Geobacillus 7349 – A new potential biosurfactant producing microorganism

Master thesis

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Popular Science Summary

Surfactant is widely used in many industrial fields such as petroleum, food, pharmaceuticals and so on as detergent, solubilizers or emulsifying agents. Currently, most of the surfactants are chemical petroleum based due to low cost but they are often toxic, irritant and non-biodegradable. Furthermore, petroleum for chemical synthesis of surfactant isn't renewable. People are trying to reduce their dependence on fossil fuels and move forward to a more renewable source. And biosurfactants which are produced by microorganisms as secondary metabolites, typically bacteria and yeast, become environmental friendly alternatives, as they are usually more biodegradable and less toxic than petroleum derived ones (Jyoti Sharma 2021). Biosurfactant can be divided into various categories. Among them, rhamnolipids are the most popularly researched one in recent years. However, production of rhamnolipids by microbial fermentation is usually very costly, due to the high substrate cost and high production cost. The carbon sources used for production are preferably cheap, such as glucose in our case. In addition, reducing the cost of downstream processing is another essential strategy to reduce the production cost. The extraction of rhamnolipids can make up 80% of the total production cost. The complexity of the extraction depends on the selected strain and the produced culture mixture and it shouldn't be over complicated.

There're quite a few strains producing rhamnolipids, nowadays. In this project, *Geobacillus* 7349 is the strain used to investigate the possibility of producing biosurfactant. *Geobacillus*, is gram-positive, rod shaped, aerobic species that is isolated from hot spring in Iceland. It can grow within a temperature range of 30 to 75 °C (Burgess, Flint et al. 2017). And it is naturally selected, without making any genetic modification. In this report, the cultivation of *Geobacillus* 7349 in a defined medium and the extraction and characterization of the product are the two main concerns. On one hand, it's important to define the culture condition of the strain. On the other hand, it's vital to extract and characterize our product, in order to know what is produced. The results can help with the further development of the production process of biosurfactant by *Geobacillus* 7349 in larger scale.

Abstract

The main aim of this master project is to find out whether strain *Geobacillus 7349* can produce biosurfactant during their growth by extraction and characterization of the product. The other goal is to optimize the cultivation condition of the strain. This work presents an evaluation of batch and fed-batch cultivation techniques for production of *Geobacillus 7349* in shake flask, using DRM medium. The growth condition of the strain was monitored by cell density (OD) measurement, cell dry weight measurement, along with glucose consumption characterized by HPAEC-PAD. Batch cultivation results in a high cell density ($OD_{600} \geq 5$) after around 14 hours of cultivation and a high concentration of biosurfactant being produced. To extract the biosurfactant from the culture broth, liquid-liquid extraction is an effective method. In both batch and fed-batch cultivations, the production of biosurfactant was detectable in both exponential and stationary phase, by the usage of high-performance liquid chromatography (HPLC), which means the cells were active in producing biosurfactant during these phases. And the fed-batch cultivation supplemented with fresh feed solution containing MgCl2, NH_4Cl, and NaCl can increase the cell density threshold and prolong the lifespan of cell generation. To conclude, the project demonstrates the production of biosurfactant from strain *Geobacillus 7349* by fermentation and the importance of the process design and nutrient source for bacterial growth. Hopefully, these findings can be used to scale up the fermentation process of *Geobacillus 7349* from lab scale to trial scale, in order to have a more substantial biosurfactant production.

Contents

Abbreviations

Defined medium – DRM medium

Biosurfactant - BS

Optical density - OD

Cell dry weight - CDW

High performance liquid chromatography – HPLC

High performance anion- exchange chromatography with pulsed amperometric detection - HPAEC-PAD

Solid phase extraction - SPE

1. Introduction

Biosurfactant, is an amphoteric compound containing hydrophilic and lipophilic groups produced by microbial metabolism (Leonie A. Sarubbo 2022). It can reduce the surface tension between two liquids, between a gas and a liquid, or interfacial tension between a liquid and a solid. Due to this property, biosurfactants are basically focused on the usage in oil recovery and bioremediation since 1980s. Although microbial based biosurfactant shows a similar and comparable surfactant's properties and capacities as chemical surfactant, they are too expensive to be used in industry, due to the high substrate cost and high production cost. In the late 1980s, the economic interest in such biosurfactant was mainly to produce unusual and valuable sugars for nutrition and pharmaceutical application (Linhardt et al., 1989). By using cheaper substrate such as renewable resources or organic waste materials rather than the relatively expensive pure hydrocarbons, the economic potential of biosurfactants has been boarded as detergent in food and cosmetic industries and bioremediation as well. Most studies on biosurfactant production were conducted on microorganisms which grow under mesophilic conditions. For instant, *Bacillus subtilis* can produce one of the most effective biosurfactant, that is surfactin, type of lipopeptides. However, some field applications of these biosurfactants require high tolerance for temperatures, pH and salts. Especially in oil reservoir where is a special ecosystem with high-temperature, high-salt, and high-pressure conditions. Lipopeptides with rheology and resistance to extreme environments have been widely used in the petroleum industry (Li et al. 2018).

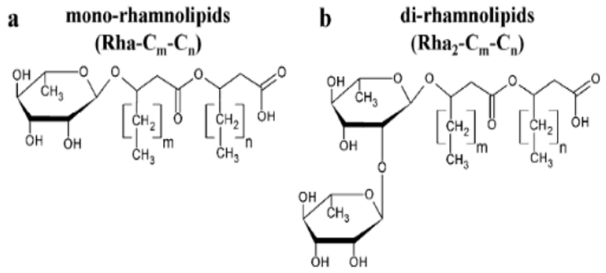
Biosurfactant can be categorized based on molecular weight and chemical structure (Drakontis 2020). The table below lists the different microbial biosurfactants based on structural classification. Glycolipids, lipopeptides, and phospholipids are low molecular weight compounds which can efficiently reduce surface and interfacial tension, called biosurfactants (BSs) (Drakontis 2020). High molecular weight compounds are complex mixtures of polysaccharides, lipopolysaccharides, lipoproteins and proteins, and complexes of biopolymers, called bio emulsifiers (Perfumo 2010).

Table 1. Structural classification of microbial biosurfactants

Class	Subclass	Microbial source
Glycolipids	Rhamnolipids	*Pseudomonas aeruginosa*
		Pseudomonas cepacia
		Lysinibacillus sphaerica
	Trehalose	*Rhodococcus sp.*
	lipids	*Nacordia farcinica*
	Sophorolipids	*Candida bombiocola*
		Candida magnolia
		Torulopsis petrophilum
		Torulopsis apiocola
Lipopeptides	Surfactin	*Bacillus subtillis*
		Kocuria marina
	Lichenysin	*Bacillus licheniformis*
Phospholipids		*Pseudomonas putida*
		Thiobacillus thiooxidans
Polymeric	Ruflsan	*Candida lipolytica*
	Emulsan	*Acinetobacter calcceticus*

Among them, glycolipids are the most worked out group. and rhamnolipids which belong to anionic glycolipid biosurfactants, are popularly researched in recent years. It consists of one or two fatty acid chains linked to one or two rhamnose molecules, those are mono-rhamnolipids and di-rhamnolipids (figure 1) respectively and are able to form highly stable emulsions of hydrophobic compounds in aqueous solutions (Abdel-Mawgoud, Lépine et al. 2010).

Figure 1. Generalized structure for mono-rhamnolipids and di-rhamnolipids (Markus Michael Müller 2012)



Rhamnolipids are always produced as mixtures of several congeners mainly from mono-and di-rhamnolipid species (Markus Michael Müller 2012). It was first produced and excreted to extracellular space by strains of P. aeruginosa in 1949 (Jarvis and Johnson 1949). Pseudomonas aeruginosa is the most studied and popular one, however it's a pathogenic bacterium (Jarvis and Johnson 1949). So the development of production in bacteria other than P. aeruginosa is also of great interest. In this project, *Geobacillus* 7349 was chosen, which can grow up to 75 degrees Celsius and has an optimal growing temperature of 55-65°C (Burgess, Flint et al. 2017) and is isolated in hot spring in Iceland. This strain belongs to genus *Geobacillus*, which consists of 20 species, of which 21 strains have been reported (Lin et al. 2019). *Geobacillus* is thermophilic, gram-positive, spore-forming, chemo-organotrophic, and can be isolated from various kinds of man made and natural thermophilic areas around the world. Thermophilic bacteria are fast growing and their molecules are thermostable over those from mesophilic or psychrophilic microorganisms. In 1920, *Geobacillus stearothermophilus* was the first species recognized under *Geobacillus,* and it has the ability of reducing nitrate to nitrogen gas (Burgess, Flint et al. 2017). Moreover, the aerobic and facultatively anaerobic *Geobacillus* species attracts lots of industrial interest as metabolic factories to produce thermophilic enzymes, biomass energy, and antibiotics. For instant, *Geobacillus thermoleovorans* JQ912239 was proved to produce bs which has a strong emulsion property and can reduce the surface tension. Facultative anaerobic bacterium *G. thermodenitrificans* NG80-2 proved to produce bio emulsifiers (Jailawi 2015). However, there is limited information on biosurfactants synthesized by *Geobacillus* species, with only four reports that *Geobacillus* sp can produce biosurfactants (Li et al. 2018). And the structural information of biosurfactants is limited too.

