**MINISTRY OF EDUCATION AND TRAINING**
**HANOI NATIONAL UNIVERSITY OF EDUCATION**
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# IMPROVING THE CONDITIONS FOR POLYHYDROXYALKANOATES PRODUCTION AND RECOVERY FROM BACTERIA ISOLATED IN VIETNAM

***Specialized Field*: Microbiology**
***Code*: 62.42.01.07**

# SUMMARY OF THESIS

**HANOI - 2017**

**The thesis was done at:**
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**The thesis was presented at Thesis Examination Board of Hanoi National University of Education at …../……./2017**

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# INTRODUCTION

## *1. Background*

Along with the development of the social, the demand for items made from plastic is also increasing day by day. For more than 50 years, from 1950 to 2012 the plastics production in the world increased rapidly from 1.5 million tones to 288 million tones and the annual everage increases of 3 % to 5 % in recent years. In Vietnam, the plastic production in the past ten years has also increased rapidly with the social demands. According to statistics from 2000 to 2010, the production of plastics has increased more than 4-folds (from 890 thousand tones to over 3.8 million tones) and also tends to increasing in the future.

The mainly source material for plastic industry now is petroleum oil that being depletetion. On the other hand, the majority of conventional plastic products derived from petroleum oil is almost non-biodegradable, so it takes hundred years to completely degradation all. Besides that, the decay process of these products in nature releases many toxic compounds. The accumulation of the plastic wates has been causing serious problems to the ecosystems in the Earth, that affect directly and indirectly on the survival of many species including human.

Bioplastics are recognized as alternative materials in oder to solve the environmental problems caused by petroleum plastic products. Among them, polyhydroxyalkanoates (PHA) emerged as one of potential material group by their significant features such as: (1) similarity in physic-chemical properties of petroleum plastics, (2) high biocompatible, and (3) biodegradable. Therefore PHA has been applied in different fields in social life.

In Vietnam market, some kinds of degradable plastic products were produced and traded. Most of them were made from petroleum plastics with additive component inside for breaking down at a certain time when exposure on light, temperature or oxygen. Besides, a number of biodegradable plastics were also interested, but most of them were made from plant materials such starch, cellulose. Meanwhile researchs on PHA are very limited.

Vietnam is located in the ecological diverse area with abundance of microorganisms. Our surveys on microbacteria in soil of some Nothern regions, especially the mangrove soil ecosystems, showed the richness and great potential of bacteria in many fields. According Wu et al. (2000), about 30 % of bacteria in soil are capable on PHA biosynthesis. There however have not been any studies on PHA production by microorganisms

from mangroves in northern Vietnam. For these above reasons, we conduct the project: ***"Improving the conditions for polyhydroxyalkanoates production and recovery from bacteria isolated in Vietnam".***

***2. Aims***

Determination of the optimal conditions for PHA production and recovery at laboratory scale using bacteria isolated from soil samples cellected in Vietnam.

***3. Objectives and study areas***

Microorganisms: Polyhydroxyalkanoates producing bacteria isolated from collected soil samples in Vietnam.

Objectives:

+ Isolation, screening of PHA producing bacteria.
+ Determination of the conditions for PHA accumulation and recovery.
+ Determination of the degradable capacity of PHA material in various conditions.

***4. Contents***

- Isolation, screening and classification of PHA producing bacteria.
- Improving the fermentation conditions for PHA production by selected bacteria.
- Developing methods for PHA extraction and purification from selected strain.
- Study on the biodegradation of PHA at various conditions.

***5. New contributions of the thesis***

- The first report on PHA production of a halophilic bacterium isolated from soil in Vietnam.
- The obtained results support more data on PHA producing bacteria from soil in Vietnam. Especially this is the first report on PHA producing bacterium belong to *Yangia* genus in the World.

# CHAPTER 1. OVERVIEW

## *1.1. Overview of plastics*

### *1.1.1. Synthetic plastics*

Production and market of plastics. Effect of synthetic plastics to environment on the Earth.

### *1.1.2. Bioplastic*

1.1.2.1. Bioplastic and their development trend

Summary on bioplastics: production, potential development

*1.1.2.2. Bioplastic and their environmental protection*

Summary on roles of bioplastic to environment on the Earth.

1.1.2.3. Biodegradable plastic

Summary on biodegradable plastic and their roles

***1.2. Polyhydroxyalkanoates (PHA)***

***1.2.1. Chemical structure and characterization of PHA granular***

Overview on chemical structure, characterization of PHA granular, and PHA accumulation conditions.

