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ORIGINAL ARTICLE

Potential application of cyclic lipopeptide biosurfactants produced by *Bacillus subtilis* strains in laundry detergent formulations

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Keywords
Bacillus subtilis, emulsification index, laundry detergent, lipopeptide biosurfactants, stain removal, surface tension reduction.

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Abstract

Aims: Crude cyclic lipopeptide (CLP) biosurfactants from two *Bacillus subtilis* strains (DM-03 and DM-04) were studied for their compatibility and stability with some locally available commercial laundry detergents.

Methods and Results: CLP biosurfactants from both *B. subtilis* strains were stable over the pH range of 7.0–12.0, and heating them at 80°C for 60 min did not result in any loss of their surface-active property. Crude CLP biosurfactants showed good emulsion formation capability with vegetable oils, and demonstrated excellent compatibility and stability with all the tested laundry detergents.

Conclusion: CLP biosurfactants from *B. subtilis* strains act additively with other components of the detergents to further improve the wash quality of detergents. The thermal resistance and extreme alkaline pH stability of *B. subtilis* CLP biosurfactants favour their inclusion in laundry detergent formulations.

Significance and Impact of the Study: This study has great significance because it is already known that microbial biosurfactants are considered safer alternative to chemical or synthetic surfactants owing to lower toxicity, ease of biodegradability and low ecological impact. The present study provides further evidence that CLP biosurfactants from *B. subtilis* strains can be employed in laundry detergents.

Introduction

Among the six groups of laundry detergent formulations, surfactants constitute one of the most important detergent components. Generally, surfactants are water-soluble surface-active agents that lower the surface tension of water and possess wetting, emulsifying, detergency and dispersing properties that enable the removal of dirt (soil particles) from fabrics. Almost all surfactants used in modern day detergents are chemically synthesized, which includes soaps and alkylbezenesulfonates (anionic), ethoxylated fatty acids (nonionic) etc.

Previous research has revealed an acute toxicity of laundry detergent components to fresh-water living organisms, such as fish and crustaceans, and these components often produce undesirable effects (Muller 1980; Warne and Schifko 1999). Surfactants and sodium silicate solutions are the main contributors to the toxicity of detergents, whereas the remaining detergent components that are present at lower concentrations contribute very little to detergent toxicity (Warne and Schifko 1999). Growing public concern about the environmental hazards and risks associated with chemical surfactants has stimulated the search for eco-friendly, natural substitutes of chemical surfactants in laundry detergents.

In recent years, natural surfactants of microbial origin, known as biosurfactants, are getting much more attention compared with the chemical surfactants owing to their lower toxicity, biodegradability, low anti-irritating effects and compatibility with skin (Kleckner and Kosaric 1993; Makkar and Cameotra 2002; Maier 2003; Mullican *et al.* 2005). Moreover, owing to the presence of one or more functional groups and chiral centers, low critical micelle concentration and higher surface activity and superior

ability to form molecular assembly and liquid crystals, biosurfactants offer some distinct advantages over the highly used synthetic surfactants, and therefore, they are considered superior to chemical surfactants (Finnerty 1994; Kitamoto *et al.* 2002). Microbial surfactants exhibit high specificity and are consequently suited to new applications as evidenced by numerous reports published during the last decade on the application of biosurfactants in various industrial sectors (Banat *et al.* 2000; Singh and Cameotra 2004) and in environment protection (Banat 1995; Das and Mukherjee 2007). Despite this, a search of the literature shows that the role of biosurfactants as substitutes of chemical surfactants in laundry detergents has rarely been explored.

Our previous study showed that two *Bacillus subtilis* strains (DM-03 and DM-04), isolated from two extremely different habitats, are efficient producers of cyclic lipopeptide (CLP) biosurfactants under thermophilic growth conditions (Mukherjee and Das 2005). These CLP biosurfactants possess some advantageous properties, such as reduction of water surface tension, micelle formation and a capability to solubilize a large number of hydrophobic compounds (Mukherjee and Das 2005). In addition, they are safe to nontarget aquatic organisms, particularly fish (Das and Mukherjee 2006), which make them good candidates for application in laundry detergents, may be as a substitute for chemical surfactants. The present study was initiated with an aim to test the compatibility and stability of *B. subtilis* CLP biosurfactants in laundry detergents and to evaluate the wash performance of laundry detergents in the presence of these CLP biosurfactants.

