OPTIMIZATION OF NUTRITIONAL AND PHYSICOCHEMICAL PARAMETERS FOR ENHANCED BIOSURFACTANT PRODUCTION BY BACILLUS SUBTILIS ISOLATES

***Dr. R. Udayasree[1] and Prof V. V. Lakshmi[2]**

1,2 Dept. of Applied Microbiology, Sri Padmavati Mahila Visvavidyalayam, Tirupathi, Chittoor, Andhra Pradesh, India.

**Corresponding author: Dr. R. Udayasree*

Abstract

Biosurfactants are surface-active microbial metabolites with considerable potential as environmentally compatible alternatives to synthetic surfactants (Desai and Banat, 1997). The present study investigated the optimization of nutritional and physicochemical parameters that influence biosurfactant production in two indigenousBacillus subtilisisolates, BS4 and BS9. The isolates were selected based on positive responses in preliminary biosurfactant screening assays and were identified by morphological, biochemical, and 16S rDNA-based characterization. Both isolates were found to produce the highest biosurfactant levels in mineral salt medium (MSM), which was therefore selected as the production medium. Optimization was performed by evaluating different hydrocarbon sources, hydrocarbon concentrations, nitrogen sources, and nitrogen concentrations, followed by optimization of pH and temperature. Among nine hydrocarbon sources tested at 1%, Castrol supported the highest biosurfactant production by BS4 (6.0 g/L), whereas BS9 produced 4.6 g/L with Castrol. Increasing the concentration of selected hydrocarbon sources demonstrated that 2% Castrol supported maximum production by BS4 (10.9 g/L), while BS9 produced 6.9 g/L under the same conditions. Sodium nitrate was identified as the preferred nitrogen source for both isolates. The maximum values obtained during nitrogen-source concentration optimization were 14.2 g/L for BS4 and 10.5 g/L for BS9 at 1.5% sodium nitrate. Optimization of physicochemical conditions showed that BS4 produced 15.7-15.9 g/L at pH 6-7, while BS9 showed maximum production of 13.4 g/L at pH 7. Both isolates exhibited maximum production at 45°C, with yields of 17.3 g/L for BS4 and 18.9 g/L for BS9. Optimized conditions resulted substantial enhancement of biosurfactant production, demonstrating the importance of nutritional and physicochemical parameters in improving biosurfactant productivity by B. subtilisisolates (Abdel-Mawgoud et al., 2008; Mukherjee et al., 2006).

Keywords: Bacillus subtilis; biosurfactant; optimization; hydrocarbon source; sodium nitrate; pH; temperature

Introduction

Biosurfactants are biologically produced surface-active compounds capable of reducing surface and interfacial tension between immiscible phases (Cooper, 1986; Rosenberg and Ron, 1999). These amphiphilic molecules consist of hydrophilic and hydrophobic moieties that partition at interfaces, enabling them to lower surface and interfacial tension and form microemulsions (Banat et al., 2000; Tadros, 2005). Microbial biosurfactants have attracted considerable attention because of their biodegradability, comparatively low toxicity, functional activity under diverse environmental conditions, and potential for production from renewable substrates (Mulligan, 2005; Santos et al., 2016; Makkar et al., 2011).

Members of the genusBacillusare particularly important biosurfactant producers they can synthesize lipopeptide compounds with surface-active and antimicrobial properties (Peypoux et al., 1999; Shaligram and Singhal, 2010). The most extensively studied lipopeptide biosurfactant is Surfactin, produced byBacillus subtilis, which exhibits exceptional surface-active properties, with the ability to reduce the surface tension of water from 72 to 27 mN/m at concentrations as low as 0.005% (Arima et al., 1969; Kowall et al., 1998). Other lipopeptides produced byBacillusspecies include iturin, fengycin, and lichenysin, each possessing unique structural and functional characteristics (Besson et al., 1976; Madslien et al., 2013; Morikawa et al., 2000).