The main purpose of the present study is to evaluate whether screened bacteria produce BS and to characterize the BSs that might be produced. To analysis the metabolic activity and substrates consumption of the strain, HPAEC-PAD is applied. To study the growth kinetics of the cells, optical density and cell dry weight measurement are implemented. To extract and purify the product, different methods are used, including solid extraction and liquid-liquid extraction. Finally, to characterize the rhamnolipid production capabilities of this strain, HPLC analysis is performed.

Obtaining high yield of biosurfactant is challenging, as the microbial growth and metabolic activities are determined by various kinds of factors. Factors that need to be consider include but not limited to carbon and nitrogen source, the concentration of macronutrients and micronutrients, temperature, ph and inoculum

size (Twigg, Baccile et al. 2021). Despite defining the culture conditions of the selected strain, finding an ideal combination of substrates is another key issue.

Cultivation techniques for rhamnolipid production include batch and fed batch in shake flask in our case. Understanding the growth kinetics of the strain is accomplished by batch cultivation. And screening of the important substrates of the medium is performed by fed batch cultivation.

1.1 *Geobacillus* sp. bacterial growth

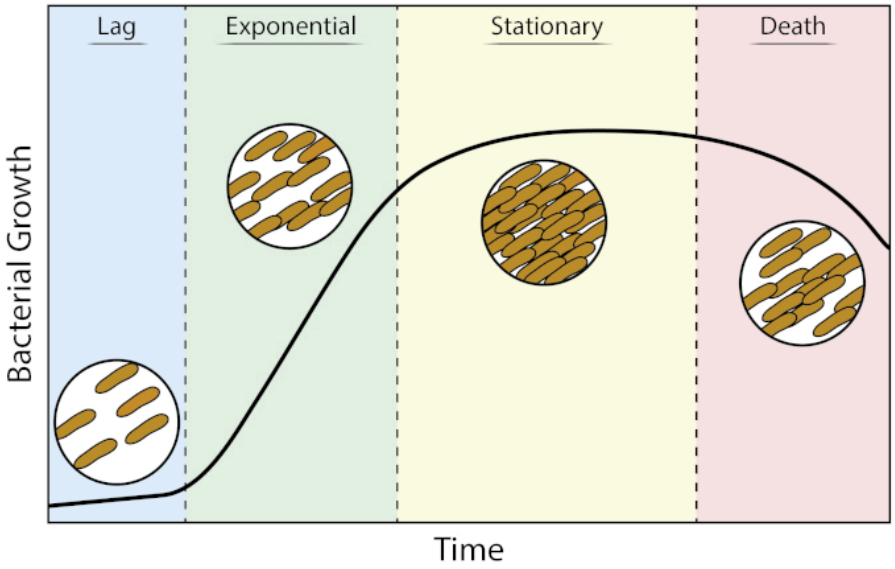


Figure 2. bacterial growth over time (Database 2023)

Bacterial growth generally follows a pattern that contains 4 phases, lag, exponential, stationary, and death (Database 2023). Lag phase is a period when bacteria adapt to the new environment and reach a state capable of rapid cell growth and division. The length of the lag phase can vary greatly, depended on the condition of the bacterial cells themselves and how different the growing condition are from the original bacteria's source. Damaged cells can have a long lag phase. As soon as the bacteria generates necessary component in lag phase, such as RNA, enzymes, and essential metabolites that might be missing from their new environment, it enters exponential or log phase. During exponential phase, cells grow and divide in a constant rapid rate. More in detail, cells doubling themselves by binary fission in which a single cell splits into two equally sized cells (Buchanan, 1918). As a result, 1 cell becomes 2 cells, becomes 4, becomes 8 and so on. However, the exponential phase isn't endless, it will reach a state where the cell growth is inhibited because of a limited growing condition. For example, some essential nutrients are exhausted or lacking space to grow or accumulation of toxic intermediates (Database 2023). In this step, the numbers of cells being produced is equal to the numbers of cells dying off (Buchanan, 1918). Therefore, the numbers of total cells remain stable and reach a stationary phase. The last phase is dead phase, where severe nutrients depletion leads to cell death (Database 2023). And it's considered that cells undergo irreparably damage under the culture condition in this stage since damaged cells fail to grow in a new fresh medium, according to JoVE science education database (Database 2023). It's better to transfer cells to new medium before stationary phase.

1.2 Optical density and absorbance

Optical density is a term that describes the ability of a material to transmit light. And absorbance is a measure of the capacity of a substance to absorb light of a specified wavelength. The difference is that optical density considers the absorption and scattering of light whereas absorbance considers only the absorption of light. And both of them are dimensionless. The use of optical density (OD) as an indication of biomass density is applicable to bacteria cultivation and has been used in laboratory scale to significant levels of success (Emeka G. Nwoba 2022). Biomass can be measured offline using OD measurements at various wavelengths. In our experiments, wavelength of 600 nanometer were applied and referred to as OD_{600}. The reason for that is because situated in the middle of the visible wavelength, the light at the wavelength of 600 nm is easy to produce and does ignorable damage to the microbials (Sutton 2006). OD_{600} values estimate of the concentration of the cells in the medium, which can be used to estimate the growth phase of the cell population and provide useful information for cell growth condition. In addition, OD_{600} measures how clear or blur a solution is in comparison to reference solution, 0.14% salt solution, a blur solution means high cell population, high light absorbance, high OD value.

2. Materials and methods

2.1. Materials

All materials and reagents were purchased from Sigma-Aldrich unless otherwise specified.

2.1.1 Bacterial strains

The strain Geobacillus 7349 obtained from the Matis culture collection (Iceland), was used in the present study.

2.2. Methods

2.2.1 Culture conditions

Geobacillus 7349 was inoculated directly from a stock culture (-80 ℃) into a sterile 250-mL baffled shake flask containing 25 mL DRM and incubated in a rotary shaker incubator at 60 ℃ and 200 rpm until OD_{600} nm ≥ 2 (17h). After preparation of DRM medium in a sterile 1L baffled shake flask, transfer 10 mL of the inoculum to a 1L baffled sake flask containing 90 mL of DRM medium (10% v/v). Details of the preparation and composition of the DRM medium can be seen in table 1. The sample (2 mL) is taken at 0 time in a sterile condition and then the culture was incubated at 60°C and 200 rpm.

2.2.2 Media preparation

The preparation of DRM medium is based on (Mukti, Sardari et al. 2022) First prepare stock solution A, B, C, D, E. The preparation condition for DRM with final concentration in the medium is shown below. If necessary, the pH was adjusted to 7.2 by NaOH (6 M) after addition of the remaining water.

Table 2. preparation of the stock solution and concentration of each solution (Mukti, Sardari et al. 2022)

Stock solution A	Concentration (g/L)
Nitriloacetic acid	10
$MgCl_2 \times 6H_2O$	10
NaCl	50
Stock solution B	Concentration (g/L)
$CaSO_4 \times 2H_2O$	2
Stock solution C	Concentration (g/L)
KH_2PO_4	27.22
Na_2HPO_4	28.39
Stock solution D	Concentration (g/L)
NH_4Cl	53.49
Stock solution E	Concentration (g/L)
glucose	100

DRM	Concentration (g/L)	volume of stock solution/ Liter DRM
Nitriloacetic acid	2	200 mL A solution
$MgCl_2 \times 6H_2O$	2	
NaCl	10	
$CaSO_4 \times 2H_2O$	0.4	200 mL B solution
KH_2PO_4	1.36	50 mL C solution
Na_2HPO_4	1.41	
NH_4Cl	0.53	10 mL D solution
glucose	5	50 mL E solution
trace element	/	5 mL
vitamin	/	5 mL

2.2.3 Cultivation condition

Development of production in *Geobacillus 7*349 can be divided into three experimental trials. The first trial was cultivating the strain in batch mode and taking samples at different time between 0 and 72 h. OD was measured during cultivation and cell dry weight was measured after that and then cell growth was plotted based on the measurement, from which we estimate the cell growth rate and condition for the subsequent cultivation.