Materials and methods

Micro-organisms

Procedures for isolation, characterization and taxonomic identification of biosurfactant-producing micro-organisms viz., *B. subtilis* DM-03 and DM-04 strains were previously described (Das *et al.* 2004; Mukherjee and Das 2005). These bacteria, isolated from a traditional fermented food and crude petroleum-oil contaminated soil sample from Northeast India, are capable of secreting biosurfactant under thermophilic growth conditions (Mukherjee and Das 2005). They were subcultured on nutrient agar plates before use as inoculums for biosurfactant production studies.

Batch fermentation and isolation of CLP biosurfactant

Production of lipopeptide from *B. subtilis* strains was carried out in a 5-l Bioflow 110 Fermentor (New Brunswick Scientific, Edison, NJ) as previously described (Das and Mukherjee 2005). The cells were harvested at exponential phase (48 h), and the clear cell-free supernatant was used to isolate crude CLP biosurfactants as previously described (Mukherjee and Das 2005).

Measurement of surface tension

The surface tension was determined using a Du-Nouy Tensiometer (Kruss 9KT Tensiometer, Kruss, Germany) at room temperature (25°C) using the ring correction mode of the instrument (Das and Mukherjee 2005).

Effect of heating and pH on biosurfactant activity

The thermal resistance of crude CLP biosurfactants at alkaline pH (10.0) was assayed by incubating aqueous solutions of biosurfactants (5 g l^{-1}) at 37–80°C for 90 min, cooling to room temperature followed by measuring the surface tension value. For assaying the pH stability of CLP biosurfactants, aqueous solutions of crude biosurfactant were adjusted to pH values ranging from 7 to 12, and the surface tension value of the resulting solutions was measured.

Emulsification assay

Emulsification assay of crude CLP biosurfactants was done by the method described by Cooper and Goldenberg (1987). Briefly, emulsification activity was measured by adding 6.0 ml of vegetable oil to 4.0 ml of aqueous biosurfactant solution and then vortexing the mixture at high speed in a SPINIX vortex for 2 min. The resulting mixture was kept at room temperature (~25°C) for 24 h and the emulsification index (E_{24}) was calculated as follows:

$$E_{24} = \frac{\text{Height of emulsion layer (cm)}}{\text{Total height of the mixture (cm)}} \times 100$$

Detergent compatibility and stability of biosurfactant

In order to confirm the potential of *B. subtilis* CLP biosurfactants as detergent additives, their compatibility and stability towards some commercial laundry detergents, available in a local market, such as Surf excel®, wheel®, Rin advanced® (Hindustan Lever Ltd, Mumbai, India), Tide® and Ariel® (Procter and Gamble, Mumbai, India) and Henko® (Henkel Spic India Ltd., Chennai, India) were examined. Detergents and biosurfactants were individually dissolved in tap water at a concentration of 10 g l^{-1} and the surface tension of tap water, detergent and biosurfactant solutions was measured. Thereafter, detergent and biosurfactant solutions were mixed in a

ratio of 1 : 1, and the resulting mixtures were incubated at two different temperatures (room temperature ~25°C and at 60°C) for 180 min, followed by measuring the surface tension of the mixtures. As a control, the detergent and biosurfactant solutions were incubated separately under identical conditions. Each experiment was repeated in triplicate to assure reproducibility.

Evaluation of wash performance

The wash performance of either the detergent solution (1.0% w/v in tap water) alone or the detergent and the biosurfactant solutions (1.0% w/v in tap water), mixed in a ratio of 1 : 1, was evaluated by subjecting the following two tests: (i) removal of vegetable oil (sunflower oil) and (ii) removal of blood stain from cotton fabrics.

