis also has an additional clinical significance, in being regarded as a virulence factor [205,206]. Furthermore, microbial haemolysins promote opportunistic infections and other clinical conditions, and also presented as risk factors in hospitals patients [202,207-209]. The expression of a haemolytic protein,

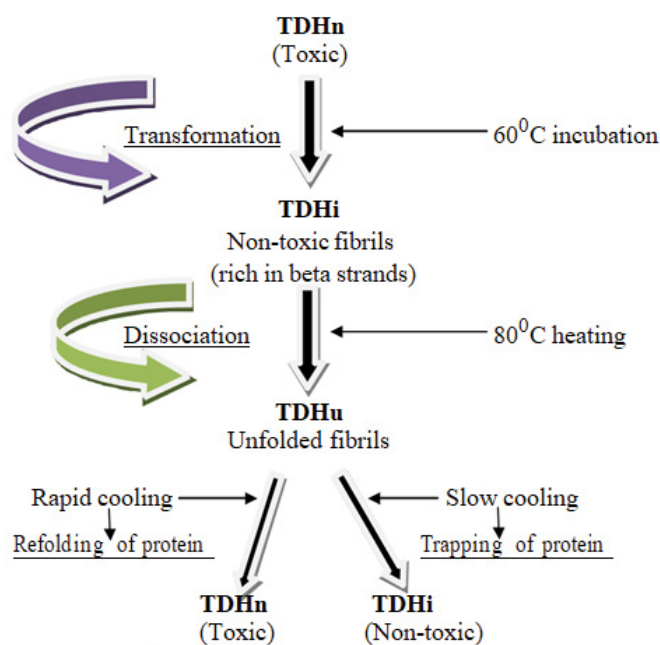


**Figure 4:** Pore-forming toxins mechanisms.

with capabilities to lyse erythrocytes, has also been suggested as providing survival strategy for fungi during opportunistic infections [210]. The haemolysin, which enabled the fungus to disrupt blood cells, contained negatively charged domains that could also be detected in infected patients [166,211-213].

Research studies have shown that another application of fungal haemolysins has been their use as biomarkers for personal exposure to fungi or species-specific identification of opportunistic fungal diseases [166,214,215-217]. There is therefore, considerable interest in the development of diagnostic assays for detecting haemolysins as biomarkers of allergic and disseminated fungal exposure [166]. In actual fact, fungal haemolysins have been useful as biomarkers for exposure to indoor fungi because they can be measured in bodily fluids and environmental samples [202].

## Extrapolations of the haemolysis screening assays in the determination of biosurfactants

According to Mulligan et al. [96] and Walter et al. [87], the technique of using blood agar plate haemolysis assay to screen for biosurfactant production on soluble substrates was shown to be quick and reliable. Some authors also believed that haemolysis screening method can be used to limit the number of samples, when selecting biosurfactant-producing microorganisms. In some cases, further screening for biosurfactant-producing microorganisms is only carried out, after screening for positive haemolytic activity [19]. The clear zone of haemolysis around the microbial colony on blood agar plates has commonly been related to the ability of the microbes to produce surfactants, while the diameter of the clear zone usually considered as a qualitative indicator of biosurfactant production [96,218]. It has however, been reported that haemolysis assay is not a specific method for biosurfactant production, since not all biosurfactants have haemolytic activities, basically due to presence of compounds other than biosurfactants [102,113]. Such other compounds include, virulence factors, toxins, and other lytic enzymes that can lyse erythrocytes [219]. It was also reported that biosurfactants that are poorly diffusible may not lyse erythrocytes nor cause haemolysis [220,221]. Furthermore, in some studies, haemolysis assay was found to exclude many good biosurfactant-producers, while in some reports, microbial strains with positive haemolytic activity were found to be negative for biosurfactant production [113]. There were a number of reports as well, which confirmed that microorganisms that were positive as biosurfactant-producing with the use other selection criteria, were negative for biosurfactant production when screened for haemolytic activity [88,113,121].

The poor specificity of haemolysis screening assay had also been confirmed, in that, it can give a lot of false-negative and false-positive results [113,117]. In addition, it was reported that the diffusion restriction of surfactant can inhibit the formation of clearing zones on blood agar plates. Likewise, over-incubation of the blood agar plates may cause microbial overgrowth, which can lead to accumulation of microbial waste-products that may lyse the blood on blood agar plates; thereby, giving false appearance of biosurfactants, which are actually not present (http://www.encyclopedia.com/science/encyclopedias-almanacs-transcripts-and-maps/blood-agar-hemolysis-and-hemolytic-reactions). Until now, haemolytic microbial strains were generally believed to be biosurfactant-producers. Whereas, from the microbiological,

clinical, pathophysiological and public health points of interpretations, the degree by which erythrocytes are haemolysed on blood-based culture media, is basically used to distinguish haemolytic and non-haemolytic microorganisms. Moreover, visualising the physical appearance of haemolysis on cultured blood agar plates has been used as a tool to determine the aetiologic (disease-causing) microbial species of various microbial infections [222].

