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Biphasic Liquid-Liquid Extraction of Biosurfactant from *Lactobacillus delbrueckii*

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HIGHLIGHTS

- Biphasic liquid-liquid extraction of *L. delbrueckii* BS from the broth is executed.
- Extraction parameters are optimized and partition coefficient calculated.
- Co-current and countercurrent extraction of BS increased its yield.
- Purified BS characterized as glycoprotein/polymeric.

Abstract: The present work is focused mainly on optimizing the extraction of glycoprotein- biosurfactant (BS) produced by *Lactobacillus delbrueckii* in a biphasic liquid-liquid extraction. The production yield of BS is significantly affected by extraction strategies instead of the whole fermentation process, so it becomes a straightforward approach to enhance BS yield while optimizing various extraction parameters. The tailoring of process parameters for BS extraction was achieved by OFAT (one factor at a time) strategy and partition coefficient (K_d) served as the calculation factor for extraction. The optimal yield of BS (5±0.1 g/L) from CFB (from cell-free broth) was achieved by solvents; chloroform, methanol, and, butanol, 1:2:1 (v/v), from cell-free broth (CFB), 30% (v/v), at pH 3.5, temperature 37°C after extraction time of 60 min. Under optimized conditions, the extraction yield was 78.5% higher and subsequently, a co-current, and counter-current system enhanced the extraction yield by 16% (5.8g/L) and 20% (6.0g/L) respectively. The purity of extracted BS (EBS) was confirmed by UV/Visible spectroscopy and HPLC (High-performance liquid chromatography). The concentration-dependent activity profile of EBS analyzed by ODA (oil displacement area; 50.24±0.3 cm^2), DCD (drop collapse diameter; 1.1±0.3 cm), and EI_{24} (73±0.3%), exhibited enhancement by 60, 52, and 55% respectively as compared to control. Thin-layer chromatography (TLC), FTIR (Fourier Transform Infra-Red) and NMR (Nuclear Magnetic Resonance) techniques confirmed the polymeric glycoprotein (65:35 protein: carbohydrates %) nature of BS.

Keywords: Polymeric biosurfactant; *Lactobacillus delbrueckii*; Liquid-liquid extraction; biphasic; co-current/countercurrent.

INTRODUCTION

Biosurfactants (BS) are surface-active, amphiphilic compounds produced by fermentation using a wide range of microorganisms [1]. They have been in limelight due to their structural, chemical, physical diversity and extensive industrial applications in pharmaceutical, food, textile, cosmetics, etc. The potential advantages of BS i.e., lower toxicity, higher biodegradability, selectivity, and specific activity at extreme conditions make them feasible to use in food (as emulsifiers) and pharmaceutical (as antimicrobial and anti-adhesive agents) industries [2, 3, 4].

To fulfill the widespread need for BS, it is widely produced by the microbial fermentation process [5, 6] and various studies have mainly focused on the optimization of medium components, production design, purification, characterization, and application of BS [7]. On the other hand, to enhance recovery of BS from the fermentation broth, its extraction happens to be the most crucial stage, because 70-80% of production cost comprises its extraction and recovery [8]. The foremost obstacles found in the BS extraction from broth are the chemical complexities of media, unknown statistics, and low BS concentration [9]. Various extraction methods mentioned in literature are: precipitation [10], solvent extraction [11], ultrafiltration [12], foam fractionation [13], dialysis [5], adsorption [14], and chromatography [15]. Precipitation is the most widely used for extraction alone [10] as well as coupled with solvent extraction [16]. The common method of BS extraction from CFB involves its acidification (pH 2-3) for 24h (for impurities removal and make the product less soluble in an aqueous mixture), followed by solvent extraction. But it is a time-consuming (24-27h) method [17, 18], henceforth there arises a need for a faster and better yielding scheme for BS extraction. There have been preliminary studies focused on the recovery of BS by liquid-liquid extraction directly from broth [9, 19], but are insufficient concerning the effect of various parameters on glycoprotein BS extraction.

The present study for the extraction of microbial glycoprotein, BS from CFB by the liquid-liquid biphasic system in the batch, followed by co-current and counter-current extraction is a novel work. A systematic overview of biphasic liquid-liquid extraction is summarized in Figure1.To the best of our knowledge, this is the first report for the comprehensive extraction of microbial polymeric glycoprotein BS which will be evidence as an easy and time-saving extraction scheme.