***1.2.2. PHA types from bacteria***

Classification of PHA

***1.2.3. Physico-chemical properties of PHA***

Describe PHA physico-chemical properties and comparing to petroleum polymers.

***1.3. Bacteria and PHA biosynthesis pathways***

***1.3.1. PHA producing bacteria***

1.3.1.1. Diversity of PHA producing bacteria species

Overview on diversity of PHA producing bacteria genus in nature.

1.3.1.2. Structure of operon pha in bacteria

Overview on diversity of *pha* operon in bacteria.

***1.3.2. Carbon sources and PHA biosynthesis pathways***

Relationship of carbon sources and PHA biosynthesis pathways in bacteria.

**1.4. Bacterial PHA production**

***1.4.1. Fermentation***

1.4.1.1. Batch fermentation

Batch fermentation - a convenient technique for studying on PHA biosynthesis.

1.4.1.2. Fed batch fermentation

Fed batch fermentation is popularly used in PHA research and production to day. Fed batch fermentation techniques could be modified depend on kind of PHA producing bacteria.

1.4.1.3. Continuous fermentation

In theory, continuous fermentation is not suitable for PHA production. Some advanced techniques are developed to improve PHA yield and productivity of fermentation.

***1.4.2. PHA extraction and recovery methods***

1.4.2.1. PHA extraction and recovery by using solvents

Summary on principles, advantages and disadvantages of this methods.

1.4.2.2. PHA extraction and recovery by digestion method

Summary on principles, advantages and disadvantages of this methods.

***1.5. Overview on study, production and application of PHA***

***1.5.1. In the world***

Summary on achievements and developments of PHA field in the world.

***1.5.2. In Vietnam***

Summary on researchs, production and application of bioplastics and PHA products in Vietnam.

## CHAPTER 2. MATERIALS AND METHODS

***2.1. Materials and mediums***

***2.1.1. Bacteria***

The bacteria were isolated from mangrove soil samples collected from Giaothuy – Namdinh, Yenhung – Quangninh; and from noodle production village Machtrang – Donganh – Hanoi.

***2.1.2. Chemicals and medium***

All chemicals are analytical grade

**2.1.3. Equipments**

The equipments used in this study belong to the following labs: Biotechnological and Microbiological Lab (Hanoi National University of Education, Vietnam), Institute of Microbiology and Biotechnology (National University, Hanoi, Vietnam), Biomaterial Lab (Biological School, Sains Universiti Malaysia, Malaysia).

***2.2. Methods***

***2.2.1. Microbiological methods***

2.2.1.1. Isolation method

Soil samples were diluted and spread on nutrient agar medium. After 48 hrs of cultivation, separated colonies were transferred to fresh medium.

2.2.1.2. Strains maintenance methods

The bacterial strains were stored by different methods such as glycerol preservation and lyophilization.

2.2.1.3. PHA producing bacteria screening methods

Screening method for PHA producing bacteria was done as described by Spiekermann et al. (1999).

2.2.1.4. Nutrient effect on growth and PHA accumulation

Bacterial strain was cultured on accumulation medium with required component changing for each experiment (carbon source, precursor, nitrogen source). Determination of cell dried weight (CDW) and PHA

content (%wt) were done after 48 hrs of cultivation.

2.2.1.5. Determination of physico-chemical characterizations

Bacterium strain was cultured on the specific medium. The extracellular enzymes were tested by using staining method.

Indole test was conducted on Tryptone water medium. After 48 hrs cultivation at 37 $^{o}$C, 1ml of ether was added to delaminate the indole. Then drop test reagent, standby and record the result.

Red methyl test was conducted on glucose – phosphate medium. After 48 hrs of cultivation, red methyl reagent was added in to culture fluid and read the result.

Voges-Proskauer test was conducted on Clarks-Lubs medium. After 48 hrs of cultivation, test reagent was added to culture fluid and the result was read immediately.

Carbon source utilization was carried out on mineral medium with different carbon source. After 48 hrs, evaluate the carbon utilization based the bacterial growth.

2.2.1.6. Observation methods for cell morphology and depot substances

*Colonial morphology:* The colonies after 24 hrs of cultivation were observed on microscopy SZ61 (Olympus, Japan) at 40x of magnification.

*Gram staining and observation method on microscopy*: Gram staining was carried out as described in Gram color Kit guider (SinhViet Co., Vietnam) and observed on microscopy at 1000x of magnification.

