Production and characterization of glycolipid biosurfactant from *Achromobacter* sp. (PS1) isolate using one-factor-at-a-time (OFAT) approach with feasible utilization of ammonia soaked lignocellulosic pretreated residues

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Abstract

With the ever growing increase in the demands of biosurfactants, the present study was focused in developing a set of parameters influencing biosurfactant production using one-factor-at-a-time (OFAT) approach in chemically defined medium from an indigenous isolate of *Achromobacter* sp. (PS1). Subsequently, the feasibility of biosurfactant production was examined using influential OFAT parameters in same medium, replacing only carbon source with lignocellulosic hydrolyzed sugars. These sugars were obtained from ammonia (15% *v/v*) soaking pretreatment of lignocellulosic residues (7.5% solid loading at 70^{o}C for 72 h) with subsequent saccharification using lignocellulolytic-enzymes. OFAT influential parameters observed were dextrose (3 – 4% *w/v*); C/N ratio 8.3 using sodium nitrate and beef extract; $2x10^{-5}$ grams equivalents Fe^{2+}; 1500 mM PO_4^{3-} in minimal salt medium (MSM) at pH 7.0, 120 rpm, 30^{o}C resulting in 4.13 ± 0.12 g/L rhamnolipid in 192 h with 30.42 mN/m surface tension and 136 mg/L critical micelle concentration (CMC). Biosurfactant was characterized using tandem-MS and NMR as rhamnolipid with six-congeners, Rha-C_{10}-C_{10} and Rha-Rha-C_{10}-C_{10} being the most abundant. Rhamnolipid showed broad range stability at temperatures (30-121°C), pH (6-12), and salinity (0.5-5% *w/v*) of NaCl. In Rice-straw (RS) hydrolysate, maximum glucan (73.10%) and xylan (91.13%) were obtained and the RS-hydrolysate medium with a total of 4.55% (*w/v*) sugars under optimum OFAT parameters (other than dextrose) showed at par production of 3.55 ± 0.06 g/L of rhamnolipid in 192 h with $Y_{BS/S}$ (biosurfactant yield per gram of sugar consumed) of 0.08 g/g and $Y_{BS/CDW}$ (biosurfactant yield per gram of cell biomass) of 0.68 g/g.

Key words: One-factor-at-a-time (OFAT); aqueous ammonia soaking (AAS); lignocellulosic residue; rhamnolipid; tandem-MS

Introduction

Microbial surfactants or biosurfactants are a heterogeneous group of surface active molecules produced by a wide range of microorganisms as secondary metabolites. They have varying chemical structures ranging from low-molecular weight glycolipids, lipopeptides, flavolipids, phospholipids to high molecular weight polymers as lipoproteins, lipopolysaccharide-protein complexes and polysaccharide protein fatty acid complexes [1]. Among the various categories of biosurfactants, the glycolipid biosurfactant "rhamnolipid" stands apart due to their low minimum surface tension values of 28mN/m, high emulsifying activity and low CMCs (10 - 150 mg/L) [2]. Rhamnolipid comprises of hydrophilic moiety of one (mono-rhamnolipids) or two (di-rhamnolipids) rhamnose molecules, while the hydrophobic part is represented by two (or more rarely one) hydroxy fatty acids, saturated or unsaturated, of different chain lengths (C_8 - C_{24}). These are mainly produced by *Pseudomonas aeruginosa* strains and in comparison to chemical surfactants exhibit higher bio-degradability, low-toxicity, increased surface activities, longer shelf life, high selectivity and specificity at extreme pH, temperature and salinity ranges [3]. Additionally, biosurfactants display anti-microbial, anti-tumorous, anti-adhesive, anti-oxidant and anti-corrosive activities [4,5]. These unique characteristics make them a multifunctional material of the 21st century with broad range of applications in various industries including in pharmaceuticals and therapeutics, cosmetics, detergents and cleaners, agriculture, bioremediation and enhanced oil recovery. Over a period of 2016 – 2023, this prominent product is projected to witness the highest compound annual growth rate of 7.5%, with a market of 250 kilotons by 2024 worth 2.7 billion USD [6]. However, its commercialization is hindered due to (i) low yields (ii) cost intensive recovery and (iii) high production costs [4]. In order to overcome these bottlenecks, research has been directed towards isolation of new isolates, media optimization and the use of renewable substrates, especially from agro-industrial wastes. The medium optimization through OFAT approach gives an insight of the essential nutritional and physical parameters and the coarse estimation of their optimum ranges. To make the process cost effective, the role of nutritional sources obtained from OFAT approach helps in designing a medium formulation comprising of renewable lignocellulosic residues. Further, an agriculture country like India which generates major portion of agro-residues in million metric tonnes (MMT) from rice-straw (112 MMT), wheat-straw (109.9 MMT) and sugarcane-bagasse (101.3 MMT) [7], the use of these renewable lignocellulosic residues will make the process cost effective especially for bioproducts as biosurfactants for which there are fewer reports.

To unlock the fermentable sugars from these feedstocks, various pretreatment methods, using dilute acid, alkali , solvent, hot water and physical disruption have been reported, but most of these methods are harsh and results in lower sugar yields with great investment risks [7]. Comparatively, ammonia pretreatment proves to be a method of choice owing to its non-corrosiveness,minimal inhibitor generation, cost, recycling considerations and high retention rates of both glucan and xylan in the pretreated solids [8]. Nowadays, aqueous-ammonia soaking (AAS) method is preferably used owing to the treatment of residues at moderate temperature range of 30-70°C under atmospheric pressure for 3-10 days. In these conditions, aqueous-ammonia reacts primarily with lignin removing 75% of it, with little effect on carbohydrates in the biomass retaining 100% glucan and 85% xylan . It is also reported to to be highly effective in improving the enzyme digestibility of the residues [9,10].

In the present study, the optimization of glycolipid biosurfactant producer has been carried out using OFAT approach in chemically defined medium from our bacterial isolate belonging to genera *Achromobacter*, reported for the first time as glycolipid producer [1]. Subsequently, the biosurfactant produced was structurally characterized based on tandem MS and NMR and its stability was monitored with respect to temperature, pH and NaCl concentration. For cost effective production,feasible utilization of lignocellulosic hydrolysed sugars obtained from different feedstocks was examined based on OFAT data.

Materials and Methods

Raw materials and chemicals

Lignocellulosic residues [rice-straw (RS), wheat-straw (WS) and sugarcane-bagasse (SB)] were procured from local sources around Uttar Pradesh, India. Prior to pretreatment the lignocellulosic residues were milled and sieved using -20/+40 American Society for Testing and Materials (ASTM) mesh screens to obtain average particle size ranging from 850 μm to 425μm. The biomass was then dried in an oven at 60°C for at least 48 h until a constant dry weight was attained**.**

All chemicals, solvents and reagents used were of analytical grade. Rhamnolipid standards JBR 215 (15% solution in water) and R95Dd Rhamnolipid (95% purity) were purchased from Jeneil biosurfactant Company (Saukville, WI, USA) and Sigma-Aldrich (India) respectively. Lignocellulolytic enzyme was purchased from Advanced enzymes (Maharashtra, India).

Microorganism

The microorganism used in the present study was isolated in our laboratory from petroleum sludge obtained from, BPCL refinery, Mumbai, India, and was identified as *Achromobacter* sp. (PS1) isolate (NCBI accession no. KT735240) showing 99% similiarity with *Achromobacter insolitus*. It showed significant hydrocarbon degradation ability of crude oil, degrading 77% of aromatic and 70% of aliphatic fractions respectively and producing glycolipid type of biosurfactant based on TLC, FT-IR and GCMS results [1].The culture was routinely sub-cultured and maintained on nutrient agar plates.

Biosurfactant production

The biosurfactant production was aerobically carried out in triplicate in 250-mL erlenmeyer flasks containing 50 mL of sterile medium. Overnight grown inoculum (1% *v/v*) in Luria broth was used to inoculate the production medium. The flasks were incubated at 30^{0}C in an incubator shaker at 120 rpm for a maximum of 10 days. Aliquots were withdrawn at 48h intervals and centrifuged at 4^{0}C, 10,000 rpm for 10 min. The resultant supernatant was monitored with respect to surface tension (mN/m), emulsification index (E_{24} %), rhamnolipid yield (g/L) and cell dry weight (g/L). The total residual reducing sugar was analyzed by dinitrosalicylic (DNS) acid method. All other experimental conditions were kept constant unless stated otherwise. All the results have been expressed as the mean of three independent replicates.

Surface Tension (ST)and Emulsification index (E_{24})

The surface tension was measured using digital surface tensiometer (SEO, instruments, korea) based on the principle of Du-Nouy ring method and the emulsification index (E_{24}%) of the culture filtrate was measured as the percentage of height (cm) of the emulsion layer divided by the total height (cm) obtained after leaving the emulsion undisturbed for 24h [1].