During the second trials of batch cultivation, samples were taken at 0 h and every 2 h from 10 to 24 h, followed by samples taken at 36 and 44 h.

For the final trial, fed batch cultivation strategy was applied after batch cultivation for 15 hours, based on the result from previous cultivations. Samples were taken at 0 and 15 h of batch cultivation and then stepwise fed-batch was started and samples were taken after 0, 24 and 48 h of fed-batch cultivation.

The batch was started with (22.5 ml of the DRM medium + 2.5 mL of inoculum) 25 mL culture broth in each 15 shake flasks. The OD is measured at 0 h and at around 15 h, when the cells growth decreased and the OD was peak, before the cells reached stationary phase. Then the fed-batch mode was initiated by addition of the 15 mL feed solution. The screening of feed solution composition was performed by this trial. Therefore, the batch cultivation was done in 15 shake flasks, separately with the supplement of 0.4 mL of 500 g/L glucose, and the added feed solution varied from flask to flask, based on the screening strategy derived from design of experiment (DOE).

2.2.4 Optimization of cultivation process to achieve a high level of biosurfactant production

To adapt the DRM medium for reaching higher yield of biosurfactant, a screening strategy was applied by using design of experiment (DOE). Modde software is used to have the experimental design and fractional factorial design method is chosen to study the most important factors/ parameters that influence critical quality characteristics (Natoli 2018). It's a type of design that allows us to study the interaction between factors. Then we know the influences of macroelements concentration within the culture broth on biosurfactant production.

The addition of eight macro-elements ($CaSO_4$, $MgCl_2$, PO_4^{3-}, NH_4Cl, NaCl, vitamin solution and trace element) during fed batch cultivation were set at two levels, either 0 (no addition) or certain amount of addition. 15 experimental sets were designed and shown in the table below.

Table 3. lists of the 15 experiments with two levels of variables

Exp No	$MgCl_2$	$CaSO_4$	NH_4Cl	KH_2PO_4	Na_2HPO_4	NaCl	Trace element	Vitamin
1	2	0	0	1.36	0	10	0	5
2	0	0.4	0	1.36	0	0	5	5
3	2	0.4	0	0	0	10	5	0
4	0	0	0.53	1.36	0	10	5	0
5	2	0	0.53	0	0	0	5	5
6	0	0.4	0.53	0	0	10	0	5
7	2	0.4	0.53	1.36	0	0	0	0
8	0	0	0	0	1.41	10	5	5
9	2	0	0	1.36	1.41	0	5	0
10	0	0.4	0	1.36	1.41	10	0	0
11	2	0.4	0	0	1.41	0	0	5
12	0	0	0.53	1.36	1.41	0	0	5
13	2	0	0.53	0	1.41	10	0	0
14	0	0.4	0.53	0	1.41	0	5	0
15	2	0.4	0.53	1.36	1.41	10	5	5

2.2.5 Biosurfactant extraction

2.2.5.1 Liquid-liquid extraction

Cell-free culture broth from 2 flasks were treated with different liquid extraction processes, one with acidification of the broth before extraction whereas the other one without acidification, in order to investigate the effect of acidification and determine whether acidification step is needed for purification. For the sample needs to have acidification, the following steps were carried out:

1. Acidify the supernatant by adding 3M of hydrochloric acid until the pH reaches 2.
2. Store it overnight to allow the biosurfactants to precipitate.
3. Centrifuge the broth to separate the precipitate.
4. Add MQ water (5 mL) to the precipitate and vortex.
5. Neutralize the suspension by barium hydroxide (0.1 M) to solubilize the biosurfactants.
6. For both flasks, the sample is extracted by the addition of ethyl acetate (5mL) (equal volumetric to water), then shaking for 30 min in a shaker and keep the sample stationary for 1 h in a separation funnel. Separate the upper organic solvent phase from the aqueous phase (lower part).
7. Repeat the extraction process twice and combine the organic phase fractions, which would be evaporated by rotary evaporator later. The residue is dissolved in mixture of acetonitrile: water (70:30) (v/v), filtered and analyzed by HPLC or kept for further experiments.

Another extraction process was without acidification and is followed the step 6 in above section (2.2.5.1)

2.2.5.2 Solid phase extraction (SPE) anion exchange column

SPE cartridges with strong quaternary ammonium modified polymeric anion exchange material (Chromabond ® HR-XA) is used for the extraction. The cartridges were conditioned with methanol (100%) and then were equilibrated with water (pH 8-9). The cell-free culture broth is filtered (using a syringe filter, 0.2 µm filter) and then loaded onto the cartridges and extracted using gravity. Cartridges were washed with NaOH (0.1 M) followed by methanol (100%). Elution was performed using methanol containing formic acid (2%). The eluted sample was concentrated using rotary evaporator or a nitrogen concentrator. The concentrated sample was dissolved in mixture of acetonitrile: water (70:30) (v/v), filtered and analyzed by HPLC.

2.2.6 Analytical methods

2.2.6.1 Determination of CDW

2 mL of sample was taken at different cultivation time. Take 1mL of each 2mL- sample (with cells) is transferred to another Eppendorf tubes, then centrifuge at 13500 rpm for 5 minutes. Supernatants are transferred into another Eppendorf tubes for biosurfactant measurement. Remaining pellets cells in each Eppendorf tube were vortexed with 1mL of MQ water to wash cells. After that, the samples are centrifuged for 5 minutes again then discarded the water. Then, another 0.5 mL ultrapure water is added to each sample again then mixed and transferred the cells to the pre-heated and pre-weighted empty aluminium boat. Dry the sample in an oven at 70 °C for 24h. After that, the dried samples are taken out from oven and put in a desiccator to cool down and after 1 h the combinational weight of boat and cells is measured using analytical balance. Each boat should be taken out one by one from desiccator and measured fast.

2.2.6.2 Total biosurfactant measurement by turbidimetry method

Add one drop of 3 M HCl (200 µm pipette) to the prepared 1mL supernatants then mix it by vortex. Fridged it at 4°C overnight. Measure the absorbance at 600nm.

2.2.6.3 Liquid chromatography method for identification of rhamnolipids (HPLC-CAD)

Reversed-phase chromatography with a charged aerosol detector (CAD) (Thermo Scientific™ UltiMate™ 3000 UHPLC system by Thermo Fisher Scientific) on Acclaim™ VANQUISH™ C18 UHPLC (2.2 µm), 2.1 × 150 mm column (Thermo Scientific™) was used at the oven temperature of 40°C. The flow rate was 0.3 mL/min. Mobile phase gradient consisting of (A) 5 mM ammonium formate (at pH 3.3 adjusted by formic acid) containing 5% acetonitrile (v/v) and (B) Acetonitrile (100%) was used. The gradient was set to 50% (B) from 0 to 2 min, increased to 100% (B) from 2 to 50 min and remained constant for 5 min at 100% (B) and then it decreased to 50% (B) in 1 min and remained constant for equilibration. Total run was 60 min. The mixture of mono-rhamnolipids, 95% purity (Sigma), 1 g/L, was used as a standard.

2.2.6.4 HPAE-PAD

Determination of glucose concentration as a remaining substrate in the culture medium was done by filtering the cell-free culture medium through a 0.2 µm polypropylene filter into a plastic vial after proper dilution and analysis by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD, Thermo Fisher Scientific, Waltham, USA) using Dionex CarboPac PA-20 guard column coupled Dionex CarboPac PA-20 analytical column. As eluents ultrapure water, 2 mM NaOH, and 200 mM NaOH were used with three separate pumps. Separation occurred at 23 min of running time by 62.5% ultrapure water and 37.5% of 2 mM NaOH mixture with an isocratic flow of 0.5 mL/min. The column was reinforced each time with 200 mM NaOH for 2 min at 0.5 mL/min flow rate. ED40 electro-chemical detector was used to detect the glucose. HPAE-PAD stands for high performance anion exchange chromatography with pulsed amperometry detection. It allows direct quantification of non-derivatized carbohydrates at low- picomole levels with minimal sample preparation and cleanup. In our case, samples with cells were diluted 50 times in the first trial and 200 times in second and third trials to avoid exceeding the maximum glucose concentration limit of the assay. And 5, 10, 20 and 30 µg/mL pure glucose solution were prepared and served as standard for the detection. Then the glucose consumption of each sample in various feed solutions at different cultivation times were detected and quantified.