The productivity of microbial biosurfactants is strongly influenced by the composition of the culture medium and physicochemical conditions of fermentation (Syldatk and Wagner, 1987; Makkar and Cameotra, 2002). Optimization of nutritional and environmental parameters is therefore essential for improving biosurfactant yield and making microbial production more efficient and economically viable (Mukherjee et al., 2006; Ghribi and Ellouze-Chaabouni, 2011). Carbon source, carbon concentration, nitrogen source, nitrogen concentration, pH, and temperature can substantially influence both microbial growth and biosurfactant synthesis (Cooper and Goldenberg, 1987; Kim et al., 1997; Wei and Chu, 1998). The selection of appropriate carbon sources is particularly critical, as biosurfactant production is often induced by hydrophobic substrates that serve as inducers of biosurfactant synthesis (Perfumo et al., 2010; Hua and Wang, 2012). Similarly, nitrogen source and concentration affect the balance between cell growth and secondary metabolite production, with inorganic nitrogen sources frequently preferred for biosurfactant synthesis (Raza et al., 2007; Joice and Parthasarathi, 2014).

The use of low-cost substrates for biosurfactant production has gained increasing attention as a strategy to reduce production costs and enhance economic feasibility (Banat et al., 2014; Makkar et al., 2011). Various waste materials, including vegetable oils, molasses, whey, and industrial by-products, have been successfully utilized as substrates for biosurfactant production (Al-Bahry et al., 2013; Joshi et al., 2008; Patowary et al., 2016b). The optimization of fermentation parameters enables maximum utilization of these substrates and improved biosurfactant yields (Najafi et al., 2010; Pereira et al., 2013).

In the present study, preliminary screening of bacterial isolates resulted in the selection of BS4 and BS9, which were subsequently identified asB. subtilisby 16S rDNA analysis. The sequences were deposited in NCBI under accession numbers KF626464 for BS4 and KF626465 for BS9 (Carrillo et al., 1996; Bodour and Miller-Maier, 1998). The objective of the present study was therefore to determine the nutritional and physicochemical conditions that maximize biosurfactant production byB. subtilisBS4 and BS9. The study specifically evaluated hydrocarbon source, hydrocarbon concentration, nitrogen source, nitrogen concentration, pH, and temperature, followed by determination of the overall optimized production conditions (Abdel-Mawgoud et al., 2008; Ghribi and Ellouze-Chaabouni, 2011).

2. MATERIALS AND METHODS

2.1 Bacterial Isolates

The bacterial isolates BS4 and BS9 were selected from previously screened biosurfactant-producing isolates (Shoeb et al., 2015; Donio et al., 2013). Both isolates exhibited characteristics consistent with the genusBacillus. They were Gram-positive, rod-shaped, aerobic, motile, and spore-forming organisms. Growth was observed between 27 and 55°C, and the isolates tolerated up to 2% NaCl (Holt et al., 1994; David Greenwood, 2008). Biochemical characterization showed that BS4 and BS9 were positive for Voges–Proskauer reaction, citrate utilization, H_2S production, and nitrate reduction. Both isolates utilized glucose, sucrose, maltose, fructose, mannitol, and xylose, while lactose and galactose were not utilized (MacFaddin, 2000; Cappuccino and Sherman, 2002).

16S rDNA analysis identified BS4 and BS9 as members ofBacillus subtilis. The corresponding sequences were deposited in the NCBI database under accession numbers KF626464 and KF626465, respectively (Weisburg et al., 1991; Janda and Abbott, 2007).

2.2 Selection of Production Medium

Biosurfactant production was compared in different liquid media, including nutrient broth, Luria-Bertani broth, and mineral salt medium (MSM) (Sousa et al., 2012; Oliveira et al., 2013). Among the tested media, mineral salt medium supported the highest biosurfactant production by BS4 and BS9 (Costa et al., 2005; Neto et al., 2008). Therefore, MSM was selected as the production medium for subsequent optimization experiments. The composition of MSM included (g/L): $Na_2HPO_4 \cdot 2H_2O$ (4.0), KH_2PO_4 (1.0), $MgSO_4 \cdot 7H_2O$ (0.5), $CaCl_2 \cdot 2H_2O$ (0.02), $FeSO_4 \cdot 7H_2O$ (0.001), and NaCl (1.0) (Haba et al., 2000; Yin et al., 2009).

2.3 Optimization of Hydrocarbon Source and Concentration

Nine hydrocarbon sources were evaluated individually at a concentration of 1% (v/v) in the selected production medium (Kumar and Mamidyala, 2011; Raza et al., 2007). The hydrocarbons evaluated were: Castrol, olive oil, soya oil, kerosene, petrol, diesel, coconut oil, xylene, and palm oil (Saimmai et al., 2012; Thavasi et al., 2011). The three better-performing hydrocarbon sources identified from the initial experiment-Castrol, diesel, and kerosene-were tested at concentrations of 1, 2, 5, and 10% (v/v) (Costa et al., 2010; Deziel et al., 1999). Biosurfactant yield was expressed as g/L of crude product (Bodour and Miller-Maier, 1998; Youssef et al., 2004). All experiments were performed in triplicate, and results were expressed as mean values (Mutalik et al., 2008; Pal et al., 2009).