Quantification of rhamnolipids (Orcinol-sulphuric acid assay)

Rhamnolipid concentration was determined by the orcinol method as described by Rahman et al. [11] with slight modifications wherein approximately, 4 mL of the culture filtrate was acidified to pH 2.0 using 6N HCl and kept overnight for precipitation. The precipitate obtained after centrifugation was extracted four times with 1 mL of diethyl ether which was evaporated to dryness and the residue was dissolved in 0.5 mL of deionized water for orcinol-sulphuric acid assay. From the correlation equation of pure rhamnolipids / rhamnose [y = (0.0139 X - 0.0058) x 0.68], the correction factor obtained ranges between 3.0 and 3.4. The value of correction factor is not

exact, since the rhamnolipid biosurfactant is not composed of single molecule rather a family of congeners that have different molecular masses. Hence, in the current work an average correction factor of 3.2 was used for multiplication [12].

Cell dry weight

Cell dry weight was determined by centrifuging 1 mL culture broth at 10,000 rpm for 10 min. The cell pellet was then washed with 1 mL distilled water and dried at 90 ^{o}C until a constant weight was recorded.

Determination of influential parameters on biosurfactant production in chemically defined media by OFAT approach

The significant nutritional and physical parameters affecting the rhamnolipid production from *Achromobacter* sp. (PS1) were analyzed by changing one-factor-at-a-time (OFAT) while keeping others constant.

Nutritional parameters

Effect of medium composition

Different media known for rhamnolipid production as minimal salt medium (MSM) [1], proteose peptone glucose ammonium salt medium (PPGASM) [13] and Kay's medium [14] were supplemented with both 1 % (*w/v*) dextrose containing 0.4 g equivalent carbon and 1 % (*v/v*) diesel (equivalent to 0.89 % *w/v*) and were analyzed for their influential effects on rhamnolipid production.

Effect of conventional carbon sources (hydrocarbons, vegetable-oils and sugars)

The effect of different hydrocarbons (paraffin, hexadecane and diesel) and vegetable-oils (palm-oil, coconut-oil, sunflower-oil, mustard-oil) at 0.89 % (*w/v*) were examined separately in the best medium both in absence and presence of dextrose (1 % *w/v*) against the control set containing only 1 % (*w/v*) or (0.4 g equivalent carbon) dextrose.

Similarly, different sugars (0.4 g equivalent carbon) as: **Monosaccharides** - xylose, dextrose, fructose, galactose; **Di-saccharides** - lactose, arabinose, cellobiose, maltose, sucrose; **Sugar alcohols** - glycerol, mannitol, sorbitol, xylitol; **Polysaccharides** - cellulose, starch were evaluated separately by replacing dextrose in the best medium.

Subsequently, different concentrations of the best carbon source supporting rhamnolipid production was examined.

Effect of different nitrogenous sources

For effect of inorganic nitrogen sources on rhamnolipid production, the cumulative effect of metal ions and their nitrogenous salts, *viz***.** $NaNO_2$; $NaNO_3$; KNO_3; NH_4NO_3; NH_4Cl; $(NH_4)_2SO_4$; $Mg(NO_3)_2$; $Ca(NO_3)_2$; $Fe(NO_3)_3$ and $Al(NO_3)_3$ was examined by replacing sodium nitrate ($NaNO_3$) and yeast extract containing total N equivalent of 0.1288 g. Similarly, organic nitrogen sources *viz*- urea, yeast extract, peptone, beef extract, tryptone and corn steep liquor (CSL) were analyzed. Finally utilizing the best inorganic and organic nitrogen sources, the carbon to nitrogen equivalent ratio (C/N) was varied from 6.2 – 12.5 keeping 3% (*w/v*) dextrose constant (*i.e.* carbon equivalent at 1.2 g).

Effect of iron and phosphate concentration

The effect of varying concentrations of $FeSO_4$ was investigated from $2x10^{-1}$ - $0.5x10^{-5}$(grams equivalent of Fe^{2+}).

Effect of phosphate concentration was studied by varying phosphate content in the medium from 100 - 1500 mM.

Physical parameters

The effect of culture conditions on rhamnolipid production was examined separately by varying the pH of the medium from pH 5 – 12, incubation temperature from 30 – 50 ^{0}C, inoculum ratio from 0.6 – 3.0 % (*v/v*) and agitation rate from 120 – 230 rpm.

Biosurfactant recovery

The culture filtrate obtained was acidified with 6N HCl to pH 2.0 and kept overnight for precipitation, centrifuged at 10,000 rpm, 4°C for 10 min and extracted twice with a mixture of chloroform:methanol (2:1 *v/v*). The pooled extracts were concentrated under vacuum conditions using a rotary evaporator resulting in a partially purified viscous honey coloured rhamnolipid product.

Critical micelle concentration

The CMC of the partially purified biosurfactant obtained from chemically defined medium was determined by performing successive dilutions of the biosurfactant dissolved in deionized water and by plotting the surface tension versus biosurfactant concentration curve.

Structural characterization

Structural characterization was conducted from partially purified biosurfactant sample obtained from chemically defined medium.

Column chromatography

Liquid column chromatography was used for the separation of rhamnolipids as described by George and Jayachandran [12] in a 45 x 2.0 cm column loaded with 5g of partially purified biosurfactant. Chloroform / methanol mobile phases were applied in sequence; 50:3 *v/v* (1000 ml), 50: 5 *v/v* (200 ml), 50:25 *v/v* (100 ml) and 50: 50 *v/v* (100 ml) at a flow rate of 1 mL /min and 50 mL fractions were collected. Active fractions containing the rhamnolipid biosurfactant were evaporated to dryness under vacuum conditions using a rotor evaporator.

Thin layer chromatography (TLC)

The partially purified biosurfactant was solubilized in methanol and analyzed by TLC on silica gel 60 F_{254} plates using a solvent system chloroform: methanol: acetic acid (65:15:2 *v/v/v*) and visualized with orcinol-sulphuric acid reagent [1].

For preparative-TLC, the column purified fractions were loaded on silica gel sheets. Corresponding portions of the non-sprayed plates with the same R_f values were scratched off, extracted with diethyl ether and centrifuged at 10,000 rpm for 10 min to remove silica gel residue. The supernatant solvent was microfiltered (pore size 3µm) and air dried for NMR studies.

Fourier Transform infrared (FT-IR) spectroscopy and Gas chromatography-mass spectrometry (GC-MS)

Fourier Transform infrared (FT-IR) analysis was carried out using a Varian-7000 FTIR. GC-MS was carried out in Shimadzu GC-MS (QP2010 ultra) for fatty acid analysis [1].

Tandem-MS analysis

To characterize the rhamnolipid homologues, 10 mg of the partially purified biosurfactant was dissolved in 1 mL methanol and subjected to tandem mass spectrometry in positive ion mode using Waters Quattro Micro Triple Quadrupole Mass Spectrometer (Waters, MA). The sample was infused into the mass spectrometer using a syringe pump at a flow rate of 30 µL/min. The capillary voltage was set at 3.5 kV, cone voltage at 30 V, extractor at 4.0 V and RF lens at 0.2 V. The source and desolvation temperature was set at 80 ^{o}C and 300 ^{o}C respectively with desolvation and cone gas flow rates at 750 L/hr and 50 L/hr respectively. The instrument was operated at a resolution of 0.7 Da and collision energy was set to 15 - 35 V for fragmentation of different molecular ions. The mass scanning range of the instrument was set to 100 - 1000 Da and the data was processed using the Masslynx 4.1 software.

NMR analysis

The column purified fractions of the mono- and di-rhamnolipid was dissolved in deuterated chloroform ($CDCl_3$). One dimensional ^1H and 2D [^{13}C,^1H] heteronuclear single-quantum coherence (HSQC) experiments were carried out at 298 K on Bruker *Avance* III spectrometers equipped with cryogenic triple-resonance 5 mm TCI probe head, operating at field strength of 500.15 MHz frequency. For 1D ^1H NMR spectra, a ^1H 90° pulse of 7.6 µs was obtained. The spectrum was measured for a spectral width of 6009.6 Hz and total acquisition time of 5.45 s. The 2D [^{13}C,^1H] HSQC spectra were measured with spectral width of 7002.8 Hz along the ^1H dimension and 18,865.6 Hz along the ^{13}C dimension. Topspin version 2.1 (Bruker AG) was used for acquisition, Fourier transformation, and processing of data. Referencing of all the spectra was done using TMS.