3. Result

3.1 Growth kinetics of *Geobacillus* in batch cultivation

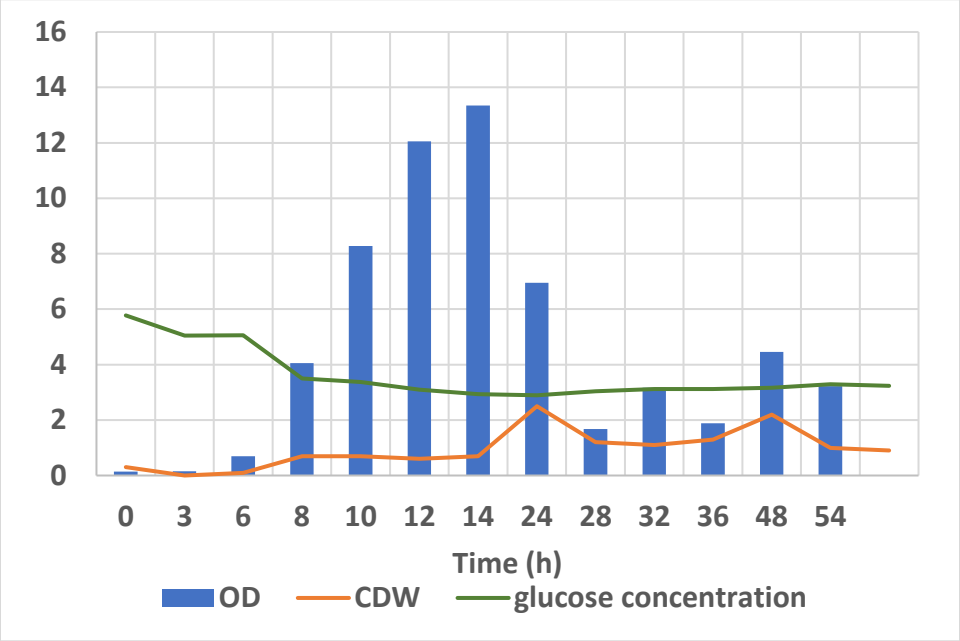


Figure 3. Growth of *Geobacillus* 7349 in DMB supplemented with 5 g/L glucose.

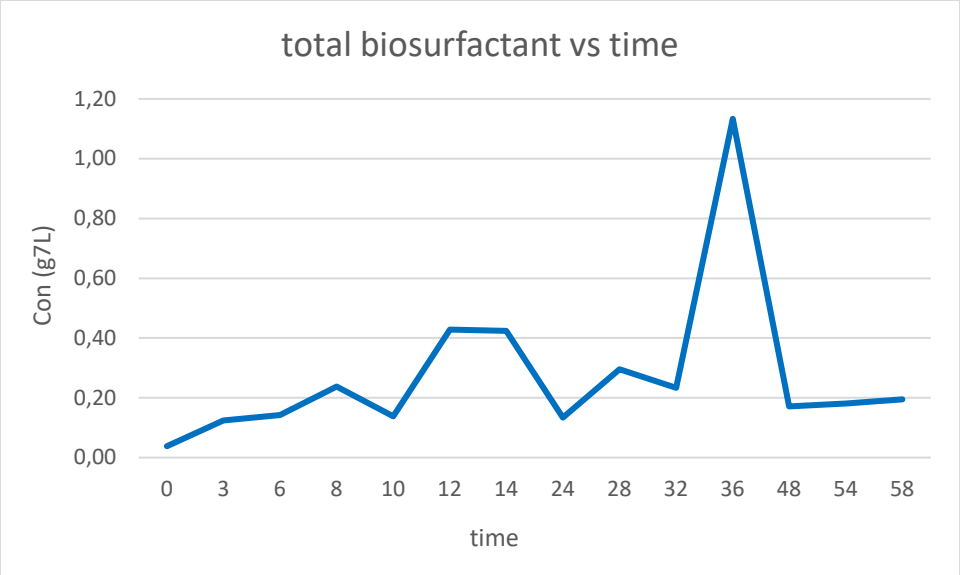


Figure 4. Total biosurfactant production over time

	biosurfutant													
time	0	3	6	8	10	12	14	24	28	32	36	48	54	58
OD	0,008	0,026	0,03	0,05	0,029	0,09	0,089	0,028	0,062	0,049	0,238	0,036	0,038	0,041
con (g/L)	0,04	0,12	0,14	0,24	0,14	0,43	0,42	0,13	0,30	0,23	1,13	0,17	0,18	0,20

Table 4. Total biosurfactant values in g/L over time

Figure 3 shows the optical density, and cell dry weight and glucose consumption of strain 7349 over time. In the beginning, the measured OD_{600} was close to zero (0), due to the low levels of bacteria in total, which was still in the lag phase. As the cultivation kept going, cells consumed glucose and continued reproducing in an exponential growth pattern. After this stage, the bacterial growth became stable then decreased. According to the curve, the OD_{600} was low in the first 6 hours and the consumption of glucose was still subtle, followed by a huge increase in cell mass and a significant decrease in glucose concentration and tremendous growth during 6 to 14 hours. The OD value peaked at 13.35 after 14 hours and declined after 24 hours of fermentation. On the other hand, the cell dry weight showed a similar but different pathway from the OD values, but one thing was obvious, that was the maximal cell mass we can get is at around 14 hours of cultivation time. Sample from cultivation at the 24^{th} hour contained the greatest number of cells, with 2.5 g/L of cells. The reason why CDW trendline behaves differently with OD trendline is caused by operating error. During sampling, even if the flask was shaken before sampling, the liquid medium cannot be homogenous thoroughly. Media with higher cell quality in the medium may still achieve lower CDW and OD values as cells aggregate at the bottom of the shake flask and only the supernatant fraction was taken. Another mistake may be caused by the improper operation during CDW measurement preparation. After the first centrifugation, supernatant was separated from cells pellet. During this process, some cells were likely to be removed with supernatant, resulting in a lower CDW in the end. Figure 2 quantify the amount of biosurfactant being produced over cultivation time. Similar to the OD measurement for biomass, the strain seems to be most active and produced most biosurfactant between 12 to14 hours cultivation. A big increase was observed at the time 36 hours which might be due to interfering some impurities in the supernatant such as exopolysaccharides that might be produced by the strain.

As no samples were taken between 14th to 24th hour in the first cultivation, the optimal timing of maximum biomass remains uncertain. So the same cultivation experiment was repeated in two flasks with different sampling times (Figure 5 and 6). However, due to some calculation mistake before the second trials, the

addition of each solution wasn't correct, resulting in a different concentration of each element in the first trial. The table below shows the deviation between the actual addition in the second trials with the correct amount.

Feed solution	The actual amount of addition (mL)	The correct amount should be added (mL)
Solution A	30	20
Solution B	30	20
Solution C	7.5	5
Solution D	1.5	1
Solution E	7.5	5
Trace element	0.75	0.5
vitamin	0.75	0.5
water	22	48

Table 5. the actual addition and the correct addition of each solution

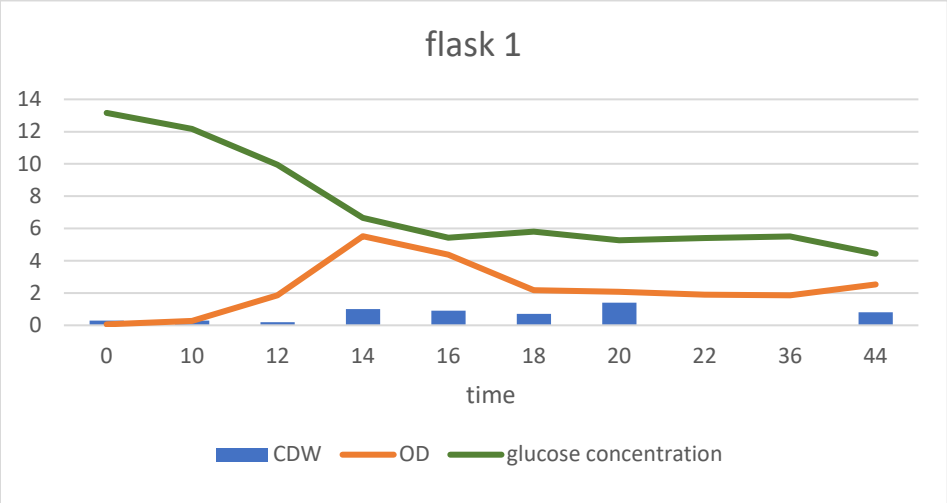


Figure 5. Optical density and cell dry weight of the strain 7349 over time at flask 1

time	0	10	12	14	16	18	20	22	36	44
CDW(g/L)	0,3	0,3	0,2	1	0,9	0,7	1,4			0,8
OD	0,06	0,28	1,86	5,52	4,38	2,18	2,08	1,9	1,86	2,54
glucose c (g/L)	13,16	12,16	9,95	6,66	5,42	5,80	5,27	5,41	5,51	4,43