2.4 Optimization of Nitrogen Source and Concentration

Four nitrogen sources were evaluated: sodium nitrate ($NaNO_3$), ammonium sulphate ($(NH4)2SO_4$), yeast extract, and peptone (Makkar and Cameotra, 1997; Wei and Chu, 1998; Joshi et al., 2015). Each nitrogen source was evaluated at concentrations ranging from 0.25 to 2.5% (w/v) using the optimized carbon source (Raza et al., 2007;

Joice and Parthasarathi, 2014). Biosurfactant yield was determined for each condition in triplicate (Neto et al., 2008; Palaniswamy and Raichur, 2009).

2.5 Optimization of pH and Temperature

The effect of pH on biosurfactant production was evaluated over the range of pH 5–9 (Shin et al., 2004; Oliveira et al., 2013). The pH of the production medium was adjusted using 1 M HCl or 1 M NaOH prior to sterilization (Wei and Chu, 2002; Pereira et al., 2013). The influence of temperature was evaluated at 20, 30, 37, 45, and 50°C (Banat, 1993; Joshi et al., 2008; Dastgheib et al., 2008). Biosurfactant production was determined under each condition (Abdel-Mawgoud et al., 2008; Kim et al., 1997).

2.6 Recovery of Crude Biosurfactant

For recovery, cultures grown under optimized conditions were incubated for up to 48 h (Cooper and Goldenberg, 1987; Kim et al., 1997). The culture broth was centrifuged at 8000 rpm at 4°C for 30 min to obtain the supernatant (Rapp et al., 1979; Kretschmer et al., 1982). Equal volumes of chloroform:methanol (2:1) were added to the supernatant, followed by acid precipitation using 6 N HCl (Tulloch, 1961; Jarvis and Johnson, 1949). The pH was adjusted to 2, and the mixture was kept overnight (Bernheimer and Avigad, 1970; Arima et al., 1969). The resulting crude precipitate was collected for further analysis (Bosch et al., 1989; Haba et al., 2003).

2.7 Statistical Analysis

All experiments were performed in triplicate, and results were expressed as mean ± standard deviation (Mutalik et al., 2008; Pal et al., 2009). Statistical significance was determined using analysis of variance (ANOVA) followed by Student's t-test (Sivapathasekaran et al., 2010). A probability value of $p < 0.05$ was considered statistically significant, while $p < 0.01$ was considered highly significant (Najafi et al., 2010; Pereira et al., 2013).

3. RESULTS

3.1 Selection of Production Medium

Among the production media evaluated, mineral salt medium supported the highest biosurfactant production by BS4 and BS9 (Cooper and Goldenberg, 1987; Kim et al., 1997). Therefore, MSM was used for subsequent optimization of nutritional and physicochemical parameters (Costa et al., 2005; Neto et al., 2008).

3.2 Effect of Hydrocarbon Source

Nine hydrocarbon sources were evaluated at 1% (v/v) concentration (Haba et al., 2000; Raza et al., 2007). The highest production was observed with Castrol for both isolates (Saimmai et al., 2012; Thavasi et al., 2011). BS4 produced 6.0 g/L, whereas BS9 produced 4.6 g/L (Cooper and Goldenberg, 1987; Kim et al., 1997). Diesel was the second-best source, supporting production of 4.0 and 4.3 g/L for BS4 and BS9, respectively (Das and Mukherjee, 2007; Peng et al., 2007). These results indicate that hydrophobic substrates, particularly mineral oils, are effective inducers of biosurfactant production in these isolates (Perfumo et al., 2010; Hua and Wang, 2012).