Biosurfactant stability

Twenty mL each of partially purified biosurfactant was dissolved in de-ionized water in screw capped vials. A vial with no NaCl content at ambient temperature of 30°C, pH 7.0 served as control and was considered as 100% stable. Relative to control, the test vials were incubated at different temperatures from 40 - 90°C for 24 h and at 100°C and 121°C for 120 min. Similarly, stability of the biosurfactant was investigated at different pH (2-12) and salinity (0.5- 20% *w/v* of NaCl) ranges by varying pH using NaOH /HCl and adding different concentrations of NaCl to biosurfactant solution respectively. Aliquots were withdrawn at regular time intervals, cooled and stability was investigated with respect to surface tension and emulsification index, with residual stability (%) calculated as:

$$\text{Loss (\%)} = [E_{24}\ control - E_{24}\ test\ ]/\ E_{24}\ control * 100$$

$$\text{Residual stability (\%)} = [100 - \text{loss}]$$

Feasibility of biosurfactant production from lignocellulosic residues

Characterization of lignocellulosic residues

The lignocellulosic residues (rice-straw, wheat-straw and sugarcane-bagasse) were subjected to compositional analysis as per the national renewable energy laboratory (NREL) analytical procedures [15].

Thermochemical pretreatment and Enzymatic hydrolysis

The raw lignocellulosic residues (7.5% *w/v*) were subjected to pretreatment by aqueous ammonia (15% *v/v*) ($NH_3.H_2O$) soaking method in a 2L PARR reactor at 70°C for 72 h without stirring. The biomass was then washed with distilled water, dried overnight at 60°C and further used for enzymatic hydrolysis. The enzymatic hydrolysis was carried out using lignocellulolytic enzyme (Advanced-sacchariSEB-enzyme, Maharashtra, India) (containing

cocktail of cellulase-365 FPU/g, β-glucosidase-571 CBU/g and xylanase 7000 ABXU/g) at 10 filter paper units /gram of pretreated biomass keeping a solid loading of pretreated biomass at 10 % (*w/v*) in a stoppered conical flask containing sodium acetate buffer (pH 5.0) and incubated in a water bath shaker at 50°C, 120 rpm for 48 h [16]. After hydrolysis, the sugar hydrolysate was separated by centrifugation at 10,000 rpm, 4°C for 10 min. The sugar concentrations were then analyzed by HPLC (Agilent 1260, Palo Alto, CA) using a Biorad Aminex HPX-87H column equipped with a refractive index (RI) detector.

The potential of each lignocellulosic hydrolysate was examined for rhamnolipid production with and without the addition of influential nutrients obtained from OFAT approach and the best hydrolysate was selected on the basis of the percentage recovery of glucan and xylan. Further, the percentage variation (*w/v*) of the total sugar on rhamnolipid production was evaluated.

Results and Discussion

OFAT approach - Nutritional parameters

Medium composition

The comparative study of different chemically defined media revealed MSM medium to support rhamnolipid (RL) production (0.29 ± 0.09 g/L) and growth (3.42 ± 0.10 g/L) as compared to 0.06 ± 0.02 g/L RL yield and 1.48 ± 0.14 g/L cell dry weight (CDW) in PPGASM and 0.09 ± 0.03 g/L RL yield and 1.85 ± 0.08 g/L cell dry weight in Kay's medium respectively (Fig.1a). Hence, MSM was selected as a basal medium for further improvement of biosurfactant yield with supplementation of various nutrients.

Carbon sources

The production of biosurfactant from *Achromobacter* sp. (PS1) was found to be constitutive as the control set containing only 1 % (*w/v*) dextrose corresponding to 0.4 g equivalent carbon showed considerable growth (2.50 ± 0.54 g/L) and rhamnolipid (RL) production (0.43 ± 0.06 g/L). Correspondingly, the (**a**) set containing hydrocarbons and (**b**) set containing vegetable oils with and without additional dextrose showed low RL production inspite of sufficient cell growth in sets supplemented with dextrose (Table 1). This constitutive mode of production can be well explained from the literature according to which biosurfactant production is strain dependent [17,18]. The surface tension and emulsification index (E_{24}) recorded in constituent control set was 31.06 ± 0.54 mN/m and 50.89 ± 1.07 respectively. From Table 1 set (**c**), it is evident that dextrose supported the maximum 0.43 ± 0.06 g/L of

rhamnolipid production followed by fructose (0.28 g/L), galactose (0.21 g/L), xylose and cellobiose (0.20 g/L) respectively. Ndlovu et al. [19] also reported maximum rhamnolipid production of 0.30 ± 0.14 g/L and 0.26 ± 0.20 g/L in dextrose and glycerol respectively from *Pseudomonas aeruginosa* ST5 in MSM.

Concentration variation of dextrose from 1% *w/v* (0.4 g equivalent carbon) to 4 % *w/v* (1.6 g equivalent carbon) showed a significant positive correlation in increase in rhamnolipid production from 0.43 ± 0.06 g/L to 3.31 ± 0.08 g/L (Fig.1b). This may be due to complete exhaustion of dextrose in 1 % and 2 % concentrations within 48 h leaving no residual sugar to divert the cell metabolism towards rhamnolipid synthetic pathway. However in 3 % and 4 % dextrose concentrations, sufficient amount of residual sugars 8.03 ± 2.95 g/L and 22.37 ± 2.00 g/L respectively beyond 48 h (Fig 1c) supported growth and normal metabolism, paving a large part of carbon towards product synthesis [20]. Beyond 4 % concentration, a decrease in rhamnolipid yield (2.77 ± 2.95 g/L) was observed which may be due to accumulation of toxic metabolites as a result of high energy demand with full oxidation via acetyl CoA and TCA cycle, thereby generating CO_2 [21]. Since there was no significant difference in the biosurfactant yield in 3 % (3.06 ± 0.06 g/L) and 4 % (3.31 ± 0.08 g/L) dextrose concentrations (*w/v)* respectively, 3% (*w/v*) dextrose concentration was considered for further optimization studies. Radzuan et al. [21] reported considerable effect of varying dextrose concentrations (% *w/v*) on rhamnolipid yield with 0.18 g/L at 2 %, 0.35 g/L at 5 % and 0.36 g/L at 10 % respectively. Higher dextrose concentrations of 10 %, resulted in early death phase of cells with significant drop in the product yield ($Y_{P/S,}$ g/g) from 0.017 at 2 % to 0.003 at 10 % (*w/v*).

Nitrogen sources

Monovalent nitrates of sodium, potassium and trivalent nitrates of iron and aluminium at 0.1288 g equivalent of total nitrogen alone were found to be influential yielding of 3.01 ± 0.17 g/L, 3.05 ± 0.02 g/L, 2.50 ± 0.20 g/L and 3.15 ± 0.12 g/L respectively (Fig. 2a). Ferric and aluminium nitrates resulted in green pigmentation hence was not considered for optimization studies. Sodium nitrate was further selected for optimization studies being most reported, efficient and cheap. Ma et al. [18] has also reported that the use of nitrates, results in its slower assimilation, thereby simulating a nitrogen limited condition favorable for rhamnolipid production as nitrate first undergoes dissimilatory nitrate reduction to nitrite and then to ammonia followed by its assimilation *via* glutamine-glutamate metabolism. The results of nutrient rich nature of organic nitrogen sources alone at 0.1288 g equivalent of total nitrogen on rhamnolipid production revealed beef extract to be effective with yield of 2.47 ± 0.18 g/L though Zhao et al. [22] reported organic nitrogen sources as unfavorable for rhamnolipid production (Fig. 2b). Cumulative

analysis of the best inorganic and organic nitrogen sources *i.e.* $NaNO_3$ and beef extract yielded 3.89 ± 0.20 g/L RL at optimum C/N ratio at 8.3. Low C/N ratio (6.2) resulted in low RL yields (2.11 ± 0.38 g/L) (Fig. 2c). This trend may be correlated with the activation of glutamine synthetase activity requiring higher consumption of carbon to meet the new energy status with the stimulation of rhamnolipid synthesis which occurs under nitrogen limiting conditions indicating its onset beyond C/N ratio of 6.2. However, the C/N ratio beyond 8.3 with low nitrogen levels showed a decrease in rhamnolipid production which may be due to the insufficient amounts of nitrates and hence glutamine thereby limiting the metabolism of dextrose [23].

Effect of iron concentration and phosphate concentrations

Varying iron and phosphate concentrations didn't show any significant enhancement in the rhamnolipid production. The optimum concentration of $FeSO_4$ was observed at $2x10^{-5}$grams equivalent of Fe^{2+} and phosphate concentration at 1500 mM keeping carbon concentration at 3% *w/v* (*i.e* 1.2 g carbon equivalent) .

Physical parameters

Achromobacter sp. (PS1) showed maximum rhamnolipid production of 4.13 ± 0.12 g/L at optimum pH 7.0, temperature 30^{o}C and optimum agitation rate of 120 rpm. Static condition yielded lower RL production (2.10 ± 0.14 g/L) (Fig. 3). No significant change in the final product yield was observed on varying inoculum ratio from 0.6 - 3.0 % (*v/v*).

Physiochemical characterization

The CMC of the partially purified rhamnolipid was determined to be 136 mg/L which lies in the range (10 - 400 mg/L) as reported by other authors [2].