*PHA morphology on electron microscopy*: The bacterium was cultured on accumulation medium. The cell mass was collected and observed on scaning electron microscopy (SEM) and transmission electron microscopy (TEM) at National Institute of Hygiene and Epidemiology.

***2.2.2. Molecular techniques***

2.2.2.1. DNA extraction method

Genome DNA extraction was carried out as described in K3032 AccuPrep® Genomic DNA Extraction guider (Bioneer, Korea).

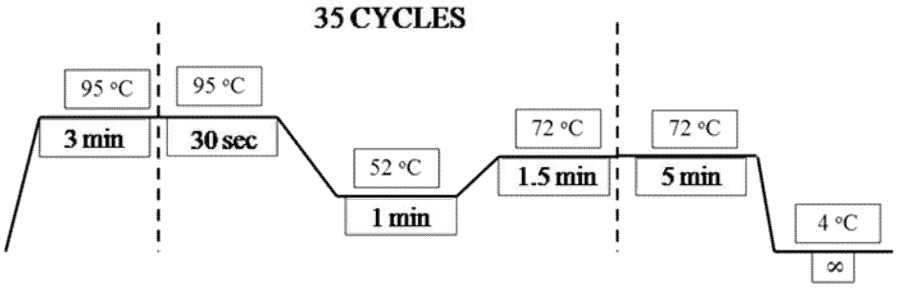
2.2.2.2. PCR

The 16S rDNA gene fragment was amplified by PCR technique base on two pair primers with following sequence:

314F (5′-CCTACGGGAGGCAGCAG-3′)
907R (5′-CCGTCAATTCCTTTGAGTTT-3′)
and: 27F (5′-AGAGTTTGATCCTGGCTCAG-3′)
1492R (5′-GGTTACCTTGTTACGACTT-3′)

The reaction mixture for PCR was prepared as described in AccuPower® PCR Premix Kit guider (Bioneer, Korea). Thermal cylcle is

performed as follows:




2.2.2.3. Sequencing and phylogenetics tree construction

The 16S rDNA samples were purified and sequenced at Bioneer company (Korea). The 16S rDNA sequences were compared to GenBank database and RDP database (Ribosomal Database Project) to determine the degree of closeness each others. The phylogenetic relationships were analyzed by using Mega5 software (Tamura et al., 2011) and neighbor-joining distance correction methods (Saitou et al., 1987).

***2.2.3. Method to study fermentation on bioreactor***

2.2.3.1. Batch fermentation method

Batch fermentation was carried out on MDL-100 (Marubishi, Japan) at 3 liters with 10 % of inoculum. The fermentation was set at 32 $^{o}$C and pH = 7.0 were adjusted automatically. Initial fructose concentration was set at 25 g/L. Sample was collected interval 3 hrs.

2.2.3.2. Fed batch fermentation method

The initial parameters were set as batch fermentation. Rich nitrogen solution was fed at first 18 hrs of cultivation. Free nitrogen solution was fed to fermentor from 18 hrs to 33 hrs of cultivation. The carbon concentration was maintained by testing and supplement interval 1.5 hrs depend on strategy of fermentation.

2.2.3.3. Two-phase fed batch fermentation method

The bacterium was cultured in two independent phase. The first phase, bacterium was cultured in rich medium in 24 hrs. The second phase, all the cell mass was washed and transferred to excessive carbon but free nitrogen medium. And then the cultivation was carried out as fed batch process.

***2.2.4. Methods for extraction and recovery***

2.2.4.1. Using chloroform method

*PHA extraction and recovery was performed as described in reports of Lau et al. (2010) and Wong et al. (2012).*

2.2.4.2. Using NaOH treatment method

The cell suspension and NaOH solution were mixed together in a

ratio of 1: 1 (v/v) to obtain cell and NaOH concentrations needed. The slurry was incubated at different temperature conditions and different time intervals. After that, samples were centrifuged, washed and lyophilized to determined the PHA purity and recovered yield.

***2.2.5. Analytical methods***

2.2.5.1. Method for carbon quantification

Carbon concentration in medium was determined according reducing sugar quantitative method using dinitrosalysilic acid (DNS) as described by Miller et al. (1959).

2.2.5.2. CDW determination

CDW was determined as described by Quillaguamán et al. (2008).

2.2.5.3. PHA content quantification method

The samples were prepared by methanolysis method (Braunegg et al., 1978). PHA content was determined by gas chromatography method as described Silva et al. (2000) on HP-7890A system (Agilent Technologies, USA) equipped HP-5 capillary column with P(3HB-co-12 mol%3HV) was used as PHA standard.