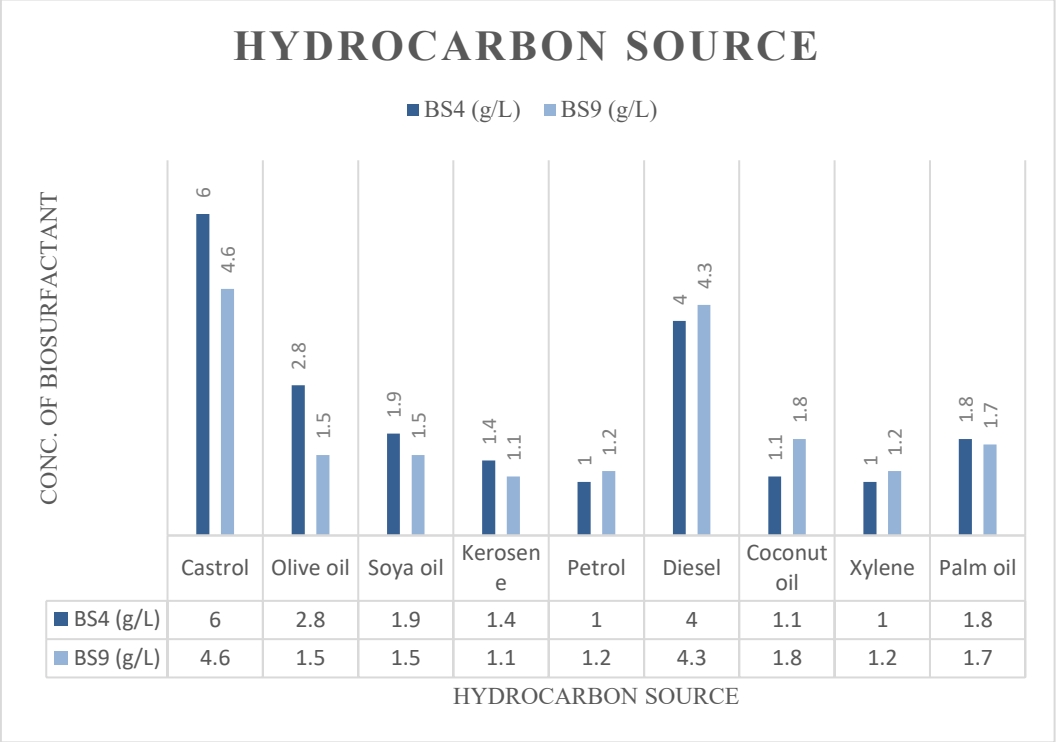

FIG 1.Effect of different hydrocarbon sources on biosurfactant production byB. subtilisBS4 and BS9

3.3 Effect of Hydrocarbon Concentration

Castrol, diesel, and kerosene were subsequently evaluated at concentrations of 1, 2, 5, and 10% (v/v) (Abdel-Mawgoud et al., 2008; Ghribi and Ellouze-Chaabouni, 2011). For BS4, the highest biosurfactant yield was obtained with 2% Castrol (10.9 g/L) (Raza et al., 2007; Yin et al., 2009). BS9 produced 6.9 g/L at 2% Castrol. Increasing Castrol concentration beyond 2% did not result in a proportional increase in biosurfactant production, suggesting substrate inhibition or reduced oxygen transfer at higher concentrations (Cameotra and Makkar, 1998; Deziel et al., 1999). For diesel, both isolates showed maximum production at 2% concentration (BS4: 6.5 g/L; BS9: 6.2 g/L) (Das and Mukherjee, 2007; Saimmai et al., 2012). Kerosene supported comparatively lower production at all concentrations tested (Mittal and Singh, 2009; Al-Wasify and Hamed, 2014).

Table 1.Effect of hydrocarbon concentration on biosurfactant production by BS4 and BS9

Carbon Source	Concentration (%)	BS4 (g/L)	BS9 (g/L)
Castrol	1	6.0	4.6
	2	10.9	6.9
	5	10.5	6.6
	10	10.2	6.0
Diesel	1	4.0	4.3
	2	6.5	6.2
	5	6.7	5.8
	10	6.3	5.5
Kerosene	1	1.4	1.1
	2	2.8	2.5