Column chromatography for rhamnolipid purification

Neutral lipids were observed at R_f value 0.96 in the first two $CHCl_3$ eluted fractions, $CHCl_3$/ CH_3OH (50:3) eluted fraction no. (3 - 12) (F_1) showed a mono-rhamnolipid spot at R_f value of 0.84 and fractions (13 – 25) (F_2) showed di-rhamnolipid spot at R_f 0.61. Other elution of $CHCl_3$/ CH_3OH (50:5; 50:25 and 50:50) did not show any spot.

Structural characterization

The TLC result of the biosurfactant suggested a glycolipid nature of the biosurfactant with two prominent spots of mono and di-rhamnolipid moieties. The FT-IR spectrum of the biosurfactant showed characteristic absorption bands (3423 cm^{-1} of O–H stretching vibrations of free hydroxyl groups, 916 cm^{-1} and 848 cm^{-1} of –O–C/ =C–H

stretching of glycosidic linkage) corresponding to specific functional groups present in the glycolipid biosurfactant. The GC-MS results showed the presence of 3-hydroxydecanoic acid ($C_{10:0}$) as the most abundant fatty acid. The results of TLC, FT-IR and GCMS of fatty acids of the biosurfactant is reported in our previous research paper [1].

The mass spectrometric analysis of rhamnolipid structures produced by *Achromobacter* sp. (PS1) showed the presence of six different types of sodium adducts $[M + Na]^+$ and their corresponding potassium adducts $[M + K]^+$ under positive ionization mode, a characteristic which is typical for samples containing carbohydrate derivatives [24]. Six-different type of congeners were observed with mono-rhamnolipids Rha-C_{10}-C_{10}, followed by the di-rhamnolipid Rha-Rha-C_{10}-C_{10} as the most abundant congeners. Similar results of rhamnolipid congeners were reported by Gudina et al. [3] and Moya-Ramirez et al. [25].

To determine the structures of rhamnolipids with respect to fatty acid chain lengths and its position and presence of rhamnose sugar, the samples were subjected to tandem MS. Based on the relative abundance, $[M + Na]^+$ adduct of mono-rhamnolipid (Rha-C_{10}-C_{10}) and di-rhamnolipid (Rha-Rha-C_{10}-C_{10}) corresponding to *m/z* of 527.5 and 673.4 were used for interpretation of the structures. $[M + Na]^+$ mono-rhamnolipid adduct of *m/z* of 527.5 exhibited daughter ions at *m/z* of 381, 357, 211, 187 and 169. The mass peak at *m/z* 169 and 187 corresponded to the rhamnose residue $[Rha_{res} + Na]^+$ and rhamnose unit $[Rha + Na]^+$, *m/z* at 357 is attributed due to the loss of terminal fatty acyl chain $[Rha\text{-}C_{10} + Na]^+$, *m/z* of 381 corresponded to middle and terminal fatty acyl chain (C_{10}-C_{10}) whereas *m/z* of 211 is attributed to single fatty acyl chain ($C_{10}H_{20}O_3$) (Fig. 4a).

The $[M + Na]^+$ adduct of di-rhamnolipid (Rha-Rha-C_{10}-C_{10}) corresponding to *m/z* of 673.4 further fragmented to *m/z* of 527, 503, 381, 315, 211 and 169. Ions at *m/z* 527 and 381 reveal the loss of one and two rhamnose residue from the parent molecule respectively. Further the product ion at *m/z* 315, was identified as $[Rha\text{-}Rha + Na]^+$ confirming that both rhamnose units are linked together. Other daughter (pseudo-molecular) ion with *m/z* 503 is due to the loss of a terminal fatty acyl chain $[Rha\text{-}Rha\text{-}C_{10} + Na]^+$ and the ion at *m/z* 211 corresponds to fatty acyl chain (Fig. 4b).

The structure of the purified rhamnolipid fractions (F_1) mono and (F_2) di-rhamnolipid was further confirmed by 1D (^{1}H-NMR) and 2D [^{13}C,^{1}H] HSQC analysis. In ^{1}H-NMR, the proton of the methyl group ($-CH_3$) of two main composition of the biosurfactant (L-rhamnose moiety and aliphatic moiety) were detected at chemical shifts δ 1.28/1.29 and δ 0.88/0.87 respectively. The methylene ($-CH_2-$) proton attached to the >C=O group in the aliphatic

chain resonated at δ 2.41/2.35 ppm along with typical signals of oxymethine (-O-CH-) protons at δ 4.17/4.16 and δ 5.25/5.08 (Table 2).

The 2D analysis showed almost similar chemical shifts in (F_1) mono and (F_2) di-rhamnolipid fractions with the carbon atom of the methyl group ($-CH_3$) of L-rhamnose moiety/ aliphatic chain of the rhamnolipid resonating at δ 17.30/17.39 and 13.94/ 14.12 ppm respectively. Carbon atoms of $(-CH_2-)_n$ group of the aliphatic chain in mono-rhamnolipid (F_1) and di-rhamnolipid (F_2) resonated in the range from δ 22.50 – 32.50 ppm and 22.40 – 34.70 ppm respectively. The HSQC analysis showed a single anomeric signal in the C-1 region at δ 4.89/96.9, suggesting a β-L-Rha (1′↔1)-hydroxy fatty acid linkage. In contrast, in (F_2) di-rhamniolipid the HSQC cross peaks in the C-1 region showed two signals at δ 4.91/94.18 and 4.90/102.40 suggesting a β-L-Rha (1′↔1)-hydroxy fatty acid and β-L-Rha (1″↔2 ′)- β-L-Rha linkages respectively. NMR observations with similar chemical shifts for rhamnolipid biosurfactant were reported by Varjani and Upasani [5] and Monteiro et al. [26].

Biosurfactant stability

Applicability of glycolipid biosurfactants in several fields depends on their stability at different temperature, pH and salinity ranges. The biosurfactant was found to be thermostable at broad temperature range with sufficient stability of approx 93% in term of emulsification index at 100°C and 121°C even after 2h of incubation compared to control (100%) . At varying temperature, pH and salinity, no significant change in the surface tension values was observed. However, stable emulsions formed over the pH range 6 – 12, while at acidic pH ranges of 2- 5 a coalescence in emulsion was observed. This stability in emulsion above pH 5.0 may be due the ionized form of the rhamnolipids with negative charge (pKa for rhamnolipis 5.6) which is counter balanced by Na^+ ions leading to an enhancement of repulsive double layer forces between micelles and thereby reducing the tendency to aggregate and favouring emulsification and emulsion stability [27]. The salinity effect on the biosurfactant emulsion showed a decrease in emulsion stability, with increase in NaCl concentration from 82% at 5% (*w/v*) to 54% at 20% (*w/v*). This decrease in stability on increasing NaCl concentration may be due to the salting-out phenomenon resulting in the preferential movement of water molecules, which immobilize and quench their role as solvents from coordination shells of biosurfactant molecules to those of salts along with the gradual decrease in the hydrophobicity of the rhamnolipid biosurfactant [28]. Zhao et al. [29] also reported E_{24} values higher than 60% of the rhamnolipid after treatment at temperatures (4–121 °C), pH values (2–10) and 0–90 g/L of NaCl .

Feasibility of biosurfactant production from lignocellulosic residues

The solid recovery on AAS pretreatment varied with the composition of the raw lignocellulosic residues. The highest solid recovery was obtained in RS (80.00%) followed by WS (78.60%) and SB (74.70%) respectively from cellulose and hemicellulose released after delignification involving the cleavage of C-O-C bonds in lignin and other ester/ether bonds in the lignin carbohydrate complex [10]. The enzymatic saccharification of the pretreated biomass resulted in maximum recovery of both glucan (73.10%) and xylan (91.13%) in RS, followed by WS and SB respectively with highest total sugar concentration of 4.55% (*w/v*) in RS .

The lignocellulosic-hydrolysate medium of each - RS, WS and SB containing approximately 4 % total sugars with no nutrient supplementation resulted in low cell dry weight of 3.55 ± 0.18 g/L (WS), 3.45 ± 0.12 g/L (RS) and 2.80 ± 0.11 g/L (SB) with corresponding lower biosurfactant yield of 1.22 ± 0.06 g/L, 1.54 ± 0.11 g/L and 0.92 ± 0.07 g/L respectively (Fig. 5a). However, both cell growth and biosurfactant yield showed better results in hydrolysate medium supplemented with nutrients with a maximum of 3.55 ± 0.06 g/L biosurfactant and 5.0 ± 0.11 g/L cell dry weight in RS (Fig. 5b). Prabhu et al. [30] also reported enhanced rhamnolipid production in wheat-straw hydrolysate supplemented with basic salts from *Pseudomonas* sp in comparison to medium with no nutrients supplemented. From Table 3, it is evident that the value of $Y_{BS/CDW}$ (g/g) is almost similar in RS (0.71 g/g) and in WS (0.65 g/g) while SB showed 0.54 g/g yield. Similarly, $Y_{BS/S}$ (g/g) value was observed same in both RS and WS (0.08g/g) with a slight lower yield (0.06 g/g) in SB-hydrolysate.