Table 6. Growing values of *Geobacillus* 7349 in DMB

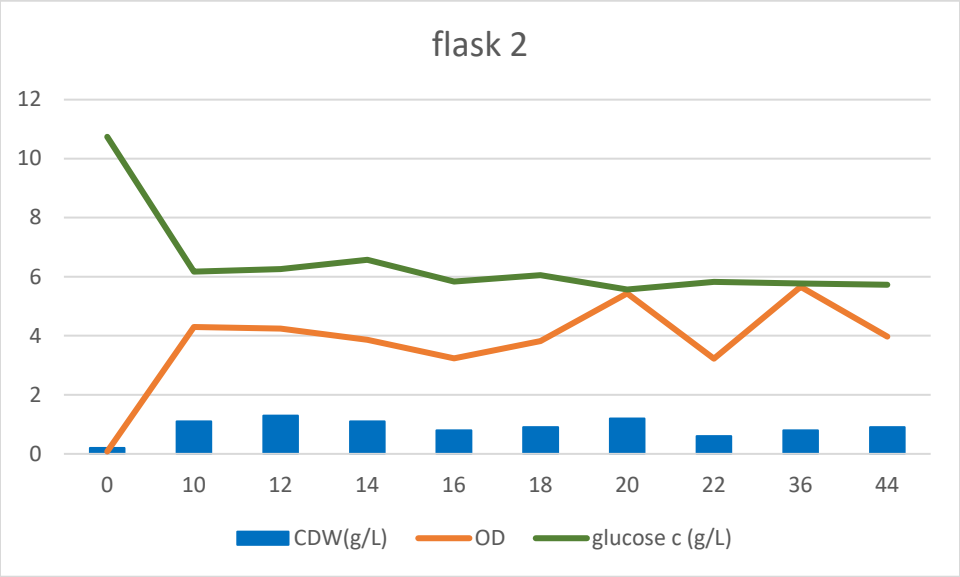


Figure 6. Optical density and cell dry weight of the strain 7349 over time at flask 21 and 2

time	0	10	12	14	16	18	20	22	36	44
CDW(g/L)	0,2	1,1	1,3	1,1	0,8	0,9	1,2	0,6	0,8	0,9
OD	0,08	4,3	4,24	3,86	3,24	3,82	5,44	3,22	5,66	3,97
glucose c (g/L)	10,74	6,18	6,26	6,58	5,84	6,05	5,57	5,83	5,77	5,73

Table 7. Growing values of *Geobacillus* 7349 in DRM

As a result, to the maximum OD_{600} in both flasks was around 5.5. These two trials cannot reach OD_{600} =13 similar to the first cultivation. And the reason for that may be caused by the high glucose concentration and other nutrients. When the substrate is high concentration, it has inhibitory effect for cells to grow. Another explanation is the individual difference among same strain. The individual difference also led to different lag phases of the bacteria. Strain in flask 1 has a longer lag phase, it grew followed by a lot in cell mass with dramatic growth until the huge consumption of glucose after14 hours. The CDW also showed a reasonable cell mass with 1 g/L after 14 hours of fermentation. For flask 2, after a fast cell reproduction process from 0 to 10 hours (OD of 4.3 and CDW of 1.1 g/L), both the OD and CDW were almost constant till 16 hours of cultivation and then went through a fluctuation till 44 hours.

Figure 7 showed the formation of biosurfactants by strain 7349 during 44 hours of cultivation.

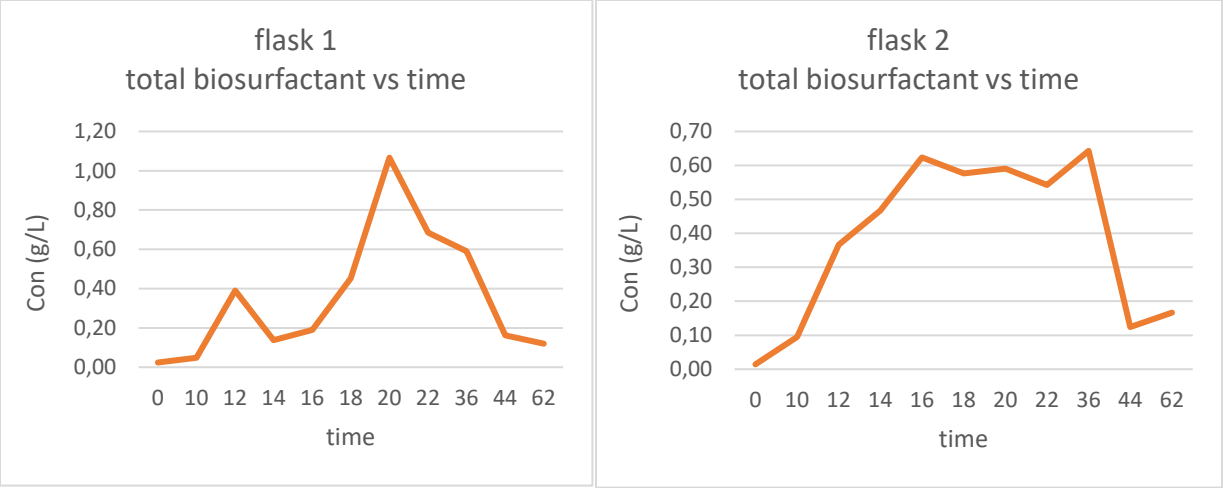


Figure 7. Total biosurfactant over time of flask 1 and 2

By considering both experimental results and different results of parameters, although each time the strain behaved differently, it still can conclude that around 14-16 hours of cultivation and not later than 16 hours, the strain 7349 has the highest cell growth and produce most biosurfactant. This conclusion can be used for the further third experiment.

Cultivation of strain 7349 in batch mode followed by stepwise fed-batch mode. The principle of applying fed batch is to prolong the bacteria growth period. Cells consumes nutrient to support their proliferation activity, and once all the nutrients are depleted, the cells will stop growing and die. With the extra fresh nutrients, the cells could theoretically continue reproducing.