Briefly, a white cotton cloth was cut into 1×10^{-1} m^2 pieces and each piece was stained with either 2.0×10^{-3} l of sunflower oil or fresh goat blood (obtained from slaughter house) and then dried at 40°C overnight. To test the wash performance, each piece of cloth, stained either with vegetable oil or with goat blood, was dipped in any one of the following flasks containing: (i) 5.0×10^{-2} l of tap water (control); (ii) 4.0×10^{-2}-l tap water and 1.0×10^{-2} l of 1.0% (w/v) detergent solution; (iii) 4.0×10^{-2}-l tap water and 1.0×10^{-2} l of 1.0% (w/v) biosurfactant (obtained from either strain) solution; or (iv) 4.0×10^{-2}-l tap water and 5.0×10^{-3} l each of 1.0% (w/v) detergent and biosurfactant solutions. Flasks were rotated at 50 rev min^{-1} for 20 min at room temperature (~25°C), followed by the removal of cloth pieces from the flasks, and the left over wash solution was decanted carefully to avoid soap bubbles. This postwash water was used to determine the removal of oil or blood stain (haemoglobin) from the cloths.

The amount of oil removed from each cloth piece was quantitated by extracting the oil from postwash water with a 2 : 1 (v/v) methanol–chloroform mixture in a final volume of 2.0×10^{-2} l. This process was repeated once more and the combined extracts were evaporated to dryness. The amount of oil recovered was determined gravimetrically. For determining the removal of blood stain (haemoglobin) from the cloth pieces, the absorbance of the postwash solution was measured at 540 nm against either detergent/or detergent + CLP biosurfactant solutions. Each experiment was repeated in triplicate to assure the reproducibility, and the standard error in all the experimental results are within 5%.

Per cent increase in the removal of oil/blood stain by detergents supplemented with crude CLP biosurfactants compared with detergent solution alone was calculated as

$$\% \text{ increase} = B - A/A \times 100$$

where A = oil/Hb removed by detergent solution alone, and B = oil/Hb removed by detergent + CLP biosurfactant solution.

Statistical analysis

The results are represented as the mean ± SD from at least three experiments. Statistical analysis was done by Student's t-test (Daniel 2000). A probability level of $P < 0.05$ was considered statistically significant.

Results

Effect of heating and pH on biosurfactant activity

As shown in Table 1, crude CLP biosurfactants from *B. subtilis* strains were stable in the entire pH range from 7.0 to 12.0. Similarly, heating the crude CLP biosurfactant solutions at 80°C up to 60 min did not result in any loss of their surface-active properties; however, heating crude CLP biosurfactants from *B. subtilis* DM-03 and DM-04 strains for 90 min at the same temperature resulted in a loss of 3.2% and 1.2% of original surface tension reduction property, respectively (data not shown).

Emulsification assay

The ability of the crude CLP biosurfactants isolated from *B. subtilis* DM-03 and DM-04 strains to form emulsion with vegetable oils is depicted in Fig. 1. CLP biosurfactants isolated from both strains were shown to be good emulsifiers, supporting their future application in laundry detergents. However, results showed that crude biosurfactant obtained from the *B. subtilis* DM-04 strain was more efficient in forming emulsion with tested oils ($P < 0.05$)

Table 1 Influence of pH on surface tension reduction property of crude cyclic lipopeptide (CLP) biosurfactants from *Bacillus subtilis* DM-03 and DM-04 strains

	Surface tension reduction by crude CLP biosurfactants (N m^{-1})	
pH	*B. subtilis* DM-03	*B. subtilis* DM-04
7·0	29·0 ± 0·5^x	26·5 ± 0·5^y
8·0	29·0 ± 0·5^x	25·8 ± 0·5^y
9·0	29·5 ± 1·0^x	28·2 ± 0·8^x
10·0	29·5 ± 0·5^x	28·1 ± 0·6^x
11·0	31·2 ± 1·1^x	30·2 ± 1·0^x
12·0	32·0 ± 1·2^x	30·1 ± 1·0^x

Values are mean ± sd of three experiments. Values in the same row within each experiment followed by different superscripts are significantly different ($P < 0.05$).