The variation of total hydrolysed sugars (1.5% - 4.5%) in the best hydrolysate (RS) showed a maximum biosurfactant production of 3.59 ± 0.09 g/L in 4.5 % closely followed by 3.18 ± 0.11 g/L in 3.5% respectively (Fig. 6). The yield of rhamnolipid (3.59 g/L) in RS-lignocellulosic hydrolysate was almost similar to chemically defined medium (3.78g/L) having same composition of respective sugars (total-sugars 4.55% -glucose 2.8%, cellobiose 0.14%, xylose 1.5 %, arabinose 0.11 %).

Conclusion

The maximum xylan and glucan sugars recovered from rice-straw using mild aqueous-ammonia soaking (AAS) pretreatment yielded 3.59 g/L of biosurfactant in minimal salt medium (MSM) in presence of optimum parameters obtained through OFAT approach. The biosurfactant was confirmed as rhamnolipid using tandem-MS and NMR with broad range stability at different temperature, pH and salinity. Thus, the rhamnolipid produced from hydrocarbonoclastic bacteria *Achromobacter* sp. (PS1) with promising tensioactive properties may find applications in various industries.

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List of Abbreviations

OFAT: One-factor-at-a-time; RS: Rice-straw; WS: Wheat-straw; SB: Sugarcane-bagasse; AAS: Aqueous ammonia soaking; MSM: Minimal salt medium; CMC: Critical micelle concentration; CDW: Cell dry weight; RL: Rhamnolipid; ASTM: American Society for Testing and Materials

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Legends of figure

Fig. 1 (a) Effect of different media on rhamnolipid production
(b) Effect of varying dextrose concentration in chemically defined media on rhamnolipid yield.
(c) Effect of varying dextrose concentration in chemically defined media on rhamnolipid yield with respect to growth (straight lines) and residual sugar (dotted lines)

Fig. 2 (**a**) Effect of inorganic nitrogen sources on rhamnolipid production
(**1**. $NaNO_2$; **2**. $NaNO_3$; **3**. KNO_3; **4**. NH_4NO_3; **5**. NH_4Cl; **6**. $(NH_4)_2SO_4$; **7**. $Mg(NO_3)_2$; **8**. $Ca(NO_3)_2$; **9**. $Fe(NO_3)_3$; **10**. $Al(NO_3)_3$; **11**. Nitrogen deficient)
(**b**) Effect of organic nitrogen sources and (**c**) Effect of varying C/N ratio

Fig. 3 Effect of (**a**) pH (**b**) temperature ($^{\circ}$C) and (**c**) agitation (rpm) on rhamnolipid yield

Fig. 4 Tandem-Ms spectrum and fragmentation pattern of (**a**) $[M+Na]^+$ ion at *m/z* 527 corresponding to mono-rhamnolipid Rha-C_{10}-C_{10} and (**b**) $[M+Na]^+$ ion at *m/z* 673 corresponding to di-rhamnolipid Rha-Rha-C_{10}-C_{10}

Fig. 5 Rhamnolipid production (straight lines) and growth (dotted lines) in different lignocellulosic hydrolysates (**a**) nutrient deficient (**b**) supplemented with influential nutrients

Fig. 6 Rhamnolipid production in different concentrations of RS-lignocellulosic hydrolysates supplemented with nutrients

List of Tables

Fig. 1

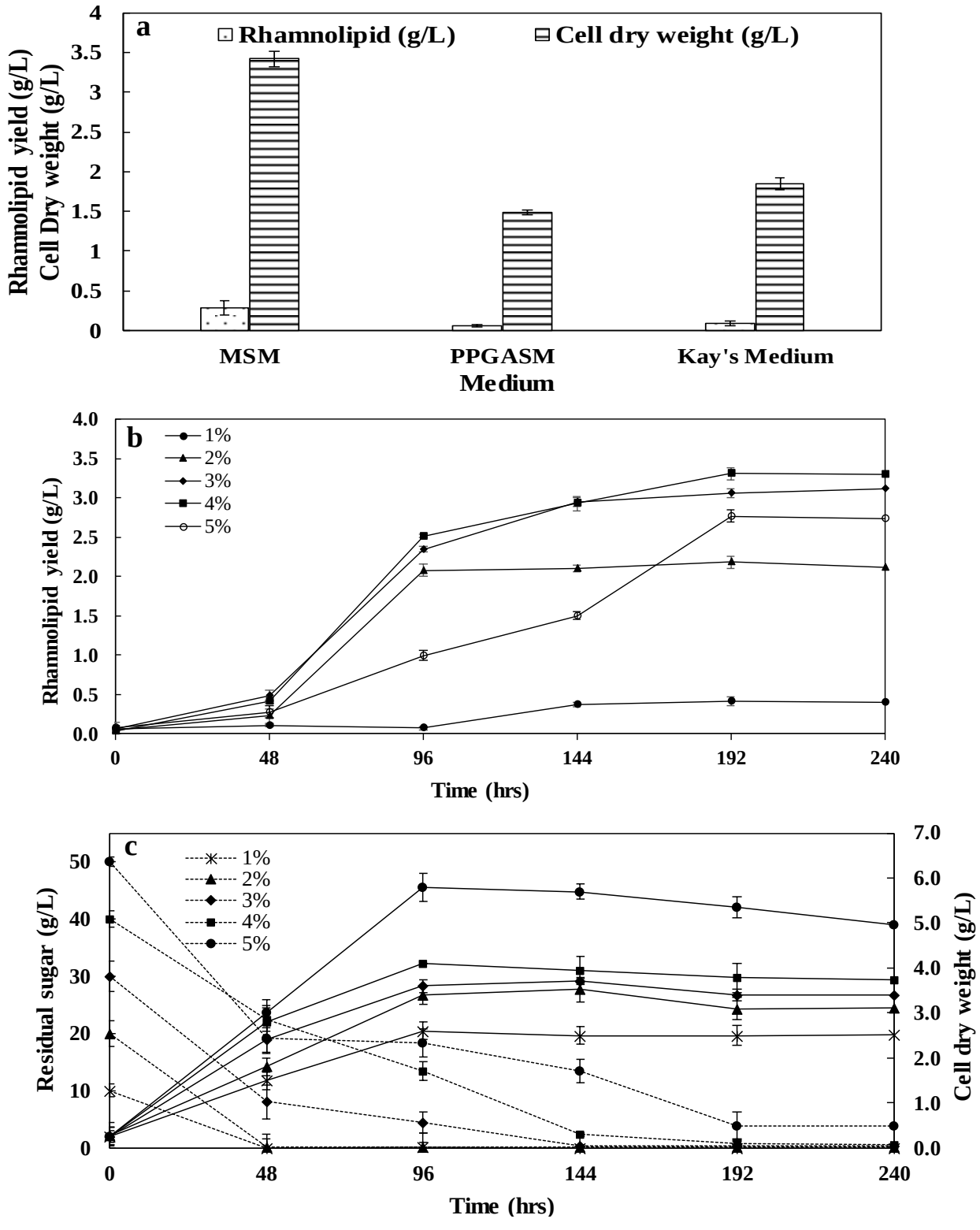

a
Rhamnolipid (g/L)
Cell dry weight (g/L)
Rhamnolipid yield (g/L)
Cell Dry weight (g/L)
MSM
PPGASM Medium
Kay's Medium
b
1%
2%
3%
4%
5%
Rhamnolipid yield (g/L)
Time (hrs)
c
Residual sugar (g/L)
Cell dry weight (g/L)
Time (hrs)