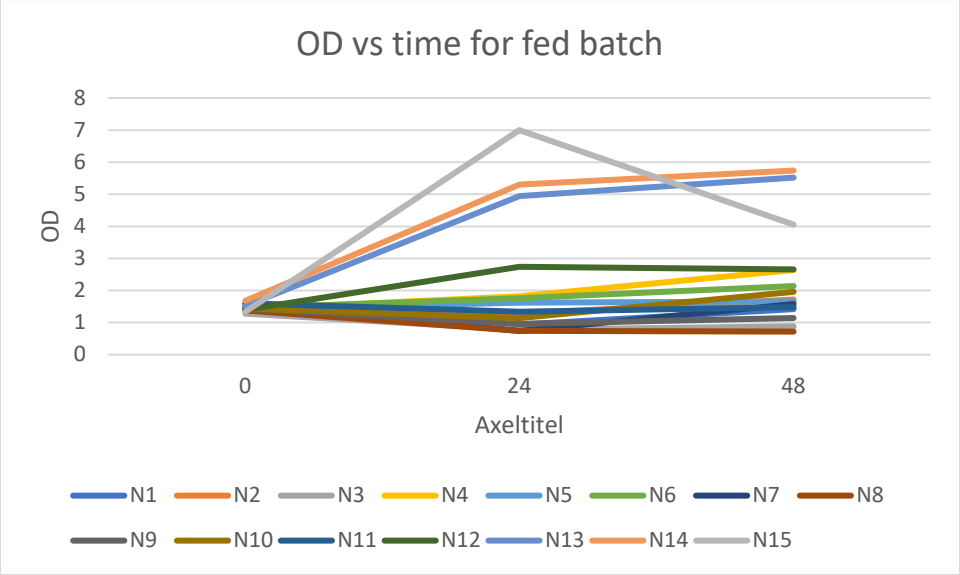


Figure 8. Optical density of the strain 7349 over time for fed batch

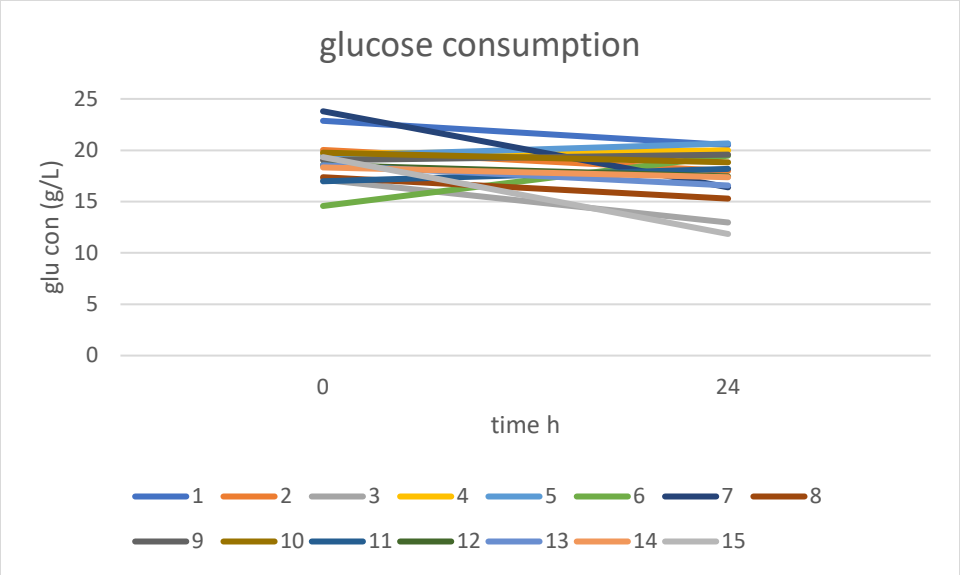


Figure 9. glucose consumption of the strain 7349 over time for fed batch

flask	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
0 h	22,87	20,03	17,12	19,65	19,44	14,57	23,79	17,37	19,03	19,76	16,98	18,56	18,55	18,33	19,33
24 h	20,51	17,99	12,96	19,99	20,66	19,48	16,39	15,29	19,57	18,82	18,18	17,45	16,57	17,37	11,84

Table 8. Glucose consumption in g/L of *Geobacillus* 7349 in DMB

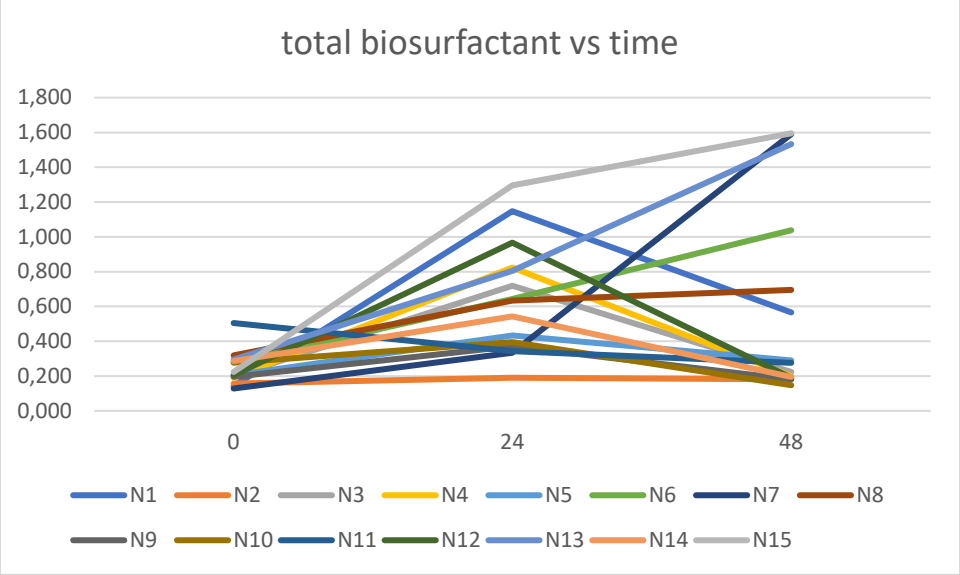


Figure 10. total biosurfactant production of the strain 7349 over time for fed batch

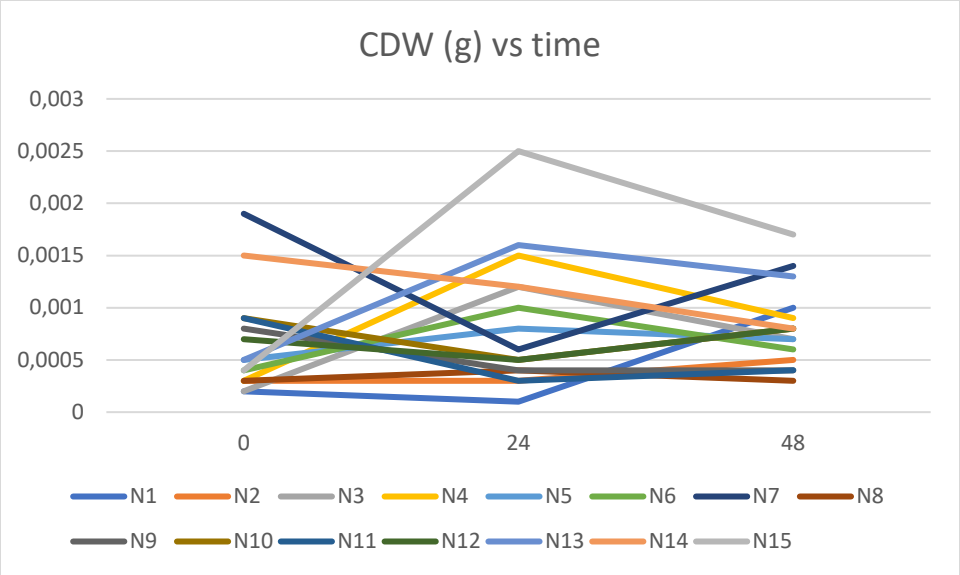


Figure 11. CDW of the strain 7349 over time for fed batch

The figures above show the optical density and biosurfactant production of the strain at fed batch cultivation. After cultivating the strains at batch model for 15 hours, based on the previous experiment, the culture media had an OD around 5, new feed solution was added according to the fractional factorial design generated by the MODDE software. As you can see, not all the flasks had an increase in cell mass and biosurfactant production. Among of all flasks, experiment no.13 and no. 15 experienced the most significant increase in both OD values (cell growth) and biosurfactant, while they had the most glucose consumption. The composition of feed solution in these two flasks can be seen in table 2. Both flasks have the addition of $MgCl_2$, NH_4Cl, Na_2HPO_4 and NaCl. Whether those elements are essential to and what's the role of other ingredient playing in the cell growth will be analyzed and explained in the following.

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3.2 Fractional factorial design

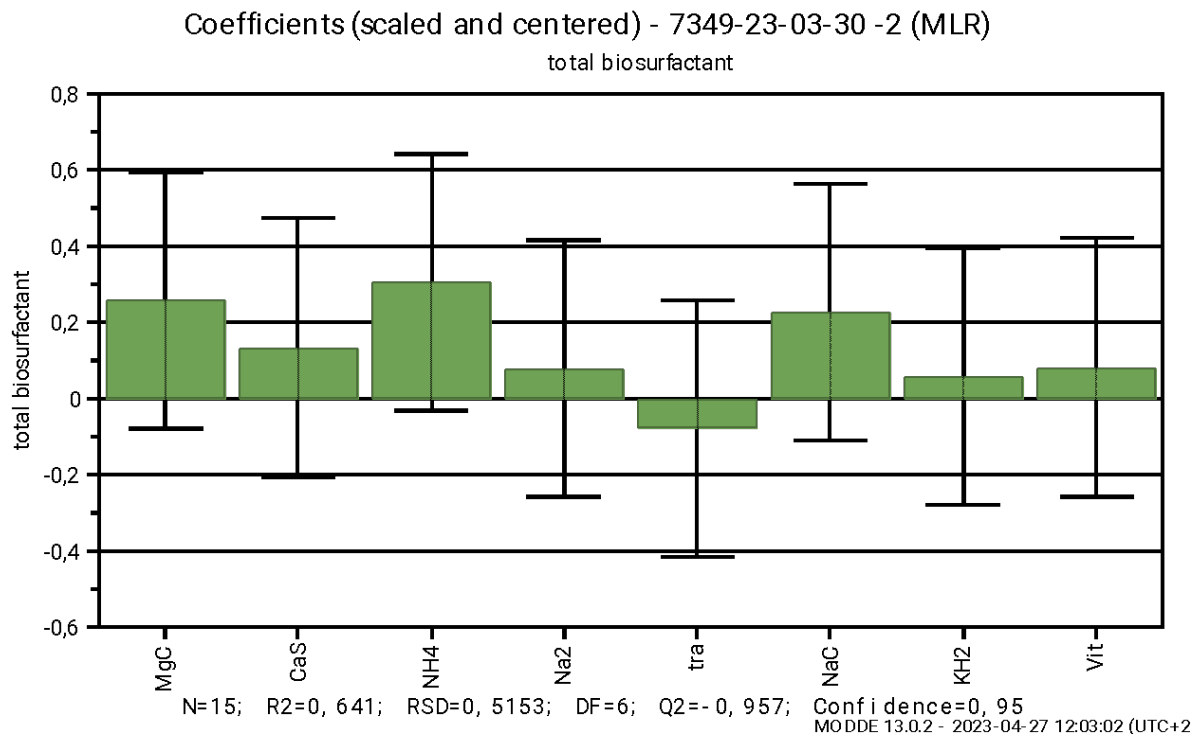


Figure 12. Coefficients of different compounds on total biosurfactant

The figure above quantifies the effect of each compound on the total biosurfactant yield, the coefficients reveal power of the effect. A positive number indicates that the ingredient has a positive effect on biosurfactant production and the negative number indicates a negative effect on the biosurfactant yield. The larger the absolute value of the coefficient, the greater the influence of the coefficient. It can be concluded that except for trace element, the addition of other ingredients during fed-batch all has positive influence of the cell growth. Among those 7 ingredients, $MgCl_2$, NH_4Cl (ammonium source) and NaCl (salt) are the most detrimental factors. We can estimate that magnesium, ammonium and salt are the 3 most important nutrient sources during strain *Geobacillus* 7349 cultivation, which can promote and prolong cell growth and metabolic activity, resulting of a greater amount of biosurfactant production.