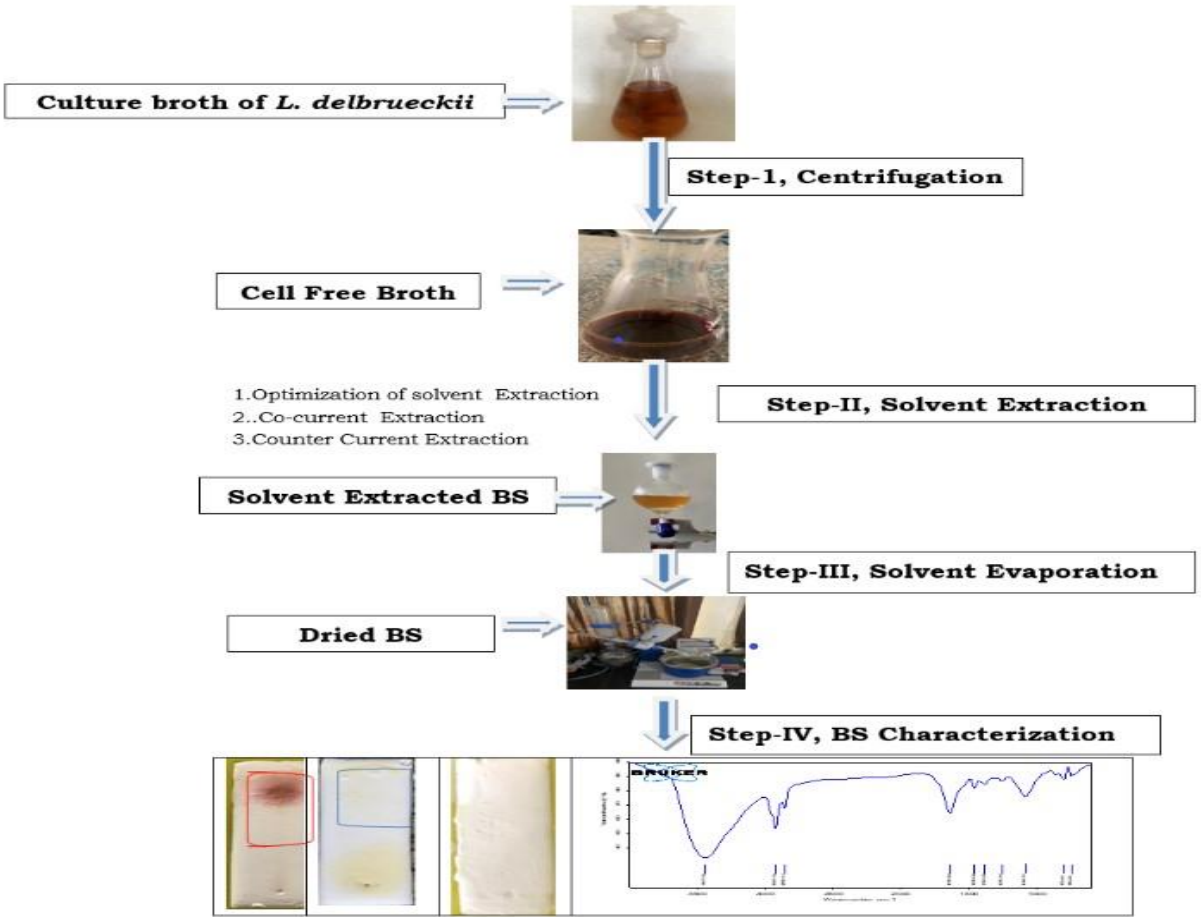

Figure 1. A systematic overview of biphasic liquid-liquid extraction**.**

MATERIALS AND METHODS

Materials

The Mann Rogosa Sharpe medium (MRS) used for culturing *L. delbrueckii* and other chemicals were purchased from Hi-Media, Mumbai, India. All chemicals were of analytical grade.

Microbial Production of BS

BS was produced by microbial fermentation from *L. delbrueckii* strain, a new isolate from a food source (Genbank accession- MW 769777). The media comprising (g/L) - xylose (10), peptone (5), K_2HPO_4 (1.6), KH_2PO_4 (0.4), $MgSO_4.7H_2O$ (0.1), $CaCl_2$ (0.02), and trace elements (mg/100ml) - $CuSO_4.5H_2O$ (0.5), H_3BO_3 (1), $MnSO_4.5H_2O$ (1), $ZnSO_4$ (0.7), MoO_3 (1), pH,6.5 was used for BS production with 5% inoculum ($1.5x10^6$ CFU/mL). The fermentation was carried out in batch mode for 48 h at 37°C (120 rpm) and the culture broth was centrifuged (10,000 rpm for 20 min, 4°C) [20] to separate CFB for further used of BS extraction.

Comparison Between Two-step and Single-step Extraction

To establish the optimum BS extraction, single and double-step extraction strategies were compared (Figure 2). In the first, CFB was mixed with solvent chloroform and methanol (2:1) and incubated (100 rpm, 37°C) for 30 min at the shaker. The separated solvent phase was vaporized (Rotary Evaporator, Popular India Ltd) to yield dry BS for further analysis [19]. In double-step extraction, CFB was acid precipitated by HCl (pH 2, 4°C) for 24h [16] and separated by centrifugation (10,000 rpm, 4°C,) for 20 min. The precipitate was mixed with a solvent and incubated (100 rpm, 37°C) for 30 min at the shaker. The solvent phase was separated and vaporized to yield dry BS for further analysis.