2.2.5.4. NMR analysis

PHA samples were dissolved in $CDCl_3$ before analyzed $^1$H-NMR at 500 MHz on Brucker ARX500 Spectrometer (Brucker, Sikertrifen, Gemany) at ambient temperature. PHA structure was determined through spectrum by using Brucker UXNMR software.

2.2.5.5. Physico-chemical properties determination

Mechanical properties of PHA material were performed on Shimadzu EZTest Power analyzer (Japan).

Thermal properties of PHA material were determined on Differential Scaning Calorimetry DSC60A (Shimadzu, Japan).

The $M_w$, $M_n$, DPI values were determined on 1200 GPC system (Agilent, USA) according method described Bhubalan et al. (2008).

***2.2.6. Method for PHA biodegradable determination***

2.2.6.1. PHA casting film method

*PHA film samples were prepared as method described by Sriwedi et al. (2006).*

2.2.6.2. Practical model

PHA films were tested for biodegradation in buried conditions. The lost weight value, color and morphology of PHA films were determine interval for 4 weeks experiment.

***2.2.7. Mathematical analysis***

# CHAPTER 3. RESULTS AND DISCUSSION

## *3.1. Isolation and screening of PHA producing bacteria*

### *3.1.1. Results of isolation and screening*

From the collected soil samples, 869 bacterial strains were isolated. Among them, 50 strains (6 % total isolated strain) exhibited PHA accumulation on selected medium. Of these, 17 strains are from Yenhung mangrove (5.67 % of total isolated strain at this area) (QN71, QN83, QN84, QN102, QN124, QN126, QN131, QN142, QN187, QN194, QN225, QN227, QN232, QN262, QN265, QN271, QN281), 13 strains are from Giaothuy mangrove (3 % of total isolated strain at this area) (NĐ97, NĐ145, NĐ153, NĐ156, NĐ199, NĐ201, NĐ203, NĐ210, NĐ218, NĐ240, NĐ325, NĐ389, NĐ355), and 20 strain s are from Machtrang (13.9 % of total isolated strain at this area) (MT15, MT21, MT29, MT33, MT56, MT65, MT70, MT71, MT72, MT84, MT85, MT88, MT90, MT97, MT112, MT123, MT129, MT132, MT133, MT144).

### *3.1.2. Growth and PHA accumulation of screened bacteria*

Among 50 PHA producing strains, 10 strains were selected. All bacteria showed good growth and PHA accumulation when cultured on glucose as carbon source. 6 of these strains accumulate P(3HB), while 4 others could form copolymer P(3HB-co-3HV) based gas chromatography and $^{1}$H NMR analysis.

*Table 3.1. Growth and PHA accumulation of screened bacteria*

| **Strain** | **CDW (g/L)** | **PHA content (%wt)** | **PHA composition (mol%)** | |
|---|---|---|---|---|
| | | | ***3HB*** | ***3HV*** |
| ND97 | 3.1 | 48 | 100 | 0 |
| ND153 | 3.1 | 65 | 100 | 0 |
| QN194 | 2.2 | 26 | 100 | 0 |
| ND199 | 2.6 | 34 | 98 | 2 |
| ND218 | 2.3 | 24 | 97 | 3 |
| QN271 | 5.1 | 48 | 100 | 0 |
| QN187 | 3.8 | 44 | 100 | 0 |
| ND240 | 3.2 | 28 | 100 | 0 |
| MT15 | 3.1 | 53 | 98 | 2 |
| MT33 | 4.2 | 51 | 97 | 3 |

### *3.1.3. Morphological cell and colony characterizations of selected bacteria*

Almost selected bacteria are rod shape and motive. Colonies are usually white ivory or dark yellow. Gram staining analysis showed that 5 strain (ND97, ND153, QN195, MT15 and MT33) are positive Gram and endospore forming. While remaining bacteria (ND199, ND218, QN187, QN271, ND240) are negative Gram and non-endospore forming.