Fig. 2

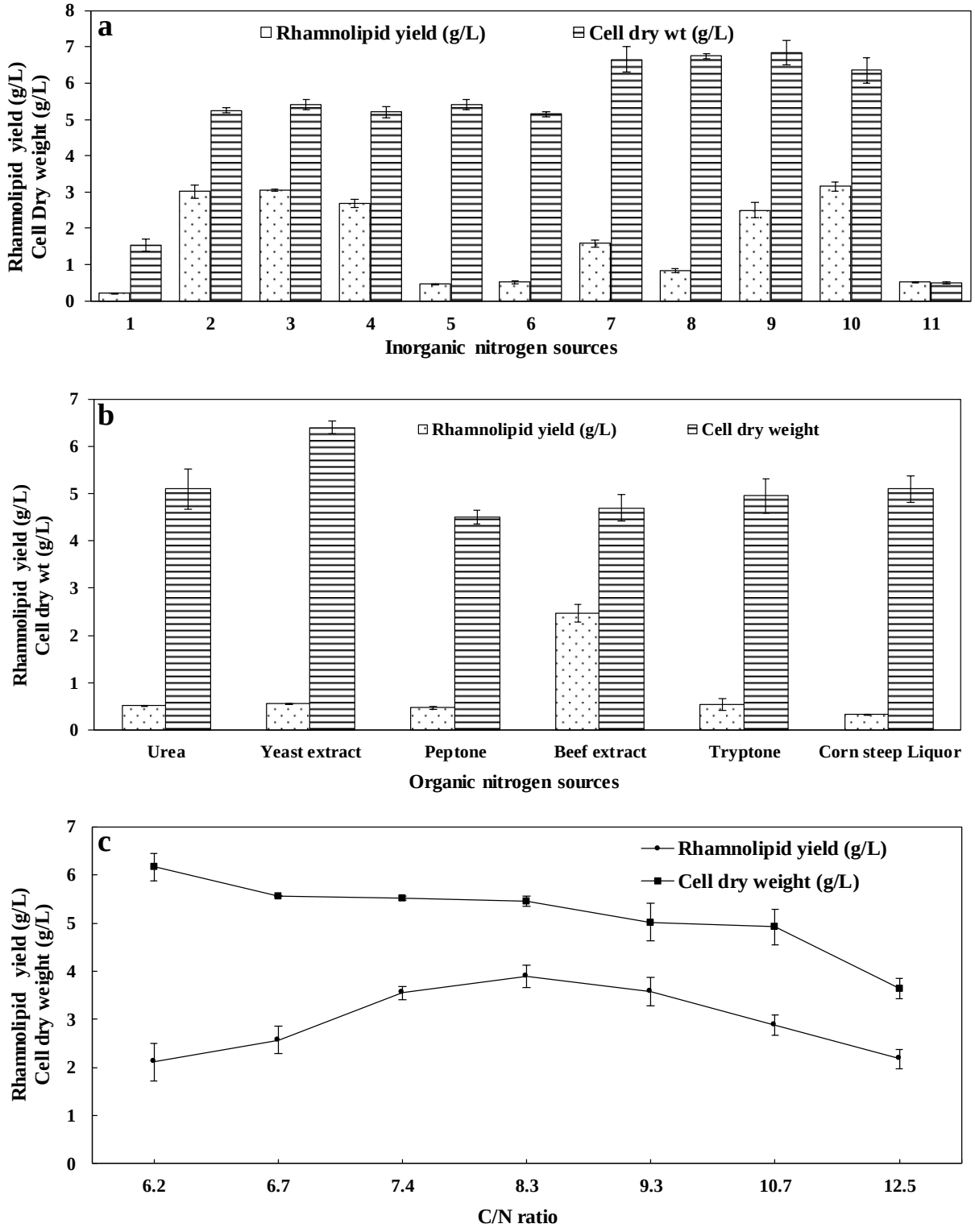

a
Rhamnolipid yield (g/L)
Cell dry wt (g/L)
Rhamnolipid yield (g/L)
Cell Dry weight (g/L)
0
1
2
3
4
5
6
7
8
1
2
3
4
5
6
7
8
9
10
11
Inorganic nitrogen sources
b
Rhamnolipid yield (g/L)
Cell dry weight
Rhamnolipid yield (g/L)
Cell dry wt (g/L)
0
1
2
3
4
5
6
7
Urea
Yeast extract
Peptone
Beef extract
Tryptone
Corn steep Liquor
Organic nitrogen sources
c
Rhamnolipid yield (g/L)
Cell dry weight (g/L)
Rhamnolipid yield (g/L)
Cell dry weight (g/L)
0
1
2
3
4
5
6
7
6.2
6.7
7.4
8.3
9.3
10.7
12.5
C/N ratio

Fig. 3

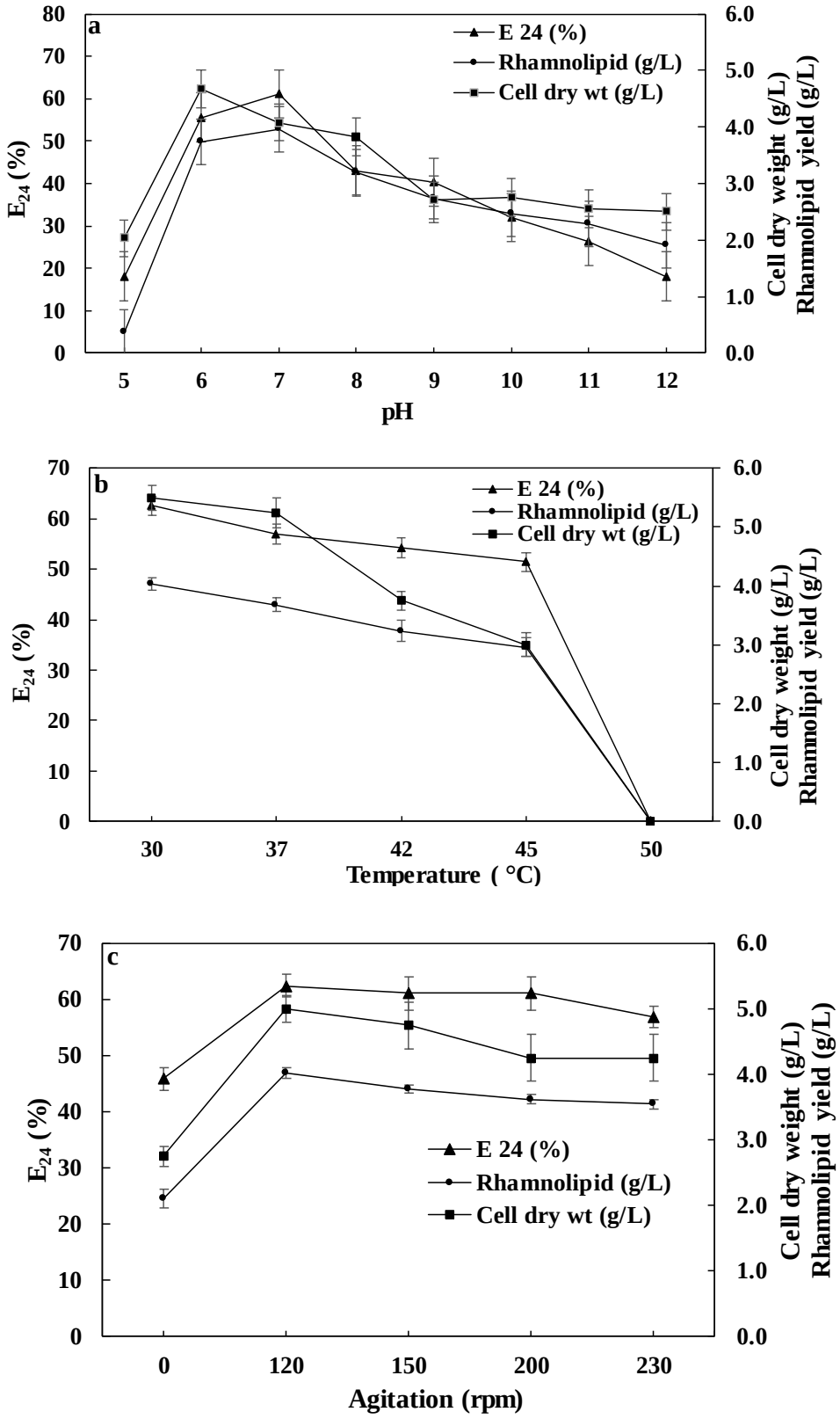
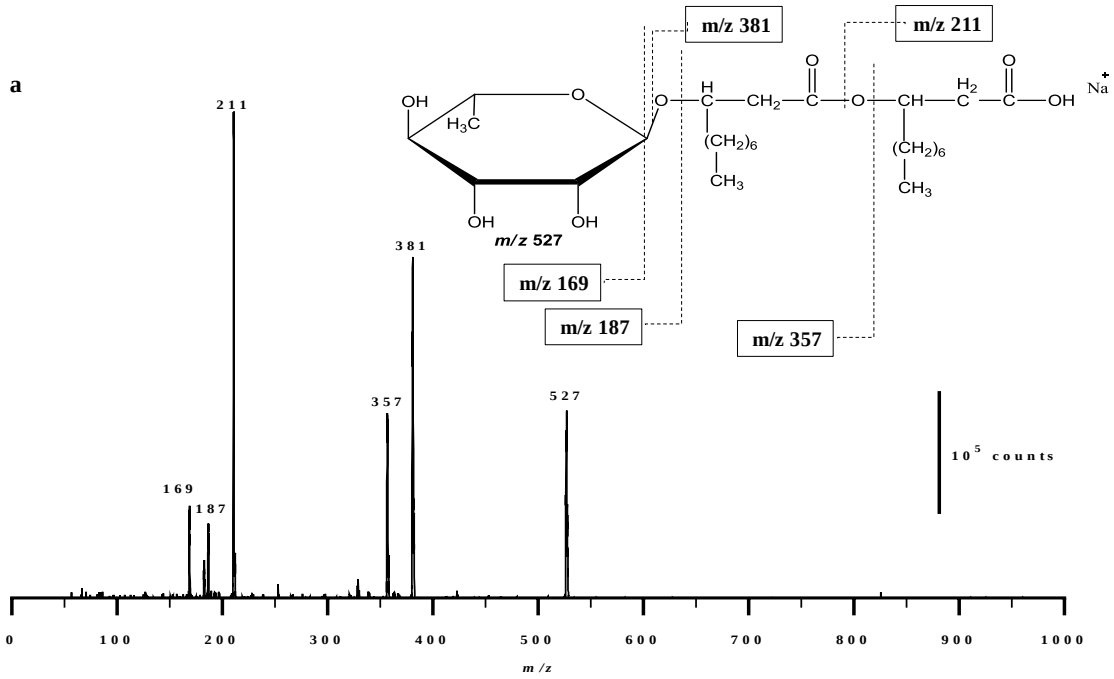

a
E 24 (%)
Rhamnolipid (g/L)
Cell dry wt (g/L)
E_{24} (%)
Cell dry weight (g/L)
Rhamnolipid yield (g/L)
pH
b
Temperature (°C)
c
Agitation (rpm)