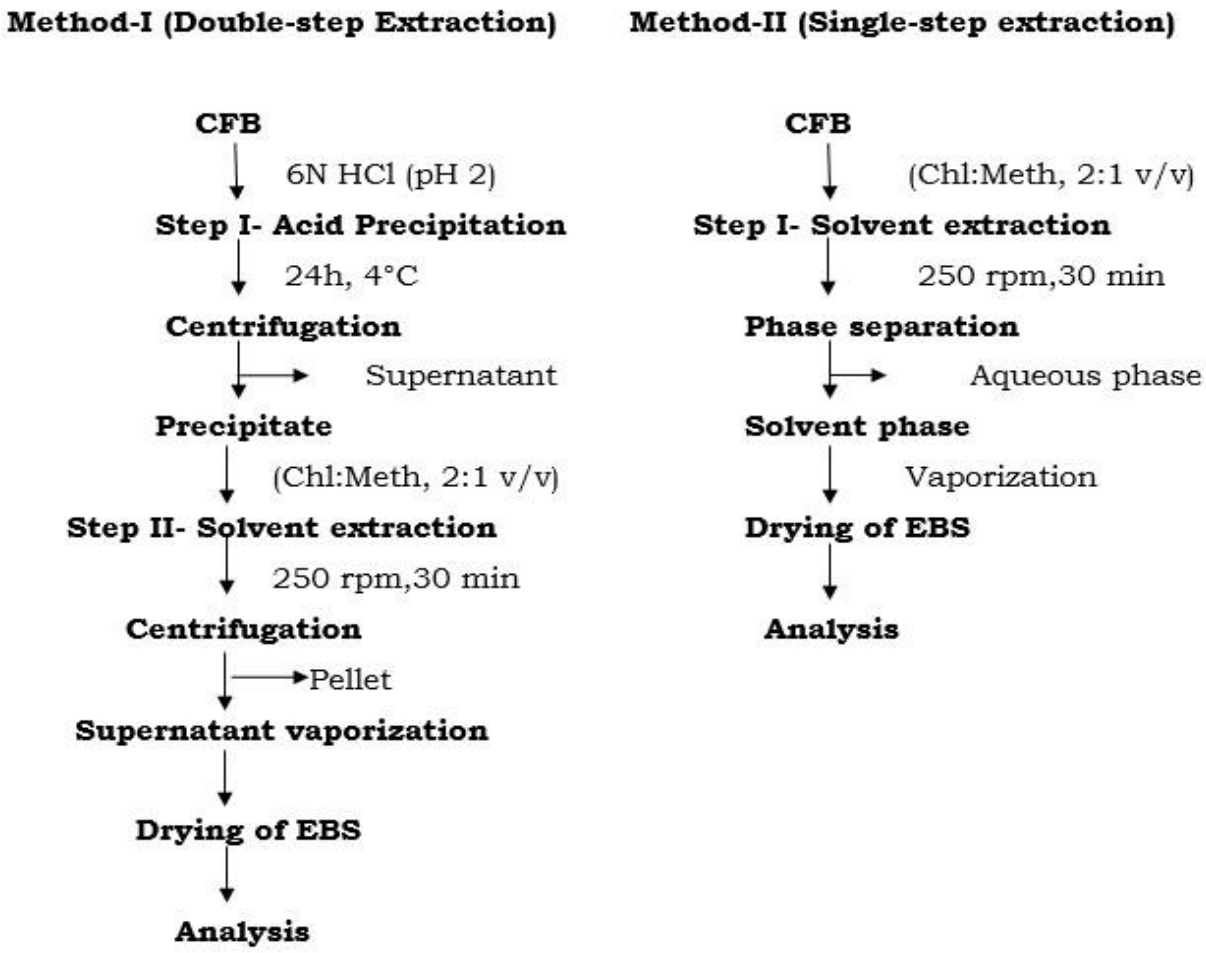

Figure 2. Flow chart for single and two-step extraction of BS.

Analysis of Biosurfactant

The degree of BS extraction was evaluated by the partition coefficient (K_d), which was calculated from the emulsification index (EI_{24}). Later was determined as per the method described by Llarch and coauthors, [21]. Briefly, two ml of each BS and a hydrocarbon (diesel) were vortexed for 2 min and after 24h, EI_{24} was calculated as per the following:

$$EI_{24}=\frac{\text{Height of emulsified layer (cm) X100}}{\text{Total Height (cm)}}$$

The partition coefficient (K_d) for extracted BS was calculated as the ratio of EI_{24} of the bottom (EI_{24Bot}) and top (EI_{24Top}) phases, as following;

$$K_d=\frac{EI_{24Bot}}{EI_{24Top}}$$

After extraction, the solvent was evaporated and the extraction yield (g/L) was calculated [22].

Optimization of Parameters in Liquid−liquid Extraction of BS: Batch System

From the preliminary experimental results, it was observed that single and double-step solvent extraction has the same yield and EI_{24} (%**).** Hence, BS extraction by a single-step system was considered for further thorough study, as it was time-saving. The extraction by partitioning of the molecule in a biphasic liquid−liquid system is influenced by various process parameters [1], so, to achieve the ideal process conditions for maximum BS extraction, the OFAT strategy was employed. In the approach, each parameter was investigated individually to determine its precise effect on BS extraction. Initially, to screen the best solvent for BS extraction; chloroform, methanol, butanol, ethyl acetate, and hexane were selected based upon previous studies [1, 8, 9] and mixed with CFB in equal volume separately in the batch process. After incubation (37°C), for 30 min the system resulted in two phases, aqueous and organic as the top and bottom, respectively. The phases were carefully separated to measured, BS activity and partition coefficient by EI_{24} and K_d respectively. After that, the combinative effect of, the selected solvents, chloroform, methanol, and butanol (C: M: B) in four different ratios; 1:1:1, 1:2:1, 1:1:2, 2:1:1 (v/v) CFB concentration (20, 25, 30 and 50% v/v), temperatures (15, 30, 37, 45, 60 and 75°C), pH (1.5-10.5) and extraction time (0, 30, 60, 90 and 120 min) on K_d was determined subsequently. Any interference from the biphasic components; broth and solvent were prevented by routinely applying control.