In more recent times, biosurfactants have been generally considered as biodegradable, non-toxic (or minimally toxic), and eco-friendly/environmental-friendly compounds that are released by microorganisms [40,54,55,61,66,223-225]. But, apparently, most of the biosurfactants proposed in literature are reportedly produced by pathogenic microbes involved in pathogenesis, while relative levels of acute toxicity have also been recorded among significant numbers of surfactant-producing bacteria and fungi. The pathogenicity associated with haemolysis is therefore, a cause for concern, considering it being a commonly adopted biosurfactant potential and /or biosurfactant screening criterion. Therefore, contrary to the reports that biosurfactants-producing microorganisms used in some studies were generally recognised as safe (GRAS) [20], most of the microorganisms referred to as GRAS may still harbour one or more pathogenic/toxigenic/virulence factor(s), which can make them opportunistic pathogens [226].

Many well-characterised biosurfactant producers have been confirmed as pathogenic microbial species [24,203,227]. Conversely, haemolysis and haemolysins are specifically indicative of pathogenic and/or virulent or/and toxigenic status, rather than biosurfactant-production. The haemolytic action of certain bacteria and fungi on blood agar is so striking that haemolysis has been classified as very significant in clinical diagnosis of microbial importance. Due to the pathogenicity of some biosurfactants-producing microorganisms [24,203,227,228], they were therefore, more recently, considered not appropriate for scaled- up production [25]. The detection of virulence genes coding for haemolysis and the determination of the antimicrobial resistance, in addition to the factors that contribute to pathogenicity and toxicity can contribute to better understanding of the need for better selection criteria of biosurfactants-producing microorganisms, and applications of their products [228], in the petroleum industries.

As earlier reported, literature on the production and analytical detection of biosurfactants is overwhelming, with assertions of high yields, and with mostly over-exaggerated estimates, due to the use of flawed or inaccurate analytical techniques [229]. However, none of the previous documented assertions on haemolysis assay, in biosurfactant determinations or contrariwise, highlighted the vivid microbiological and safety implications of haemolysis assay in biosurfactants-producing microorganisms. Based on the tremendous afore-mentioned intriguing justifications, haemolysis assay more appropriately identifies microbial strains with haemolytic (pathogenic/toxigenic/virulent) potentials. It can then be extrapolated that haemolysis assay (i.e., lyses of erythrocytes) on blood agar plates are more of diagnostic or determinative tools for microbial pathogenicity, rather than biosurfactants productions, and can therefore, not be conclusively confirmatory of biosurfactant-production, nor considered an appropriate selection criterion for biosurfactants. Thus, the likely or real pathogenicity, and/or virulence and toxicity of biosurfactants-producing microbes need to be appropriately assessed by haemolysis assay, prior to their potential applications in various petroleum industries, more especially, as they may be multi-antimicrobial resistant haemolytic.

From the petroleum industries perspectives, polycyclic aromatic hydrocarbons (PAHs) and naphthenic acids (NAs) are well-known to be toxic contaminants of environmental concern [230]. It is therefore, of necessity to ensure that additional hazardous concerns associated with petroleum activities are not introduced into the environment. A variety of microbial taxa are able to synthesize biosurfactants but it is ideal to isolate biosurfactants-producing microorganisms from appropriate and safe sources. From the ideal petroleum microbiology, public health, and hydrocarbon-processing points of view, the microbial strain profile and the ecological niche matter, as they determine the physiological status and metabolites production of the putative microorganisms. Therefore, it is proper to isolate biosurfactants-producing microorganisms from same or closely related ecological nich(es), for same physiological characteristics, extended survival, and maximal production of biosurfactant metabolites. Furthermore, toxic agents can cross microbial membranes [231], into the hosts; so, haemolysis assay, is hereby, suggested as, a highly determinative and qualitative screening assay indicative of the biosafety potentials, for the determination of pathogenic, toxigenic and/or virulent biosurfactant-producing microorganisms in the petroleum industries.

## CONCLUSION

Biosurfactants are highly important microbial compounds of tremendous benefits but their significant public health concerns, especially regarding their haemolytic potentials serving as biosurfactant property are presently misconstrued. Bacterial and fungal haemolysins have been used as diagnostic tools, and/or biomarkers but microbial toxicity is undesirable in selected microbial candidates for various beneficial activities, such as, biosurfactants-productions. Based on the intriguing afore-listed justifiable reasons, it can be noted and extrapolated that haemolysis assay; using blood agar is not so reliable, sensitive or suitable for determination of biosurfactant production, as it does not correlate particularly with specific characteristics of biosurfactants' production. However, haemolysis assay is quite appropriately as, a reliable and sensitive safety bioassay, in routine monitoring, for pathogenic/toxigenic, and virulence determinations and regulations, as well as for selecting safe and environmental-friendly biosurfactant-producing microbial candidates.

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