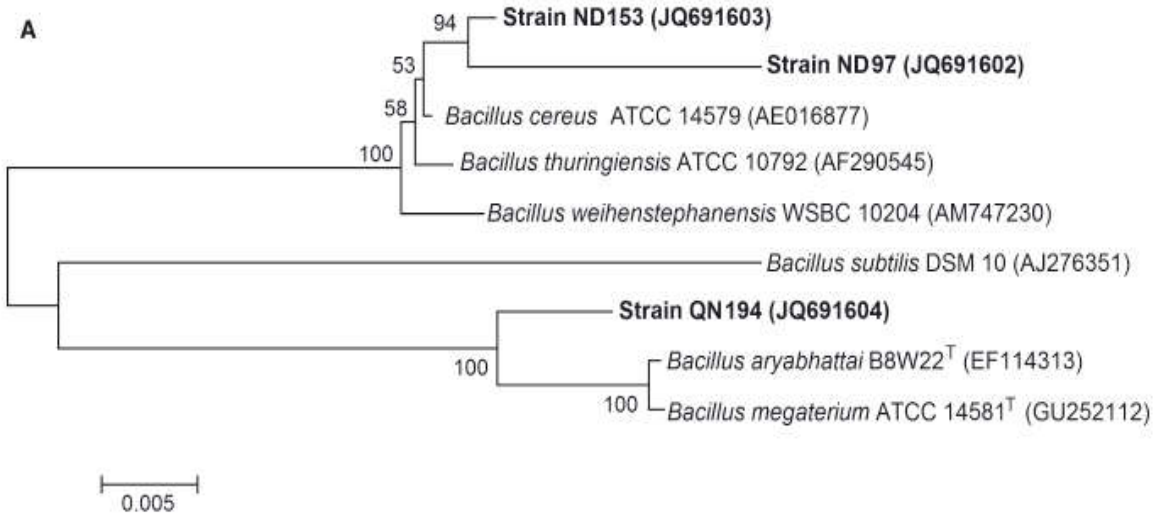
8 bacterial strains from mangrove soil were chosen for futher studies.

### *3.1.4. Physiological and biochemical characterizations of selected bacteria*

8 selected strains from mangrove soil are aerobic bacteria. They are catalase, oxidase positive and non-indole forming. All of them are mesophilic bacteria with optimal temperature for growth between 30 $^{o}$C to 40$^{o}$C. pH optimum for these strains is neutral. Among them three positive Gram strains are halotolerant bacteria and have biochemical characteristics similar with *Bacillus cereus*. Five negative Gram strains are halophilic bacteria and have biochemical characteristics similar to strain *Yangia pacifica* DX5-10T.

### *3.1.5. Phylogenesis relationship of selected bacteria*

The 8 selected bacterial strains were classified into genus of *Bacillus* and *Yangia* based on morphological, phisico-biochemical characterizations and genetic analysis. The *Yangia* sp. ND199 was selected for further studies.



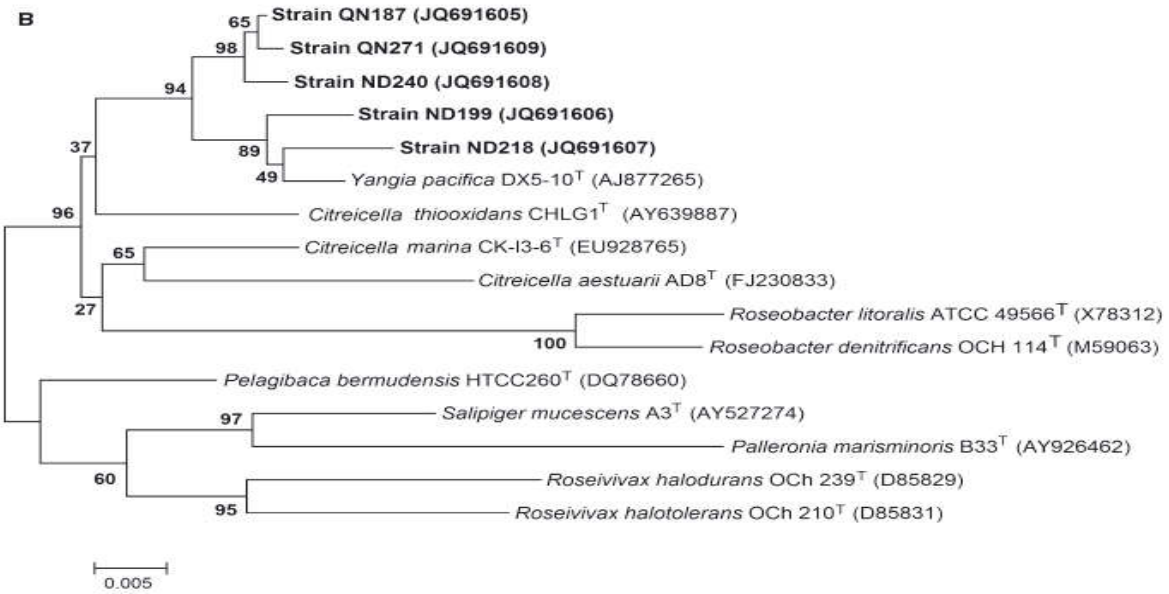



*Figure 3.1. Phylogenetic relationship of selected bacteria isolated from mangrove soil*