Simulation of Multistage Co-current and Continuous Counter-current Extraction System

For co-current extraction of BS in a three-stage system (Figure 3) the CFB (pH-3.5) and solvent (C: M: B, 1:2:1 v/v) mixture (1:2 v/v) was incubated at 37°C. After 60 min, the solvent phase was separated to transfer the raffinate at stage-II, then fresh solvent was added and repeated the first step (incubation at 37^0C for 60 min). The solvent was separated and the raffinate was transferred to stage III [23] to repeat the process. The EI_{24}, K_d, and yield (g/L) of solvent extract A, B, and C were determined. Furthermore, the liquid-liquid extraction of BS from CFB was also carried out in three stages, counter-current extraction [24] as shown in Figure 3(b), where CFB and solvent move in the opposite direction. In every stage, BS solution and solvent were mixed (1:2) for 60 min (37°C, 3.5 pH) and the aliquot was analyzed for EI_{24} and K_d.

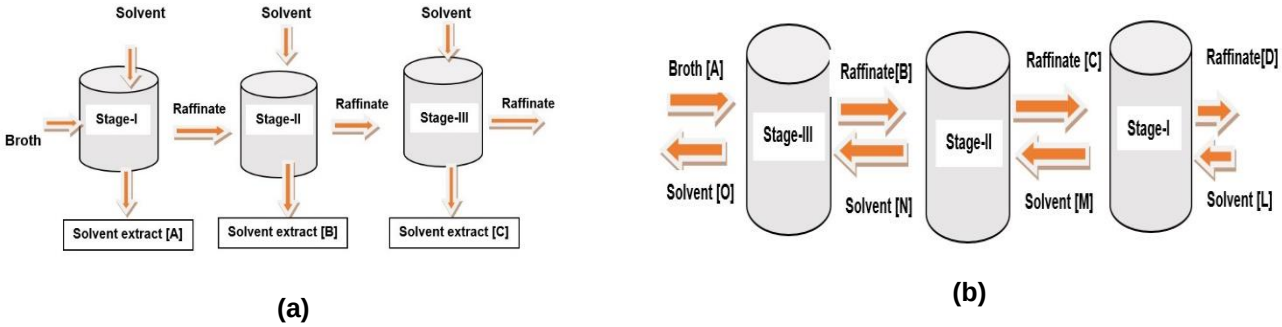

Figure 3. Co-current (a) and countercurrent (b) extraction schemes of BS in the biphasic system.

Evaluation of BS

The homogeneity of EBS was confirmed by UV/VIS spectroscopy [25] and HPLC [26]. The ultraviolet absorbance spectrum of EBS [25] at a range of 190-400 nm (UV-VIS Spectrophotometer, PERKIN-ELMER) was analyzed. Further, EBS was examined with HPLC (Shimadzu, USA) using, a reverse-phase column (Lichrosorb C18-5 µm; Merck, Germany) and UV assay detector (280). The mobile phase; acetonitrile, Methanol in the ratio of 80: 20 (v/v) at a flow rate of 1 mL/min was applied. Moreover, to evaluate the purity, the concentration-dependent activity profile of EBS was performed by the ODA (oil displacement area; cm^2) [27], DCD (drop collapse diameter; cm) [28], and emulsification index (EI_{24}) [21].

To confirm the chemical nature of EBS the TLC, NMR, and FTIR techniques were employed. A BS (5 µl) was applied at the point of origin of the TLC plate [29] and separation was achieved by the solvent system of chloroform: methanol: water (65:25:15; v/v) After running the mobile phase, plates were sprayed with anisidine HCl, ninhydrin, and iodine vapors and dried at 110°C for revealing carbohydrate, protein, and lipid moieties respectively. The colored spot illustrated the type of moieties present in the BS. For infrared spectroscopic analysis 1mg of EBS was mixed with 100 mg of KBr and pressed at 134 MPa for 3 min to obtain a transparent pellet. The IR spectrum of the pellet from 400 to 4000 wavenumber (cm^{-1}) an average of 24 scans were obtained using an FTIR (BRUKER ALPHA, USA) spectrometer [30]. Also, the chemical nature of EBS was confirmed with NMR spectroscopy (BRUKER, GERMANY), using H^1 spectra recorded at 400 MHz in D_2O at room temperature [17]. The total protein and carbohydrate content of EBS was quantified by Lowry and coauthors, [31] and the phenol sulphuric acid method [32] respectively.

Statistical Analysis of Data

All analyses and experiments were performed in three independent replicates, and results are given as mean ± standard deviation (SD). Data were subjected to variance analysis using the software SPSS16.0. One-way Analysis of Variance (ANOVA) and Least Significant Difference (LSD) tests were used to detect significant differences among various optimization parameters, and differences were considered statistically significant when $P< 0.05$.

RESULTS AND DISCUSSION

Optimization of Parameters in Liquid−Liquid Extraction of BS: Batch System

The evaluation for BS extraction in terms of single and double-stage was accomplished. The BS yield (2.8 g/L) and EI_{24} (55%) in single-stage was attuned to the double-stage extraction method (Figure 4). Therefore, the result was significant to develop the liquid-liquid single-stage system, which will be time-saving, as compared to the double stage [33].