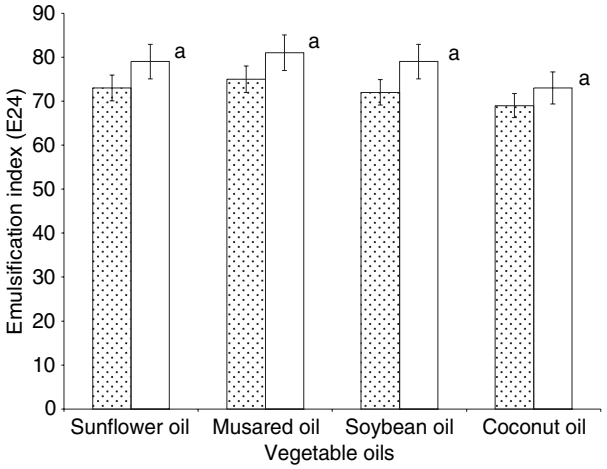

Figure 1 Emulsification property (E_{24}) of crude cyclic lipopeptide (CLP) biosurfactants isolated from *Bacillus subtilis* DM-03 (▨) and DM-04 (□) strains. Each value represents mean ± sd of three experiments. The emulsification index of CLP biosurfactants from *B. subtilis* DM-04 was significantly higher than those from *B. subtilis* DM-03 strain – $^{a}P < 0.05$.

compared with the biosurfactant from the *B. subtilis* DM-03 strain (Fig. 1).

Detergent compatibility and stability of biosurfactant

Present-day commercial laundry detergent formulations include anionic surfactants, bleaching agents, water-softening builders and enzymes. A prerequisite for any substance to be included in the detergent formulation is its stability and compatibility in the presence of detergent components over a wide range of temperatures generally applied for washing purposes.

As shown in Table 2, the stability of *B. subtilis* crude CLP biosurfactants in laundry detergents demonstrated a promising result. More interestingly, surface-tension reduction shown by a 1 : 1 (v/v) mixture of detergent: biosurfactant solution was significantly higher ($P < 0.05$) compared with the same property exhibited by either detergent solution or crude CLP biosurfactant solution alone in the tested temperatures (25 and 60°C). These results clearly indicate that CLP biosurfactants produced by both *B. subtilis* strains have remarkable stability and compatibility with various commercial laundry detergents.

Wash performance

The results of the present investigation clearly demonstrated the improved wash performance of detergents in the presence of CLP biosurfactants obtained from either *B. subtilis* strain. This was evident from the enhanced removal of oil (9–14%) and blood stain (23–26%) from the cotton fabrics when the washing was performed in the presence of detergents containing CLP biosurfactants compared with the detergent solution alone (Fig. 2).

The biosurfactant from *B. subtilis* DM-04 strain was more efficient than DM-03 strain ($P < 0.05$) in improving the wash performance of the laundry detergents (Fig. 2). However, the removal of oil/blood from cotton fabrics by crude CLP biosurfactants from either *B. subtilis* strain was significantly lower ($P < 0.05$) than the removal of oil/blood stain by detergent solution alone (data not shown).

Table 2 Stability and compatibility test of *Bacillus subtilis* crude cyclic lipopeptide (CLP) biosurfactants in commercial laundry detergents at two different temperatures