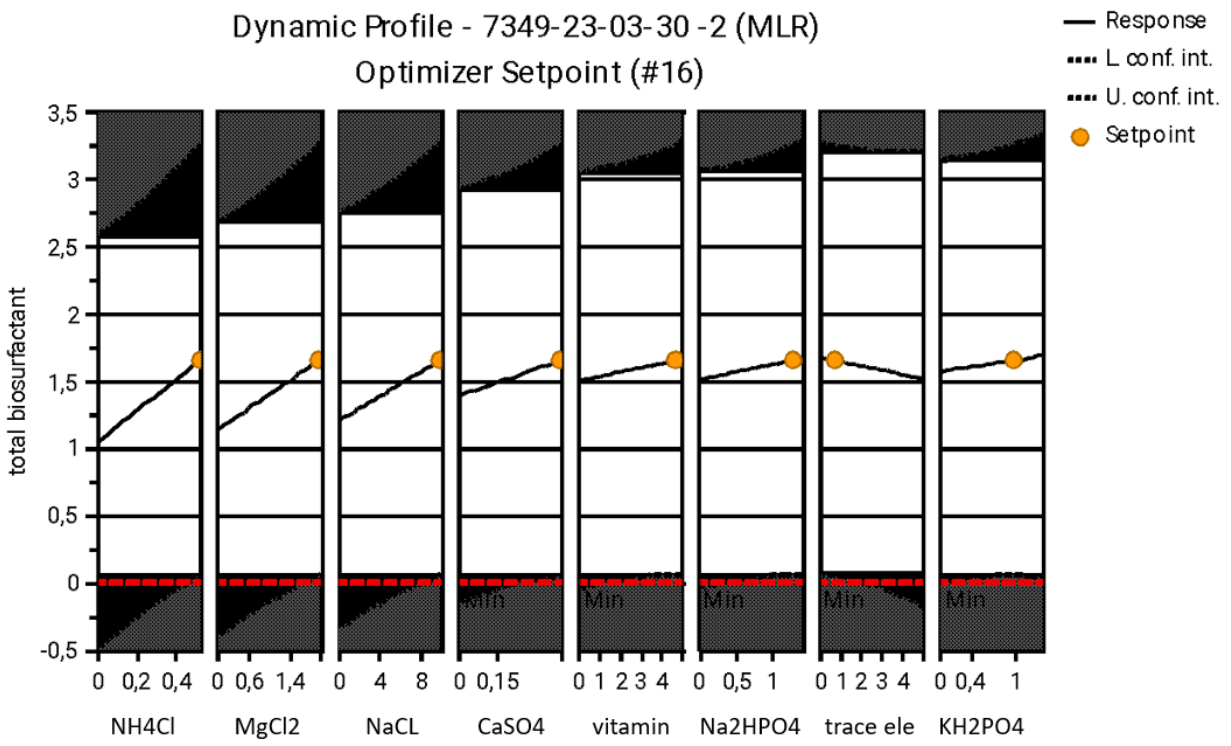
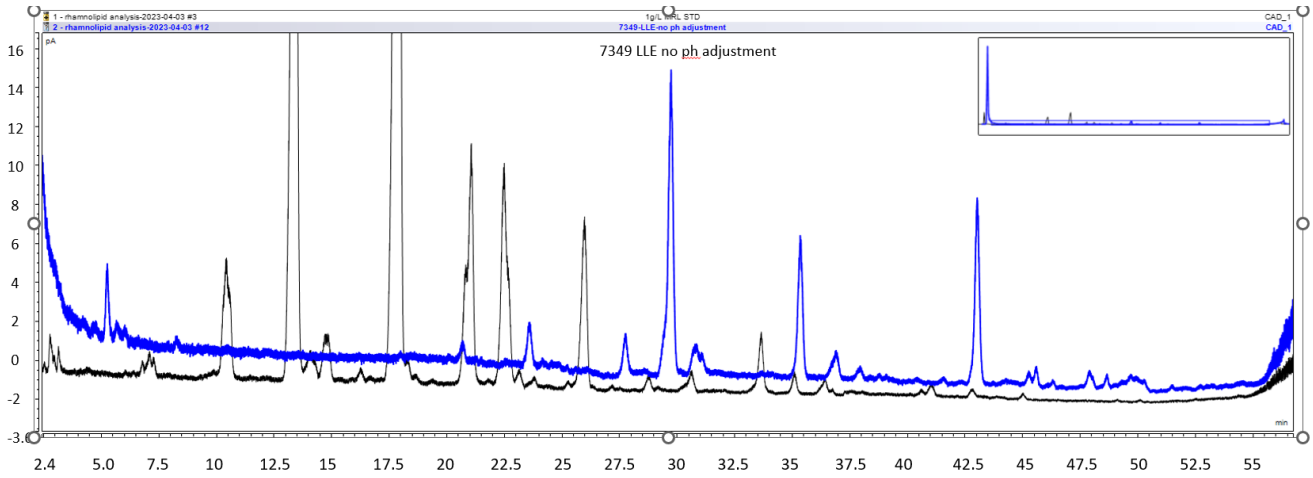
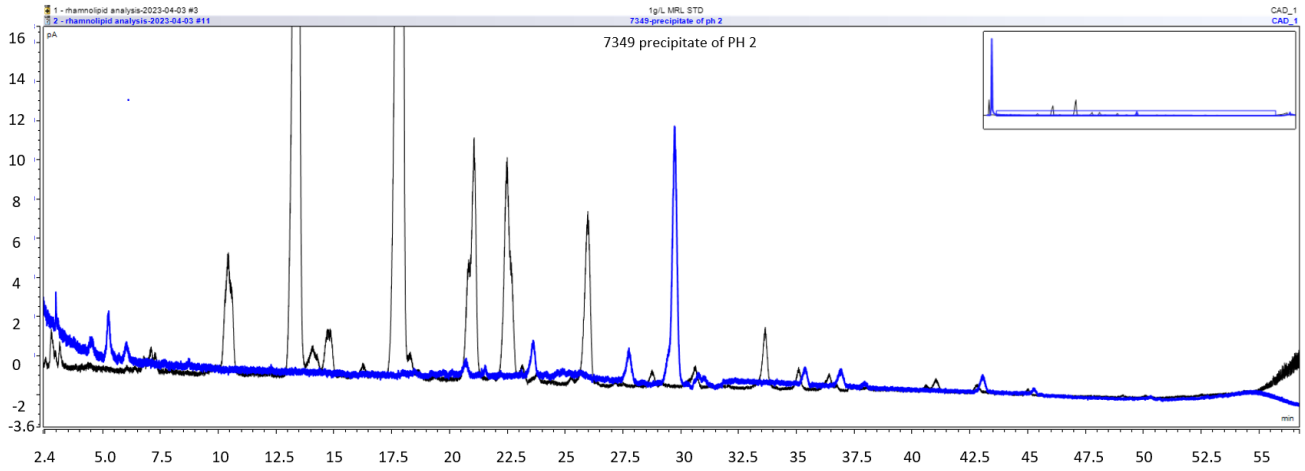