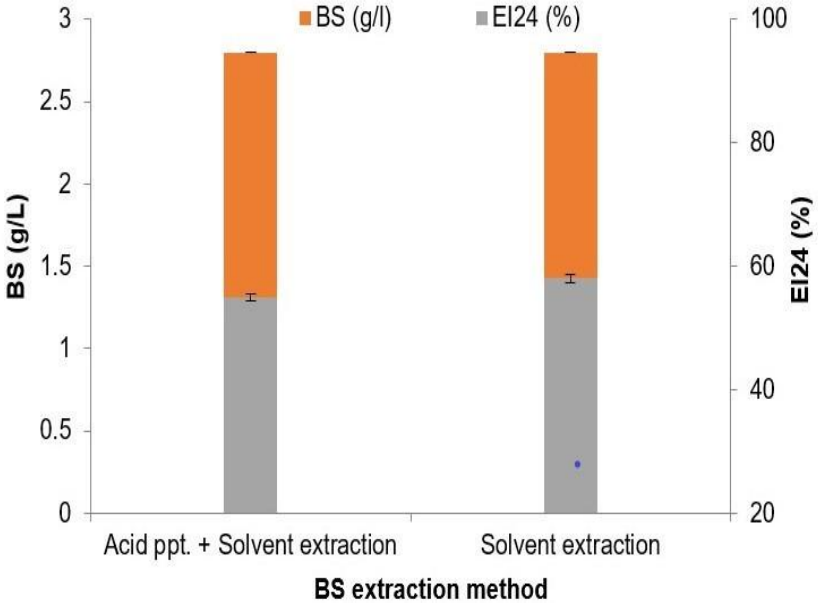

Figure 4. BS extraction in two-step and single-step method.

Hence, BS extraction from the CFB by single-step solvent extraction was considered for further thorough study. To achieve optimal BS extraction, the responsible process conditions were augmented in biphasic

liquid−liquid extraction. The solvents; chloroform, methanol, butanol, ethyl acetate, and hexane were applied and the extraction efficiency (Figure 5a) of chloroform, methanol, and butanol, was similar having a relative $K_{d,}$ 117**,** which was significantly higher by 17, 22, and 13 % as compared to control ethyl acetate, and hexane respectively. The lower K_d in hexane and ethyl acetate might be due to their physicochemical properties, as it has been observed in the literature, the solvent polarity index and, solubility factor, etc. affect the extraction of a solute greatly [9]. Chloroform and methanol solvents have been used in the coupled manner [34, 35] as well as individually [36, 37] for the BS extraction, so different ratio of chloroform, methanol, and butanol was applied to determine the combinatory solvent effect on partition coefficient. The higher proportion of methanol as compared to the chloroform and butanol was more effective and a K_d, 6.5±0.1 (Figure 5b) was attained. In comparison to the previous literature, chloroform and methanol (2:1) were employed for the extraction of glycolipoprotein and glycolipid [33, 34].