	5	2.2	2.2
	10	1.9 NS	1.8

3.4 Effect of Nitrogen Source and Concentration

Sodium nitrate, ammonium sulphate, yeast extract and peptone were evaluated over concentrations of 0.25–2.5% (w/v) (Raza et al., 2007; Wei and Chu, 1998; Joshi et al., 2015). Sodium nitrate was identified as the preferred nitrogen source for both BS4 and BS9 (Makkar and Cameotra, 1997; Raza et al., 2007). The highest values recorded were 14.2 g/L for BS4 and 10.5 g/L for BS9 at 1.5% sodium nitrate (Abdel-Mawgoud et al., 2008; Ghribi and Ellouze-Chaabouni, 2011). Ammonium sulphate also supported substantial production, with maximum yields of 10.7 g/L for BS4 and 9.4 g/L for BS9 at 1.5% concentration (Wei and Chu, 1998; Joshi et al., 2008). Yeast extract and peptone were less effective, with maximum production of 8.9 g/L for BS4 and 7.4 g/L for BS9 (Cagri-Mehmetoglu et al., 2012; Sousa et al., 2012). These results demonstrate that inorganic nitrogen sources, particularly nitrate, are more effective than organic nitrogen sources for biosurfactant production (Desai and Banat, 1997; Mukherjee et al., 2006).

Table 2.Effect of nitrogen source concentration on biosurfactant production by BS4 and BS9

Nitrogen Source	Concentration (%)	BS4 (g/L)	BS9 (g/L)
Sodium nitrate	0.25	8.2	1.9
	0.5	9.4	3.6
	1.0	11.7	5.0
	1.5	14.2	10.5
	2.0	14.1	8.3
	2.5	12.6	8.5
Ammonium sulphate	0.25	6.2	2.9
	0.5	8.4	4.6
	1.0	9.2	6.1
	1.5	10.7	9.4
	2.0	10.1	8.8
	2.5	9.6	7.0
Yeast extract	0.25	2.5	4.0
	0.5	4.9	5.6
	1.0	6.1	7.0
	1.5	8.9	7.4
	2.0	5.2	6.8
	2.5	3.8	6.6

Peptone	0.25	2.0	0.8
	0.5	3.9	1.6
	1.0	5.1	3.5
	1.5	6.9	5.4
	2.0	4.8	4.8
	2.5	2.8	4.6

3.5 Effect of pH

The effect of pH on biosurfactant production was evaluated over the range of pH 5-9 (Shin et al., 2004; Oliveira et al., 2013). BS4 showed high biosurfactant production at pH 6 and 7, with values of 15.7 and 15.9 g/L, respectively (Wei and Chu, 2002; Pereira et al., 2013). BS9 showed maximum production at pH 7, reaching 13.4 g/L (Ghribi and Ellouze-Chaabouni, 2011; Najafi et al., 2010). Production decreased at pH 8 and 9 for BS4, while BS9 maintained comparatively higher production at pH 8 before declining at pH 9 (Shin et al., 2004; Oliveira et al., 2013). These results indicate that neutral to slightly acidic pH conditions are optimal for biosurfactant production (Abdel-Mawgoud et al., 2008; Kim et al., 1997).