	Surface tension reduction (N m^{-1})*	
Detergent and/or CLP biosurfactant	25°C	60°C
CLP from *B. subtilis* DM-03	30·5 ± 0·5	31·0 ± 0·6
CLP from *B. subtilis* DM-04	28·5 ± 0·8	29·0 ± 1·0
Surf excel®	31·8 ± 0·3	31·6 ± 0·4
Surf excel® + CLP from *B. subtilis* DM-03	26·2 ± 0·4^{x}	26·5 ± 0·4^{x}
Surf excel® + CLP from *B. subtilis* DM-04	25·0 ± 0·3^{x}	25·4 ± 0·6^{x}
Rin advanced®	31·6 ± 0·4	31·9 ± 0·6
Rin advanced® + CLP from *B. subtilis* DM-03	27·5 ± 0·3^{x}	28·3 ± 0·4^{x}
Rin advanced® + CLP from *B. subtilis* DM-04	25·8 ± 0·4^{x}	26·2 ± 0·6^{x}
Tide®	31·3 ± 0·6	31·9 ± 0·5
Tide® + CLP from *B. subtilis* DM-03	27·3 ± 0·4^{x}	28·0 ± 0·3^{x}
Tide® + CLP from *B. subtilis* DM-04	25·6 ± 0·4^{x}	26·1 ± 0·5^{x}
Wheel®	32·4 ± 0·7	33·1 ± 0·9
Wheel® + CLP from *B. subtilis* DM-03	27·4 ± 0·5^{x}	28·1 ± 0·8^{x}
Wheel® + CLP from *B. subtilis* DM-04	25·4 ± 0·3^{x}	27·2 ± 1·1^{x}
Henko®	31·6 ± 0·4	31·9 ± 0·5
Henko® + CLP from *B. subtilis* DM-03	27·4 ± 0·6^{x}	28·0 ± 0·4^{x}
Henko® + CLP from *B. subtilis* DM-04	25·6 ± 0·5^{x}	26·4 ± 0·8^{x}
Ariel®	30·6 ± 0·6	31·0 ± 0·7
Ariel® + CLP from *B. subtilis* DM-03	27·3 ± 0·4^{x}	28·2 ± 0·6^{x}
Ariel® + CLP from *B. subtilis* DM-04	25·5 ± 0·5^{x}	26·4 ± 0·7^{x}

*Surface tension of water was 70.2 ± 0.6 N m^{-1}.
Experiment was performed as described in the text. Each data point represents the mean ± SD of three experiments.
Significance of difference with respect to surface tension reduction by detergent alone is $^{x}P < 0.05$.

Discussion

The basic prerequisite for any component to be included in laundry detergents should be its stability at the alkaline pH range and the ability to withstand the washing temperature. Thermal resistance of lipopeptide biosurfactants in the present study is in good agreement with earlier reports demonstrating the thermostable nature of biosurf-