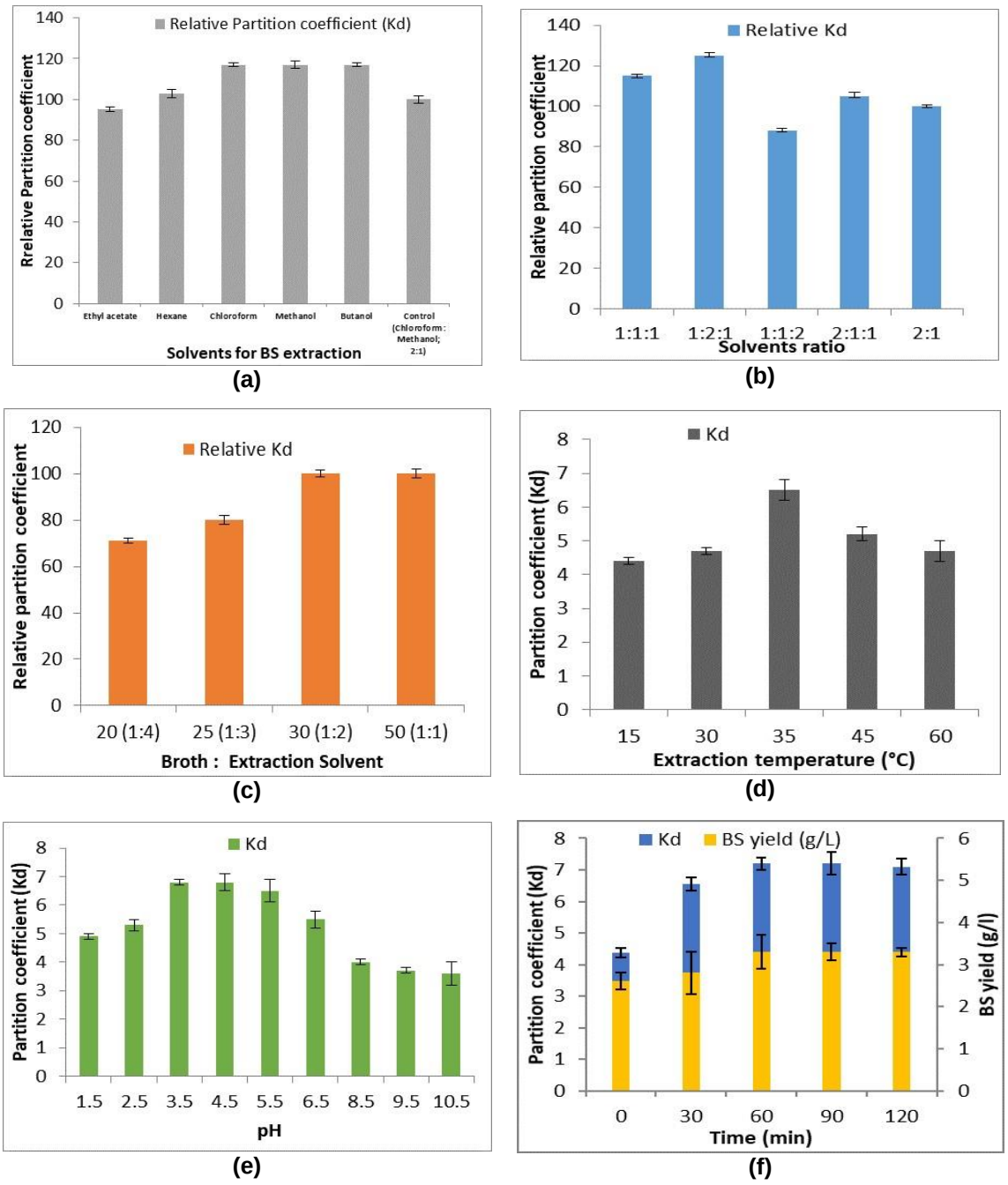

Figure 5. Extraction optimization of (a) solvents, (b) solvents ratio, (c) broth: solvent ratio, (d) temperature, (e) pH, (f) time for *L. delbrueckii* BS in the single-step biphasic system by OFAT with analysis of relative partition coefficient, K_d, and extraction yield.

According to a study by Shen and coauthors [37], three solvent systems (methanol: water: hexane, 2:1:1 v/v) lead to 80% recovery of mannosylerythritol (MEL) BS from *Pseudomonas aphidis*, hence it was evident that high methanol concentration assists in BS extraction. Afterward, the impact of broth-solvent ratio on K_d was investigated at different broth concentrations (20, 25, 30, and 50%) and maximum K_d, 6.5±0.2 (Figure 5c) was obtained at CFB concentration 30%, which was higher as compared to 20 and 25%, but, above (30%) it remains constant. The results illustrate the significant effect of broth to solvent ratio which is reliable with mass transfer principles where the driving force depends on the concentration gradient between the broth and solvent [8]. Similarly, the various reports have also stated that extraction solvent, facilitates the extraction of target product from CFB if mixed in a specific ratio [9,38,39]. As the temperature is a crucial factor in any extraction, so its effect on the K_d of BS was also calculated. The K_d increases with the increase in temperature and was maximum at 35°C, but above that, it decreases (Figure 5d) because the phase composition, electrostatic interactions, and hydrophobic interactions are temperature-dependent [22].

The extraction process is influenced by pH [34], so the effect of pH on the BS extraction was investigated. The extraction was higher in acidic (3.5-5.5) as compared to basic pH (8.5 to 10.5), with K_d of 6.9±0.1 and 3.6±0.5 respectively (Figure 5e)**.** The literature also evidences that acidic pH provides a better yield in BS extraction [19, 33, 34]. For instance, in a study by George and Jayachandran [16], the pH, 3 of CFB was applied for the rhamnolipids extraction with ethyl acetate. In another study, Felix and coauthors [18] extracted BS from 12 different strains of *Bacillus* species in acidic conditions (pH 2). The extraction time has also a significant effect on the removal of components [40]. The optimal BS extraction through K_d, 7.2±0.2, and yield 5.0±0.2g/L were attained after 60 min and afterward, it remains constant (Figure 5f) whereas. extraction time of 24 and 12 h is reported in literature, for glycopeptide [41] glycolipid [39] respectively.

Co-current and Countercurrent Extraction of BS

To enhance the BS recovery from the CFB, a three-stage co-current extraction system (Figure 3a) was accomplished. The overall K_d (7.1±0.2) was almost similar to the single-stage (Table, 1) but, on the other hand, the overall extraction yield (5.8±0.2 g/L) was 16% higher. Furthermore, to improve BS extraction, a three-stage counter-current approach was applied [24] and an increasing trend of K_d and extraction yield through each stage was observed (Table 1). In the first stage, the K_d was 6.9±0.2 but enhanced in the second (7.6±0.1) and third stage (7.8±0.2) by 10 and 13 % respectively. The overall K_d and yield of 7.4±0.2 and 6.0±0.3 g/L respectively, ensures that continuous flow of the organic phase is free from any cross-contamination of impurities from the aqueous phase. Similarly, a three-phase counter current extraction system was applied where K_d and recovery, 0.8-0.9 and 50% were respectively achieved [42].

Table 1. Co-current and Counter current extraction of BS

Stages	Co-current		Counter current	
	K_d	Extraction yield (g/L)	K_d	Extraction yield (g/L)
I	7.2±0.1	5.0±0.2	6.9±0.2	3.5±0.4
II	1.8±0.3	0.5±0.1	7.6±0.1	4.8±0.2
III	1.5±0.1	0.2±0.1	7.8±0.2	5.9±0.4
Overall	7.1±0.2	5.8±0.2	7.4±0.2	6.0±0.3

Evaluation of BS Purity and Structure

The purity of EBS was confirmed by UV/VIS and HPLC techniques by evaluating the extent of homogeneity. UV spectrum (Figure 6a) revealed a single peak (280 nm) for EBS, therefore concluded that BS was extracted precisely from CFB which is supported by. Meena and coauthors [26] also described the purity of BS through UV/VIS spectra (200-800 nm). In the next step, the purity of EBS was further confirmed by HPLC spectra, which have a peek at a retention time of 9.11 min other than the solvent peak of 3.024 (Figure 6b).