Fig. 4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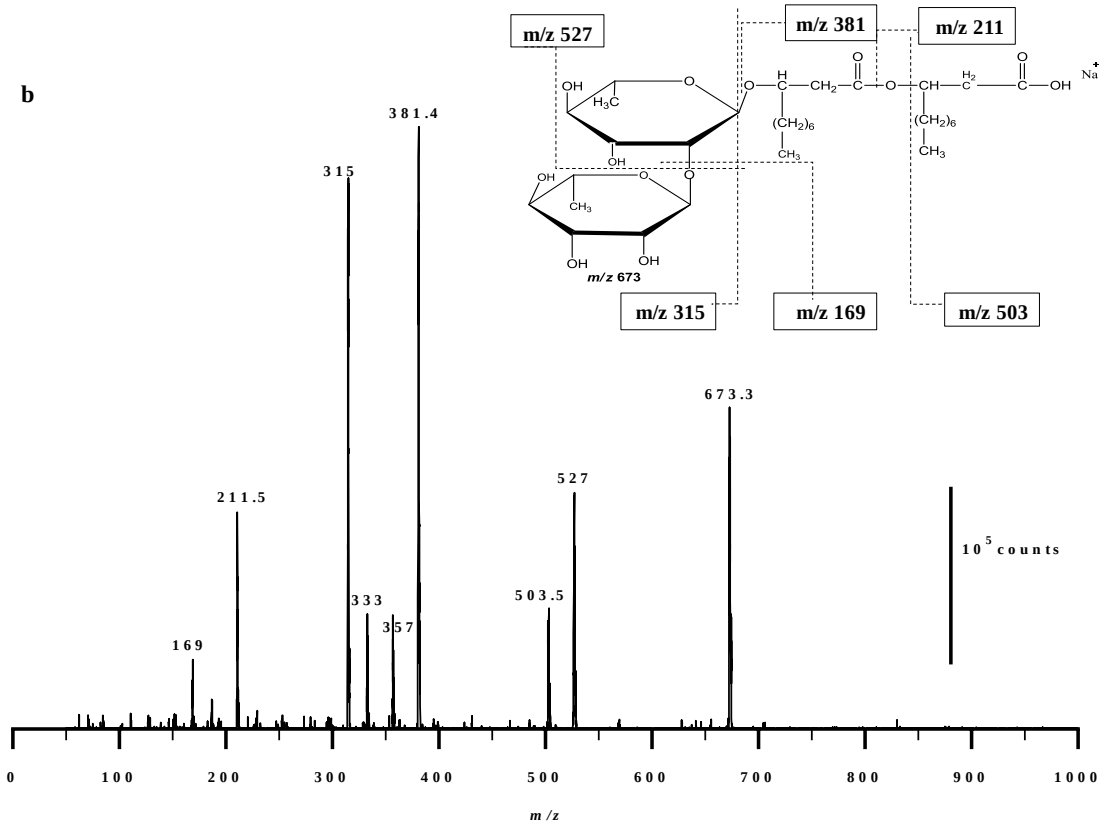
m/z 381
m/z 211
m/z 527
m/z 169
m/z 187
m/z 357
211
381
357
527
169
187
10^5 counts
0 100 200 300 400 500 600 700 800 900 1000
m/z


b
m/z 527
m/z 381
m/z 211
m/z 673
m/z 315
m/z 169
m/z 503
381.4
315
673.3
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10^5 counts
0 100 200 300 400 500 600 700 800 900 1000
m/z

Fig. 5

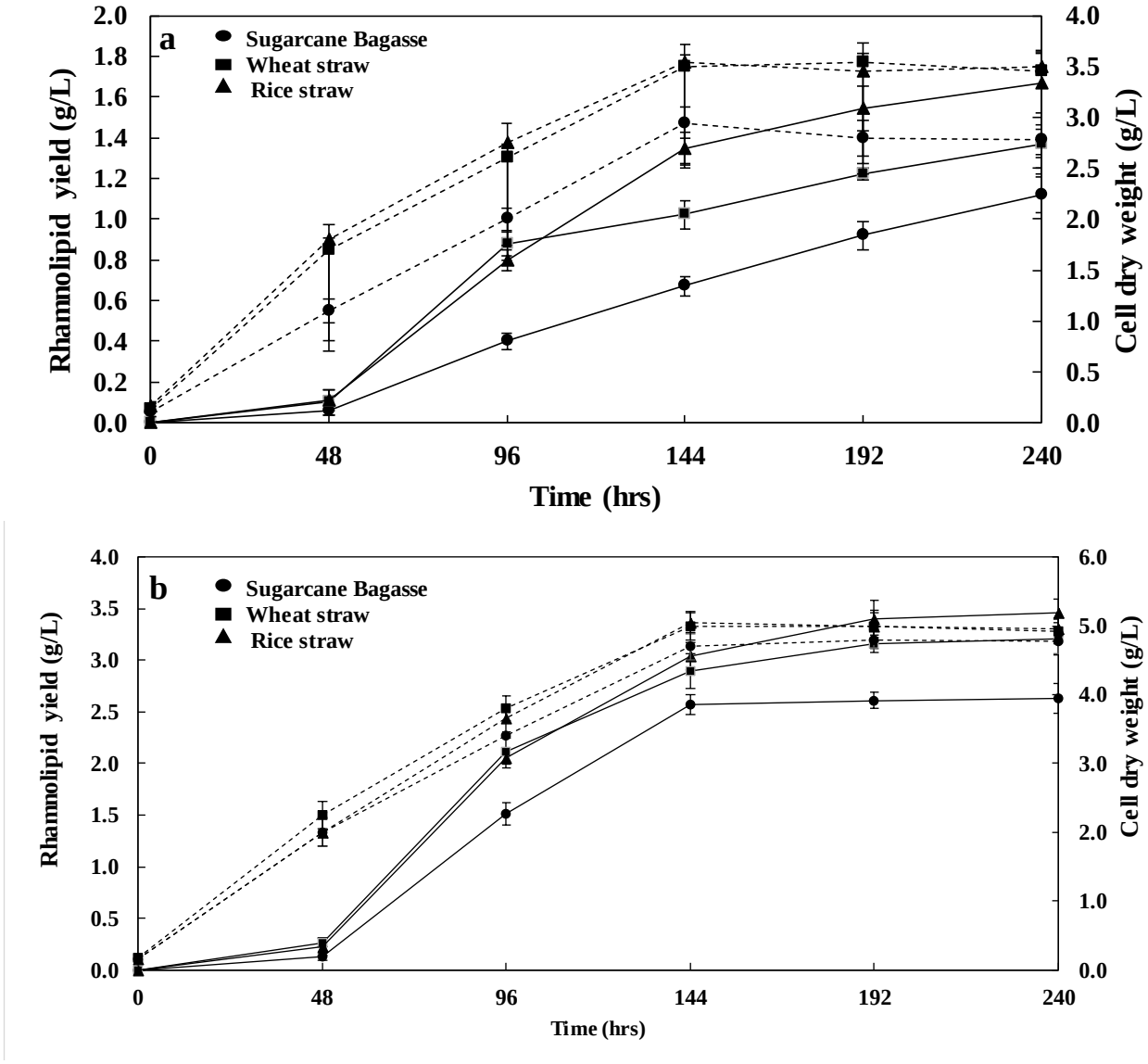
a
Sugarcane Bagasse
Wheat straw
Rice straw
Rhamnolipid yield (g/L)
Cell dry weight (g/L)
Time (hrs)
0
48
96
144
192
240
b
Sugarcane Bagasse
Wheat straw
Rice straw
Rhamnolipid yield (g/L)
Cell dry weight (g/L)
Time (hrs)