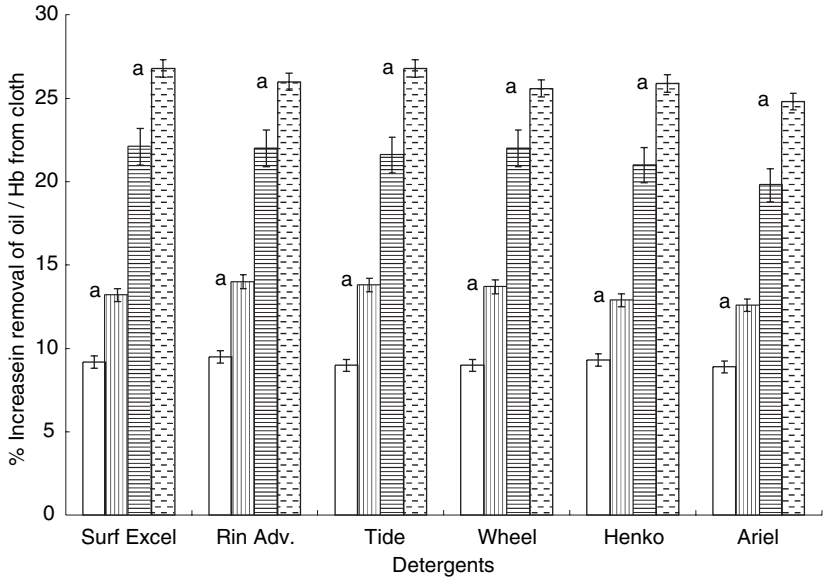

Figure 2 Improvement of wash performance of some commercial laundry detergents in the presence of *Bacillus subtilis* cyclic lipopeptide (CLP) biosurfactants at room temperature (~25°C). Per cent increase in the removal of oil from cotton fabrics by detergents in presence of CLP biosurfactants from *B. subtilis* DM-03 (□) and *B. subtilis* DM-04 strain (▥); and per cent increase in removal of blood stain from cotton fabrics by detergents in presence of CLP biosurfactants from *B. subtilis* DM-03 (▤) and *B. subtilis* DM-04 strain (▨). The values are mean ± SD of three washing experiments. Significance of difference with respect to CLP biosurfactants from *B. subtilis* DM-03 strain, $^{a}P < 0.05$.

actants from other *B. subtilis* strains (Makkar and Cameotra 1998). In general, detergents are subjected to wash performance within a temperature range of 25–60°C, and therefore, the thermal resistance and extreme alkaline pH stability of *B. subtilis* CLP biosurfactants favour their inclusion in laundry detergent formulations.

In molecular terms, surface-active agents are amphiphilic compounds containing both hydrophilic and lipophilic moieties, which are essential for forming and stabilizing dispersive systems, such as foams and emulsions (Maier 2003). Their efficiency in foaming and emulsifying depends on their amphiphilic structure (Deleu *et al.* 1999). The lipopeptide molecules, owing to their hybrid structure and intermediate size in comparison with small surfactant molecules and high molecular weight proteins, diffuse and orient rapidly at water–oil interfaces, thus efficiently reducing the interfacial tension and promoting the formation of a dispersion system (Deleu *et al.* 1999; Maier 2003). Furthermore, it has been reported that the foaming property of surfactin, a lipopeptide biosurfactant form *B. subtilis*, was superior in comparison with the same property exhibited by sodium dodecyl sulfate (SDS) and bovine serum albumin (BSA) (Deleu *et al.* 1999). However, the significantly higher emulsification property shown by CLP biosurfactants from *B. subtilis* DM-04 compared with the DM-03 strain may be related to the differences in the biochemical composition of these CLP biosurfactants. The biosurfactant secreted by *B. subtilis* DM-03 is rich in iturins, whereas surfactins represent the predominant lipopeptide isoforms in the *B. subtilis* DM-04 strain (Mukherjee and Das 2005). It is well known that among the lipopeptide families, surfactin is the most effective in terms of emulsion formation with oils and lowering the surface tension of water (Deleu *et al.* 1999; Peypoux *et al.* 1999; Mukherjee and Das 2005).

The results of the present study shows that CLP biosurfactants from *B. subtilis* strains act additively with other components of detergents to further improve the wash quality of detergents. Superior wash performance of the laundry detergents in presence of lipopeptide biosurfactant from *B. subtilis* DM-04 strain, compared with *B. subtilis* DM-03 strain, might be attributed to the presence of higher amounts of surfactin isomers in the former biosurfactant. It is well established that surfactin molecules have higher emulsion formation property and perform better compared with iturin isomers at higher alkaline pH (Morikawa *et al.* 2000).

The stability and compatibility of any component in detergent should not be the only prerequisite for its inclusion in detergent formulation. To save energy from heating the water to be used for washing, the ability of the detergent components to perform wash function at lower (room) temperature should also be addressed (Krik *et al.* 2002). Therefore, as shown in the present study, the better wash performance of detergents in the presence of *B. subtilis* CLP biosurfactants at room temperature has great relevance, because the supplementation laundry detergents with these CLP biosurfactants may overcome the problems with efficient cleaning and stain removal of detergents at low temperature.

In conclusion, the compatibility and stability of CLP biosurfactants from *B. subtilis* in various commercial laundry detergents, and above all, their additive interaction with the components of tested laundry detergents to improve the wash performance by efficiently removing oil and blood stains, leads us to conclude that the future use

of CLP biosurfactants from *B. subtilis* strains as laundry detergent additives is highly promising.

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