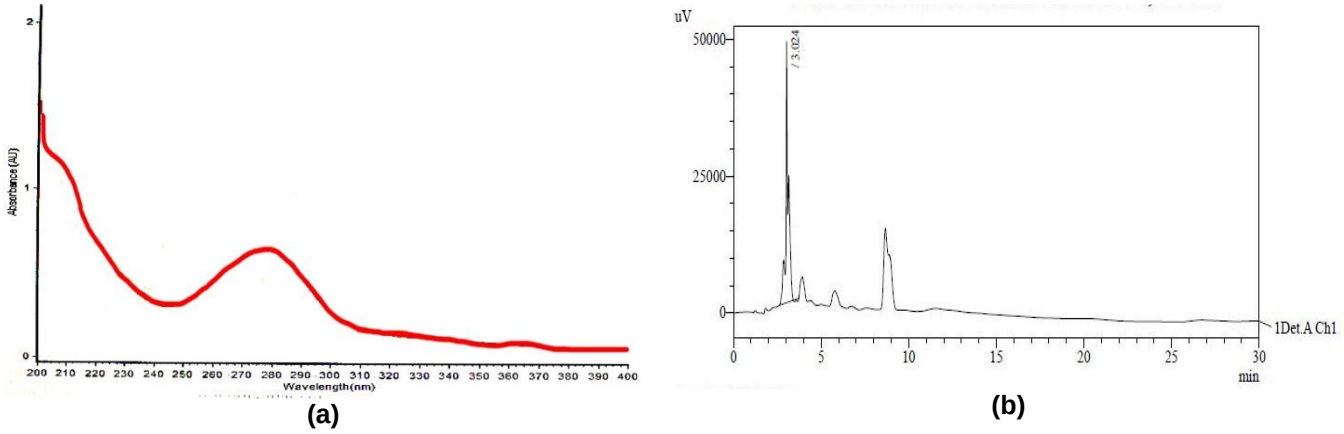

Figure 6. (a) UV and **(b)** HPLC spectra of solvent EBS.

After establishing EBS homogeneity, the concentration-dependent activity profile was investigated. The ODA (oil displacement area; 50.24±0.3 cm^2), DCD (drop collapse diameter; 1.1±0.3 cm), and EI_{24} (73±0.3%) increased by 60, 52, and 55% respectively as compare to CFB (Fig. 7) which is owing to the higher BS concentration in EBS than CFB. The literature also provides evidence that the ODA [2] and EI_{24} [26] anticipate the quantitative information about the BS concentration, i.e., the larger the value, the higher the BS concentration, and vice versa.

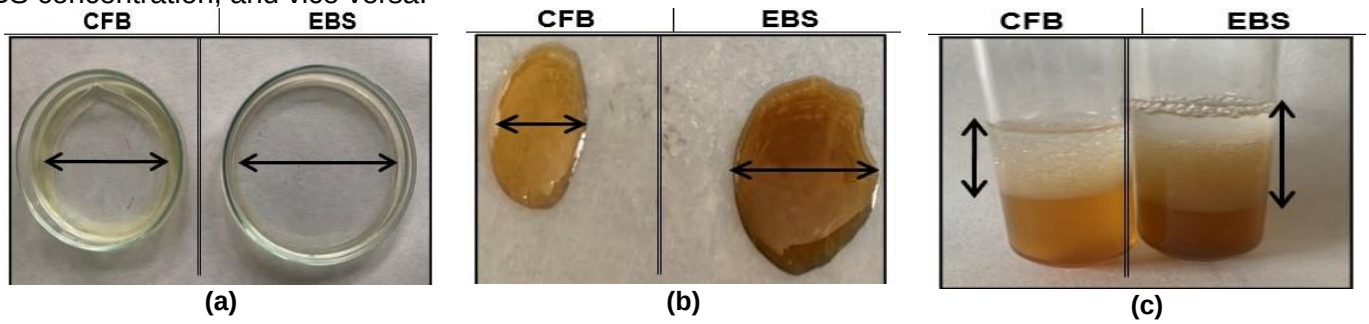

Figure 7. Quantification of CFB and extracted BS using (a) Oil displacement area, (b) Drop collapse diameter, and (c) Emulsification index EI_{24} % of CFB and solvent extracted polymeric BS.

The developed chromatogram of TLC plates exhibited, R_f values of 0.78 and 0.47 for carbohydrate and protein respectively, but no spot for lipid moiety (Figures 8, a, b, and c) which are comparable to literature R_f values, 0.73 [38] and 0.44 [29] of polysaccharide and protein moieties respectively.

Figure 8(d) shows the FT-IR spectra of the EBS of *L. delbrueckii which is* characteristic of polysaccharides, related to O–H stretching and overlapping NH vibration (3454cm^{-1}) and a slightly weak C–H stretching i.e., CH_2-CH_3 (2934cm^{-1}) [30]. It is worth remarking that characteristic bands at 1639 and 1459 cm^{-1} are equivalent to Amide I (C=O stretching) and Amide II (NH bending) vibrations of protein structure, respectively [40, 43]. The relative peak intensities in the region 1200–950cm^{-1}, generally known to be a typical characteristic of the polysaccharides, with the C–O-C stretching bands at 1084cm^{-1} [44]. The spectra also exhibited complex vibrational intensities at low wavenumbers (below 799cm^{-1}) due to the glucose pyranose ring. NMR spectra of EBS (Figure 8e), in comparison with literature (Table 2) illustrated the glycoproteinaceous nature of the substance. Hence FTIR and NMR spectrum results were in conjunction with TLC results, suggesting the EBS as a glycoprotein with protein and carbohydrates composition of 65:35 % (w/w).