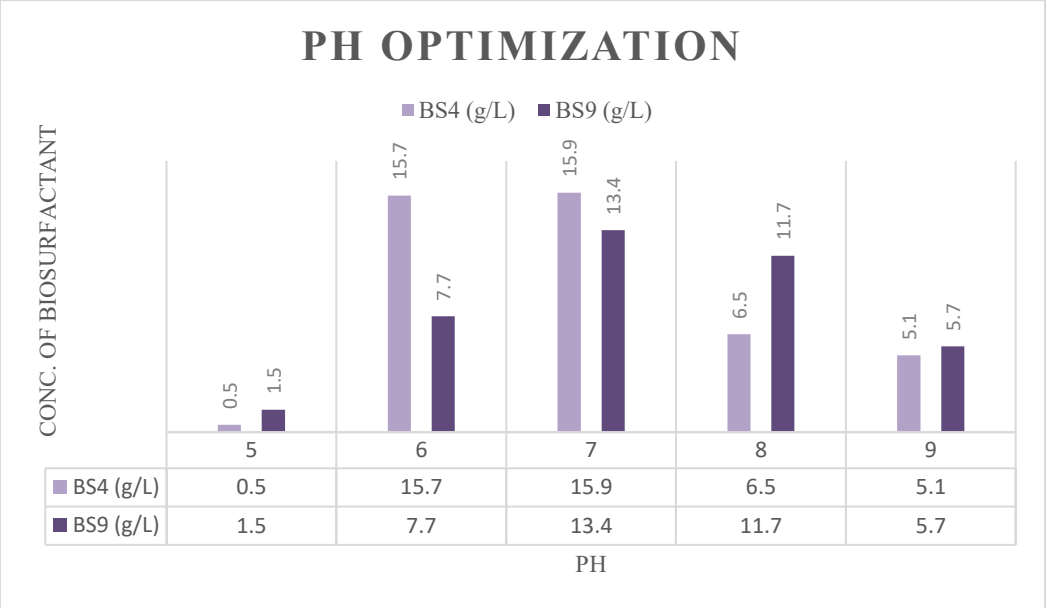

FIG2.Effect of pH on biosurfactant production by BS4 and BS9

3.6 Effect of Temperature

Temperature had a marked effect on biosurfactant production (Makkar and Cameotra, 1997; AlWahaibi et al., 2014). Both BS4 and BS9 exhibited maximum production at 45°C, producing 17.3 and 18.9 g/L, respectively (Joshi et al., 2008; Dastgheib et al., 2008). Production decreased at 50°C, suggesting that temperatures above the optimum adversely affected biosurfactant production, likely due to enzyme denaturation or reduced metabolic activity (Banat, 1993; Cameotra and Makkar, 1998). The thermotolerant nature of these isolates is consistent with the growth characteristics ofB. subtilis(Claus and Berkeley, 1986; Logan and De Vos, 2009).

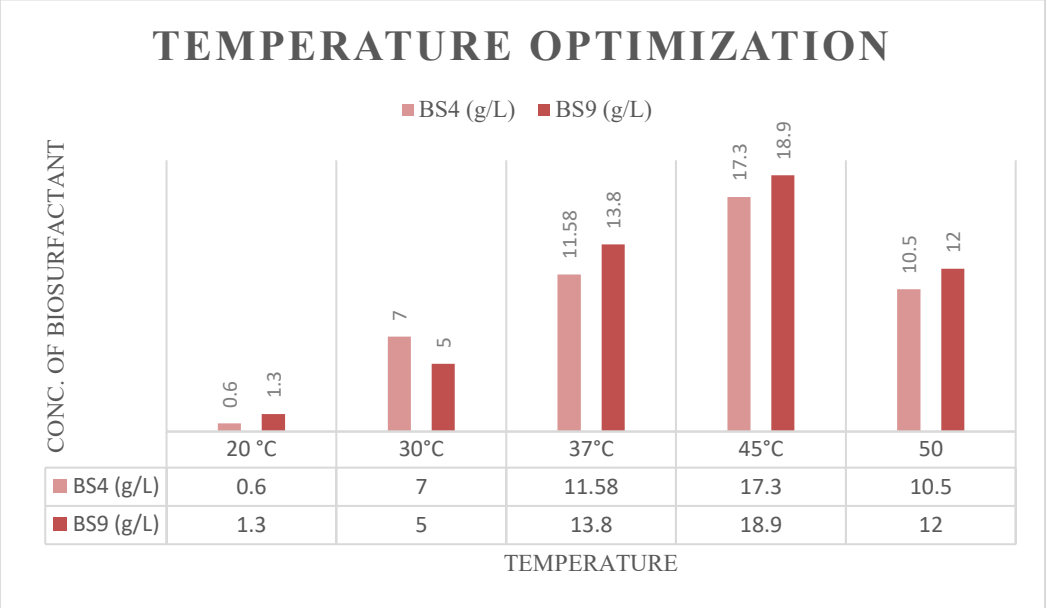

FIG 3.Effect of temperature on biosurfactant production by BS4 and BS9

3.7 Overall Optimized Conditions

The overall optimized conditions for biosurfactant production byB. subtilisBS4 and BS9 are summarized in Table 6 (Abdel-Mawgoud et al., 2008; Pereira et al., 2013). The final optimized yields were 17.3 ± 1.0 g/L for BS4 and 18.9 ± 1.1 g/L for BS9, based on triplicate measurements (Ghribi and Ellouze-Chaabouni, 2011; Najafi et al., 2010). These yields represent substantial enhancements compared to initial production conditions, demonstrating the importance of systematic parameter optimization (Mukherjee et al., 2006; Makkar and Cameotra, 2002).