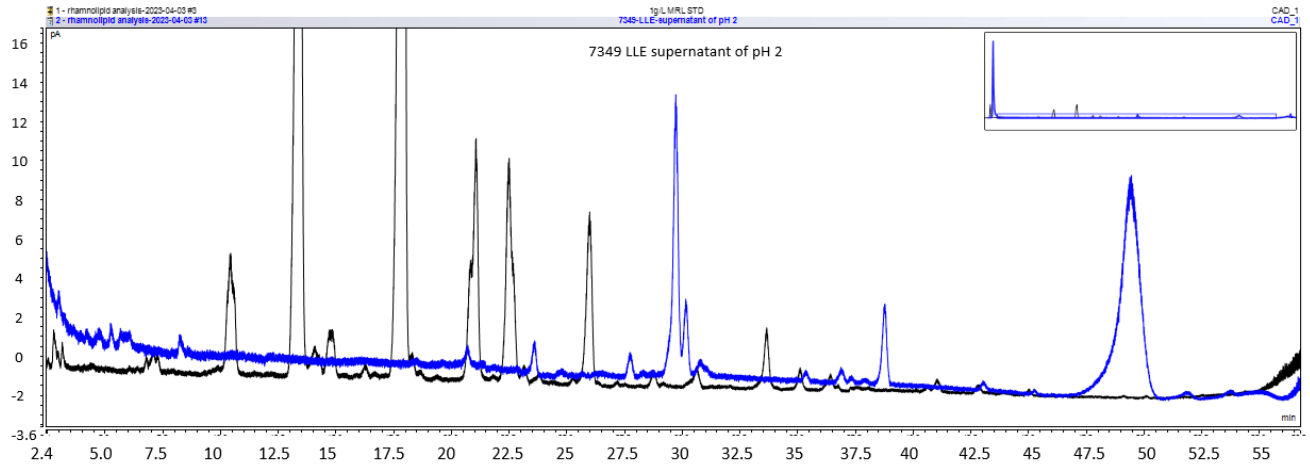
Figure 13. Dynamic profile of different compounds on total biosurfactant

Figure 13 here draws a dynamic profile of the total biosurfactant versus different concentrations of each compound and the corresponding biosurfactant yields at different concentrations are plotted. Figure 13 shows a similar result as Figure 12. Trace element is the only negative factor that inhibits the production of biosurfactant, based on the fact that it has a negative slope, which means a higher concentration of the trace elements leads to a lower yield. The slopes of the rest of all other compounds were all positive. The larger the slope, the greater the impact of the substance on the biosurfactant yield. Moreover, $MgCl_2$, NH_4Cl and NaCl are still the most powerful factors that affect the total biosurfactant. The addition of these 3 ingredients in a certain concentration all results in the highest biosurfactant yield according to the curve. However, not all the ingredients were added in a concentration that leads to a maximum biosurfactant production yield, for instance the 1.36 g/ L KH_2PO_4 in the medium. A greater amount of total biosurfactant can be achieved if more KH_2PO_4 was added based on the trendline. However, the concentration of each ingredient in the final total medium can't be changed (table X), otherwise the medium won't be in stable condition and precipitation will occur. Additionally, the aim of this fed batch test is to screen out the most influential ingredients within the solution instead of having an optimization of each ingredient.

3.3 HPLC


7349 LLE no ph adjustment
pA
min


7349 precipitate of PH 2
pA
min


7349 LLE supernatant of pH 2
pA
min

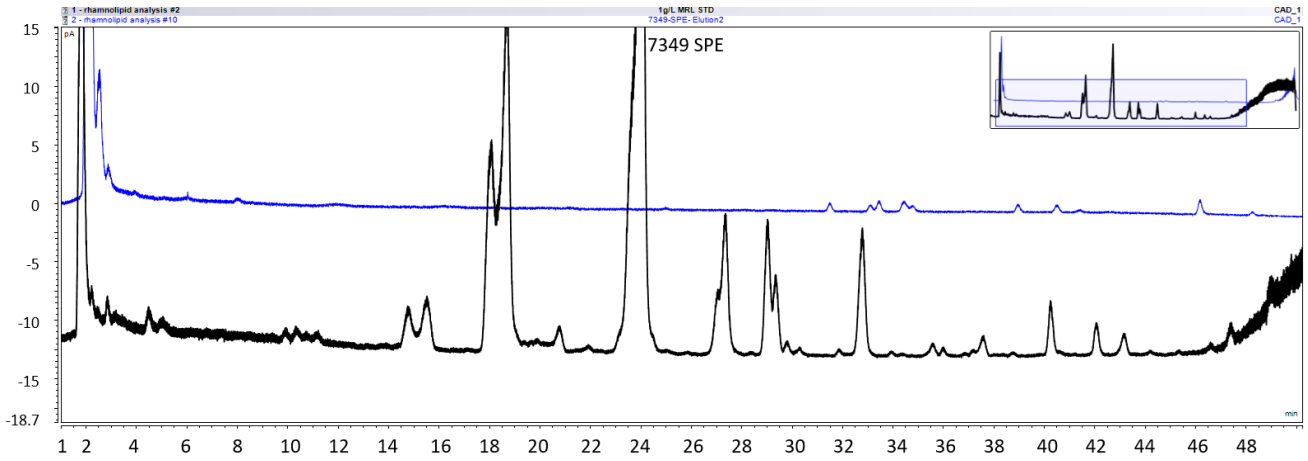


Figure 14. Hplc of different samples from liquid-liquid extraction

The blue lines are our sample and the black lines represent the standard solution. Rhamnolipid solution containing different types of rhamnolipids (mono and di-rhamnolipid) as mentioned in the theory section was used as standard to compare with our samples. In graph 8, the chromatograms of samples and standard were overlaid on top of each other on the same time scale. Injection volumes were the same in both runs. If chromatograms of sample show a peak at same retention time as standard solution, indicating that the sample contains rhamnolipid. The area under the peak is a measure of the concentration of the compound it represents.

As mentioned in the method section, samples were treated with different liquid-liquid extraction processes, one with acidification whereas the other one without acidification. The first graph is the one undergoes only acetyl acetate extraction, and second graph is the precipitated biosurfactant and the third one is the one undergoes both acidification and acetyl acetate extraction. For the one without acidification, both chromatograms show a peak at retention time 31, 35 and 43 minutes. And our sample shows a much larger rhamnolipid peak, which means our sample contains high concentration of certain types of rhamnolipid or biosurfactant. For the precipitated biosurfactant, it also shows peaks at time 35 and 43 minutes and contains some biosurfactant, but with much lower concentration compared with the first one. Even if it contains low concentration and purity of biosurfactant, it's still applicable in some cases, depending on the application and requirement of the company. Moreover, the precipitated biosurfactant can be sold as product in powder or granule form at a lower price because the production process doesn't have the acetyl acetate extraction step thus saving money. As for the supernatant after acidification, it has a similar profile as the other two chromatograms, except for a strong peak at retention time 31. To identify what type of rhamnolipid is and how is the chemical structure, further mass spectrometry can be applied. From this detection, we can have several conclusions. Firstly, the strains produce significant amounts of biosurfactant during fermentation. Secondly, liquid-liquid extraction is an effective method to extract biosurfactant from culture broth. Thirdly, acidification is not a crucial step within liquid-liquid extraction but acetyl acetate extraction is a critical step to apply in order to get high purity of biosurfactant. Last but not least, low purity of biosurfactant can also be sold as a product in a lower price, according to market demand and specific application.

Finally, the final graph plots the chromatograms of samples extracted by solid phase extraction. And there're few small peaks which indicates a low concentration. What can be concluded here is solid phase extraction is less effective than liquid- liquid extraction.

4. Conclusion and discussion

In this study, *Geobacillus* 7349 was cultivated in defined medium (DRM) with glucose as sole carbon source. And it succeeded in propagating cells and producing biosurfactant during exponential and stationary phases. The selected strain can reach OD_{600} at least 5.5 after 15 hours of cultivation, when cell yield reach the highest amount and biosurfactant is actively produced. However, due to some deviation of the substrates' concentration in first and second trials, it's better to perform more experiments to get more accurate data. Moreover, due to the fact that liquid medium cannot be homogenous thoroughly before sampling, the OD and CDW values fail to reflect the real cell growing conditions in the medium. Future cultivations can be performed in bioreactor where the culture medium will be in a more homogenous condition and the taken sample will be a more homogenous liquid. In addition, in order to find out the most influential factors in the medium, fractional factorial design strategy was applied. And fed batch cultivation with fresh extra $MgCl_2$, NH_4Cl and NaCl succeeded in prolonging the lifespan of the cells, resulting in a longer exponential and stationary phase to produce greater amount of biosurfactant. However, this fractional factorial design strategy doesn't show the full picture of all the combinations of different substrates. It's a reduced version that some substrate combinations were eliminated. Moreover, the experiment we performed doesn't mean to change the final concentration of each substrate (Table 2). If people want to further optimize the medium with different substrate in different concentrations, full factorial design strategies can be applied and more experiments should be done.

Liquid-liquid extraction is an effective method to obtain high purity of biosurfactant. And whether applying acidify the sample before liquid extraction depends on the application and requirements of the company. The acid precipitated biosurfactant can be sold directly as product in powder or granule form at a lower price.

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