Fig. 6

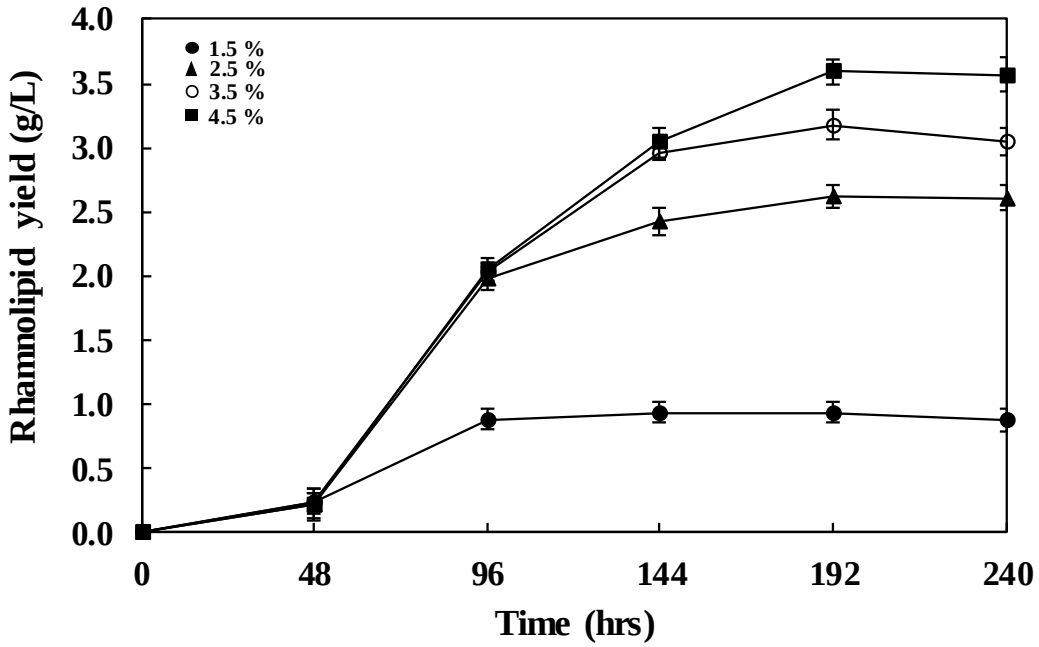
Rhamnolipid yield (g/L)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1.5 %
2.5 %
3.5 %
4.5 %
0
48
96
144
192
240
Time (hrs)

Table 1. Effect of different carbon sources on biosurfactant production from *Achromobacter* sp. (PS1) in MSM

(a). Hydrocarbon (1% *v/v*)

	Without Dextrose			With Dextrose (1% *w/v*)		
	ST (mN/m)	**RL (g/L)**	**CDW (g/L)**	**ST (mN/m)**	**RL (g/L)**	**CDW (g/L)**
Paraffin	53.85± 1.06	0.10 ± 0.03	1.70 ± 0.04	34.63 ± 0.31	0.20 ±0.03	3.15 ± 0.07
Hexadecane	46.51 ± 1.05	0.05 ± 0.02	0.85 ± 0.07	33.62 ± 0.24	0.13 ± 0.02	3.35 ±0.08
Diesel	47.58 ± 0.91	0.07 ± 0.02	1.30 ± 0.04	33.48 ± 0.82	0.34 ±0.06	3.40 ±0.07

(b). Vegetable oil (1% *v/v*)

	Without Dextrose			With Dextrose (1% *w/v*)		
	ST (mN/m)	**RL (g/L)**	**CDW (g/L)**	**ST (mN/m)**	**RL (g/L)**	**CDW (g/L)**
Palm oil	56.89 ±0.92	0.06 ± 0.02	2.05 ± 0.05	34.87 ± 1.02	0.18 ± 0.08	3.25 ± 0.09
Coconut oil	58.57 ± 0.84	0.08 ± 0.02	1.90 ± 0.03	34.33 ± 0.78	0.22 ± 0.06	3.39 ± 0.05
Sunflower oil	58.15 ±0.73	0.05 ± 0.02	1.35 ± 0.09	45.19 ± 0.69	0.19 ± 0.04	2.85 ± 0.09
Mustard oil	49.30 ± 0.98	0.04 ± 0.01	1.05 ± 0.04	35.45 ± 1.31	0.18 ± 0.07	2.65 ± 0.05

(c). Sugars (0.4g *w/v* equivalent carbon)

Monosaccharide				Disaccharide			
	ST (mN/m)	**RL (g/L)**	**CDW (g/L)**		**ST (mN/m)**	**RL (g/L)**	**CDW (g/L)**
Xylose	37.43 ±1.13	0.20 ±0.05	1.85 ± 0.07	**Lactose**	45.54 ± 1.15	0.11 ± 0.06	1.35 ± 0.04
Dextrose (control)	**31.06 ± 0.54**	**0.43 ±0.06**	**2.54 ± 0.08**	**Cellobiose**	42.77 ± 1.04	0.20± 0.05	2.23± 0.06
Fructose	36.43 ± 0.79	0.28 ± 0.04	1.37 ± 0.05	**Maltose**	51.05 ± 1.16	0.11 ± 0.06	0.93 ± 0.05
Galactose	44.01 ± 1.05	0.21± 0.05	1.25 ± 0.09	**Sucrose**	47.13 ± 1.05	0.18 ± 0.03	1.40 ± 0.04
Arabinose	38.02 ± 0.76	0.19 ± 0.03	1.37± 0.09				
Sugar alcohol				**Polysaccharide**			
	ST (mN/m)	**RL (g/L)**	**CDW (g/L)**		**ST (mN/m)**	**RL (g/L)**	**CDW (g/L)**
Glycerol	35.45 ± 1.12	0.19 ± 0.06	2.70 ± 0.05	**Cellulose**	53.87± 0.95	0.08± 0.04	1.30± 0.05
Mannitol	50.46 ± 1.13	0.14 ± 0.03	1.05 ± 0.04	**Starch**	53.81 ± 1.13	0.08± 0.02	1.20± 0.07
Sorbitol	50.37 ± 1.07	0.08 ± 0.07	1.10 ± 0.06				
Xylitol	51.18 ± 1.32	0.09± 0.03	0.95± 0.03				

ST= Surface tension, RL= Rhamnolipid yield, CDW=Cell dry weight

Table 2. ^{1}H and ^{13}C NMR spectroscopic data of glycolipids from fractions F_1 (mono-rhamnolipid) and F_2 (di-rhamnolipid) fractions produced from *Achromobacter* sp. (PS1)

	Carbon	F_1 (Mono-rhamnolipid) $\delta\ ^{1}H$	F_2 (Di-rhamnolipid) $\delta\ ^{1}H$	F_1 (Mono-rhamnolipid) $\delta\ ^{13}C$	F_2 (Di-rhamnolipid) $\delta\ ^{13}C$
Rhamnose-1	C-1′	4.89*	4.91*	96.9	94.18
	C-2′	3.86	3.71	71.29	79.71
	C-3′	3.73	3.69	71.45	70.41
	C-4′	3.40	3.61	73.71	70.45
	C-5′	3.80	3.93	71.66	70.30
	C-6′	1.28	1.28	17.30	17.39
Rhamnose-2	C-1″	-	4.90*	-	102.40
	C-2″	-	3.70	-	70.25
	C-3″	-	3.71	-	70.23
	C-4″	-	3.59	-	70.38
	C-5″	-	3.94	-	71.88
	C-6″	-	1.29	-	17.39
Lipid	C-1	4.17	4.16	72.74	70.21
	C-2	2.41	2.35	39.52	34.25
	C-3	5.48	-	-	-
	$-CH_3-$	0.88	0.87	13.94	14.12
	C-4	5.25	5.08	70.07	72.15
	C-5	2.54	2.35	39.01	36.19
	$-(CH_2)_n-$	1.25 - 1.36	1.28 - 1.33	22.50 - 32.5	22.40 - 34.70

* HSQC cross-peaks

Table 3. Results of biosurfactant production (8thday) from different lignocellulosic hydrolysates supplemented with influential nutrients

Ligno-cellulosic hydrolysate	Initial Surface Tension (mN/m) [a]	Final Surface Tension (mN/m) [b]	E_{24} (%)	Biosurfactant concentration [BS] (g/L)	Total sugar consumed [S] (g/L)	Cell dry wt. [CDW] (g/L)	$Y_{BS/CDW}$ (g/g)	$Y_{BS/S}$ (g/g)
RS	63.30 ± 0.18	31.12 ± 0.05	58.33 ± 1.96	3.55 ± 0.06	42.0	5.0 ± 0.11	0.71	0.08
WS	63.32 ± 0.22	31.16 ± 0.89	56.94 ± 2.02	3.16 ± 0.04	40.93	5.0 ± 0.16	0.65	0.08
SB	65.50 ± 0.52	33.24 ± 0.10	50.00 ± 0.97	2.61 ± 0.03	41.39	4.8 ± 0.14	0.54	0.06

[a] Initial Surface tension (0 day); [b] Final Surface tension (8th day); $Y_{BS/CDW}$ = Biosurfactant yield per gram of cell biomass; $Y_{BS/S}$ =Biosurfactant yield per gram of sugar consumed