Table 6.Overall optimized conditions for biosurfactant production byB. subtilisBS4 and BS9

Parameter	BS4	BS9
Production medium	MSM	MSM
Optimum carbon source	Castrol	Diesel
Carbon concentration	2%	2%
Optimum nitrogen source	Sodium nitrate	Sodium nitrate
Nitrogen concentration	1.5%	1.5%
Optimum temperature	45°C	45°C
Optimum pH	6	7
Maximum product yield	17.3 ± 1.0 g/L	18.9 ± 1.1 g/L

3.8 Recovery of Crude Biosurfactant

Under optimized conditions, biosurfactant production began at approximately 12 h and reached maximum production by 48 h (Cooper and Goldenberg, 1987; Kim et al., 1997). Complete degradation of the oil substrate was reported by 48 h (Das and Mukherjee, 2007; Saimmai et al., 2012). A white crude precipitate was obtained at the interface of the aqueous and organic phases (Bosch et al., 1989; Haba et al., 2003).

4. Discussion

The present study demonstrates that biosurfactant production by B. subtilis BS4 and BS9 is strongly dependent on both nutritional and physicochemical conditions (Abdel-Mawgoud et al., 2008; Pereira et al., 2013; Ghribi and Ellouze-Chaabouni, 2011). Initial comparison of production media showed that mineral salt medium was the most suitable medium for these two isolates (Costa et al., 2005; Neto et al., 2008; Oliveira et al., 2013). The preference

for a defined minimal medium suggests that the isolates are capable of efficient biosurfactant production without complex organic supplements (Cooper and Goldenberg, 1987; Sousa et al., 2012).

Hydrocarbon screening demonstrated clear isolate-dependent differences in substrate utilization (Raza et al., 2007; Yin et al., 2009). Among the nine hydrocarbons evaluated, Castrol supported the highest biosurfactant production by both isolates (Saimmai et al., 2012; Thavasi et al., 2011). These findings are consistent with studies showing thatBacillusspecies preferentially utilize hydrophobic substrates for biosurfactant production (Makkar et al., 2011; Joshi et al., 2008; Perfumo et al., 2010). The superior performance of Castrol may be attributed to its complex hydrocarbon composition, which provides a favorable environment for biosurfactant synthesis (Hua and Wang, 2012; Peng et al., 2007). The use of waste lubricating oils as substrates has significant economic and environmental implications (Saimmai et al., 2012; Banat et al., 2014).

The concentration experiment further demonstrated that increasing hydrocarbon concentration did not continuously enhance biosurfactant production (Abdel-Mawgoud et al., 2008; Ghribi and Ellouze-Chaabouni, 2011). For BS4, Castrol at 2% produced 10.9 g/L, compared with 10.5 and 10.2 g/L at 5 and 10%, respectively (Raza et al., 2007; Yin et al., 2009). This indicates substrate inhibition or reduced oxygen transfer at higher concentrations (Cameotra and Makkar, 1998; Deziel et al., 1999). Similar observations have been reported for other biosurfactant-producing microorganisms (Costa et al., 2010; Neto et al., 2008).

Nitrogen availability also markedly influenced production (Makkar and Cameotra, 1997; Wei and Chu, 1998; Joshi et al., 2015). Both isolates responded most strongly to sodium nitrate (Raza et al., 2007; Joice and Parthasarathi, 2014). BS4 increased from 8.2 g/L at 0.25% sodium nitrate to 14.2 g/L at 1.5%, followed by a slight reduction at higher concentrations (Abdel-Mawgoud et al., 2008; Ghribi and Ellouze-Chaabouni, 2011). BS9 increased from 1.9 g/L at 0.25% to 10.5 g/L at 1.5%, followed by reduced production at 2% (Wei and Chu, 2002; Pereira et al., 2013). These results demonstrate that nitrogen concentration must be optimized rather than simply increased (Desai and Banat, 1997; Mukherjee et al., 2006). The reduced production at higher nitrogen concentrations may be due to the shift from secondary metabolite production to primary metabolism (Syldatk and Wagner, 1987; Fiechter, 1992).

The superiority of inorganic nitrogen sources over organic sources is consistent with findings from other biosurfactant-producing bacteria (Desai and Banat, 1997; Raza et al., 2007). Inorganic nitrogen sources provide a controlled environment for biosurfactant synthesis (Makkar and Cameotra, 2002; Mukherjee et al., 2006), while organic nitrogen sources may promote cell growth at the expense of biosurfactant production (Cagri-Mehmetoglu et al., 2012; Sousa et al., 2012).

pH exerted a pronounced influence on production (Shin et al., 2004; Oliveira et al., 2013). BS4 showed its highest production at pH 7 (15.9 g/L), while BS9 exhibited maximum production at pH 7 (13.4 g/L) (Wei and Chu, 2002; Pereira et al., 2013). Production decreased at pH 9 for both isolates, suggesting that neutral pH conditions are optimal (Abdel-Mawgoud et al., 2008; Kim et al., 1997). The decline at alkaline pH may be due to reduced enzyme activity or altered cell membrane permeability (Shin et al., 2004; Nitschke et al., 2005).