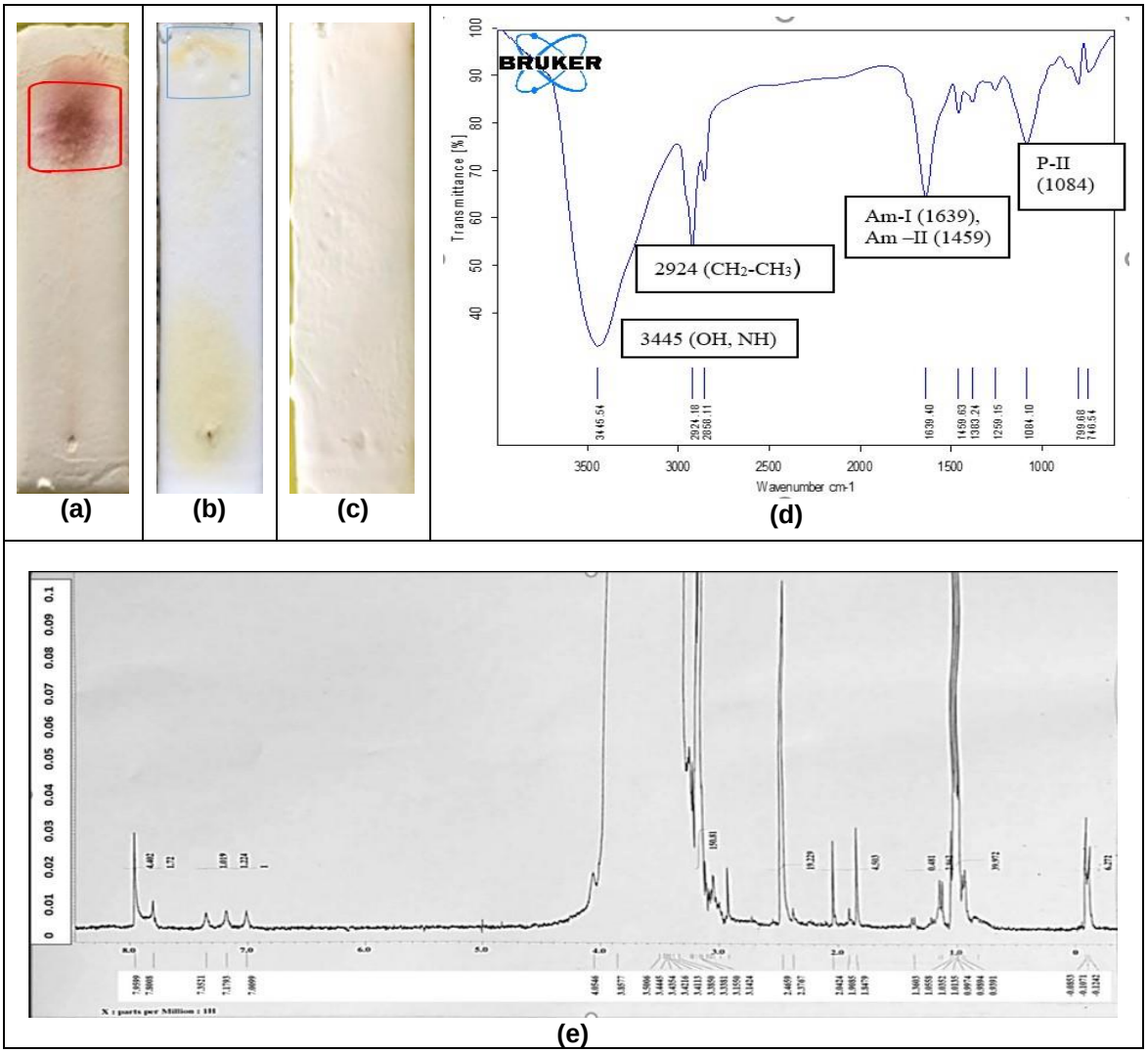

Figure 8. TLC (a) Protein; (b) Carbohydrates and (c) Lipids (no spots observed), FTIR (d) and H^1NMR (e) of extracted BS.

Table 2. NMR peaks assignment of EBS.

Assignments	H^1- NMR (ppm) Peak values	Reference
CH_2=CH	7.35	[29]
Proton attached to C-1 of sugar moiety	4.05	[45]
Proton attached to C-2 of sugar moiety	3.50	[46]
Proton attached to C-3 of sugar moiety	3.50	[40]
Proton attached to C-4 of sugar moiety	2.37	[29]
Protein group of glucan-protein structure	1.03,1.05,1.36	[47]; [25]

CONCLUSION

The biosurfactant produced from *L. delbrueckii* was subjected to biphasic liquid-liquid extraction for optimal removal from cell free broth. The process variables, solvent type, solvent combination, temperature, pH, and broth concentration affect the BS extraction in the single-step method and he maximal BS extraction 5.0±0.2g/L was achieved under a defined set of conditions. A co-current and counter-current system enhanced the extraction yield by 16 and 20% respectively. The HPLC and UV/VIS spectra verified the purity of solvent extracted BS with enhanced activity profile after purification with biphasic liquid-liquid extraction. The structural nature of extracted BS was polymeric glycoprotein. Hence it can be concluded that glycoprotein extraction was enhanced after optimization which can further be applied to commercial BS productions.

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