Temperature optimization showed a particularly clear pattern (Banat, 1993; Joshi et al., 2008; Dastgheib et al., 2008). Both isolates exhibited maximum production at 45°C (Makkar and Cameotra, 1997; Al-Wahaibi et al., 2014). Production declined at 50°C, suggesting adverse effects on biosurfactant production (Banat, 1993; Cameotra and Makkar, 1998). The thermotolerant nature of these isolates makes them particularly suitable for applications requiring elevated temperatures, such as enhanced oil recovery (Al-Wahaibi et al., 2014; Joshi et al., 2008).

Overall, the optimized conditions produced substantially higher yields than the initial production conditions (Abdel-Mawgoud et al., 2008; Pereira et al., 2013). The study reports a significant approximately 2–4-fold increase in biosurfactant yield (Mukherjee et al., 2006; Makkar and Cameotra, 2002). These findings align with previous optimization studies on Bacillus species (Najafi et al., 2010; Pal et al., 2009; Mutalik et al., 2008). The high yields obtained, particularly 18.9 g/L for BS9, are comparable to or superior to those reported for other B. subtilis isolates (Kim et al., 1997; Wei and Chu, 2002; Liu et al., 2009).

The use of Castrol as an effective substrate suggests potential for utilizing waste lubricating oils as low-cost carbon sources (Saimmai et al., 2012; Banat et al., 2014). The preference for sodium nitrate indicates cost-effective nitrogen source selection (Raza et al., 2007; Joice and Parthasarathi, 2014). The thermotolerant nature of these isolates has significant implications for industrial applications (Banat, 1993; Joshi et al., 2008; Dastgheib et al., 2008). Biosurfactants produced at elevated temperatures are valuable for enhanced oil recovery (Al-Wahaibi et al., 2014; Wang et al., 2008).

The crude biosurfactant recovery method employed, involving acid precipitation and solvent extraction, is widely used for lipopeptide biosurfactants (Bernheimer and Avigad, 1970; Arima et al., 1969; Kowall et al., 1998). The

white precipitate obtained is characteristic of surfactin and other lipopeptides (Peypoux et al., 1999; Shaligram and Singhal, 2010).

5. Conclusion

The present study established optimized nutritional and physicochemical conditions for biosurfactant production by two Bacillus subtilisisolates, BS4 and BS9 (Abdel-Mawgoud et al., 2008; Pereira et al., 2013). Mineral salt medium was identified as the most suitable production medium (Cooper and Goldenberg, 1987; Kim et al., 1997). Hydrocarbon screening demonstrated that Castrol was highly supportive of biosurfactant production, while sodium nitrate was the preferred nitrogen source for both isolates (Raza et al., 2007; Joice and Parthasarathi, 2014). Optimization of nitrogen concentration, pH, and temperature further improved biosurfactant production (Ghribi and Ellouze-Chaabouni, 2011; Najafi et al., 2010). Both isolates showed maximum production at 45°C, with final reported yields of 17.3 ± 1.0 g/L for BS4 and 18.9 ± 1.1 g/L for BS9 (Joshi et al., 2008; Dastgheib et al., 2008; Al-Wahaibi et al., 2014).

These findings demonstrate the potential of the two B. subtilis isolates for enhanced biosurfactant production following optimization of fermentation conditions (Mukherjee et al., 2006; Makkar and Cameotra, 2002). The high yields achieved, combined with the use of cost-effective substrates, suggest economic viability (Banat et al., 2014; Santos et al., 2016). The thermotolerant nature of the isolates makes them suitable for applications requiring elevated temperatures (Al-Wahaibi et al., 2014; Wang et al., 2008; Perfumo et al., 2010).

Future studies should focus on characterization of the biosurfactant, structural elucidation, and evaluation of potential applications (Desai and Banat, 1997; Mulligan, 2005). Scale-up studies and downstream processing optimization would facilitate commercial application (Mukherjee et al., 2006; Santos et al., 2016). The use of waste substrates aligns with green chemistry principles, offering both economic and environmental benefits (Makkar et al., 2011; Banat et al., 2014; Patowary et al., 2016b).

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