### *3.2. Cultivation for growth and PHA accumulation of* Yangia *sp. ND199*

#### *3.2.1. Effect of nutrient sources on growth and PHA accumulation*

3.2.1.1. Effect of carbon sources

The strain *Yangia* sp. ND199 could grow accumulate PHA on all carbon sources. These carbon sources, fructose exhibited as suitable carbon source for growth and PHA accumulation of *Yangia* sp. NĐ199 strain. Morever, molasses showed as potential carbon source for PHA production by this strain.

*Table 3.2. Effect of carbon sources on growth and PHA accumulation of Yangia* sp. ND199 *strain*

| Carbon source | CDW (g/L) | PHA (%wt) | RCM (g/L) | PHA composition (mol%) | |
|---|---|---|---|---|---|
| | | | | 3HB | 3HV |
| Molasses | 3.85 ± 0.13 | 35.0 ± 1.32 | 2.50 ± 0.21 | 96.8 | 3.2 |
| Glucose | 3.78 ± 0.1 | 47.5 ± 3.2 | 1.98 ± 0.07 | 96.9 | 3.1 |
| Glycerol | 3.61 ± 0.53 | 40.1 ± 1.8 | 2.16 ± 0.11 | 93.9 | 6.1 |
| Fructose | 4.87 ± 0.48 | 66.9 ± 2.1 | 1.61 ± 0.17 | > 99 | trace |
| Saccharose | 3.43 ± 0.06 | 18.2 ± 0.43 | 2.81 ± 0.02 | 83.8 | 16.2 |
| Maltose | 2.68 ± 0.06 | 13.6 ± 1.52 | 2.32 ± 0.05 | 79.5 | 20.5 |
| HFCS | 4.24 ± 0.27 | 58.3 ± 0.77 | 1.77 ± 0.23 | 98.4 | 1.6 |
| Unsterile HFCS | 4.13 ± 0.17 | 54.6 ± 1.36 | 1.88 ± 0.08 | 98.6 | 1.4 |

(Note: HFCS – high fructose corn syrup)

3.2.1.2. Effect of some precursors to growth, PHA accumulation and PHA composition

The P(3HB) was obtained when fructose and its combination with some precursors such as 1,5-pentanediol, 1,6-hexanediol, sodium hexanoate, and sodium octanoate were used as carbon sources. PHA containing 4HB could be produced by adding of 1,4-butanediol, γ-butyrolactone, or sodium 4-hydroxybutyrate. While P(3HB-co-3HV) were formed by adding of sodium heptanoate or sodium valerate.

Effect of various carbon precursor concentrations to growth and PHA biosynthesis of *Yangia* sp. ND199 strain were also determined. 3HV fraction in PHA composition increased while polymer content were not different when sodium heptanoate supplement increased. The 4HB fraction and PHA content were not difference significant when concentration of 1,4-butanediol and sodium 4-hydroxybutyrate increased. While both values tend to decreased when γ-butyrolactone as carbon source. Almost precursors inhibited to cell growth of this strain.

3.2.1.3. Physico-chemical properties of PHA from Yangia sp. ND199 strain

PHA product from *Yangia* sp. ND199 on fructose as carbon source has force properties similar to P(3HB) was reported by Doi et al. (1995). Physico-chemical properties of PHAs extracted from *Yangia* sp. ND199 were summaried in table 3.3.

*Table 3.3. Physico-chemical properties of some PHAs from Yangia* sp. ND199 *growth on different carbon sources*

| **Carbon** | | **PHA type** | $M_w$ (x10$^6$ Da) | $M_n$ (x10$^5$ Da) | **DPI** ($M_w/M_n$) | $T_m$ (°C) | $T_g$ (°C) |
|---|---|---|---|---|---|---|---|
| Fructose | | P(3HB) | 2.2 | 6.4 | 3.4 | 175 | 4 |
| Fructose | γ-butyrolactone | P(3HB-*co*-5,2%4HB) | 1.7 | 5.4 | 3.2 | 163 | -20 |
| | 1,4-butanediol | P(3HB-*co*-4,3%4HB) | 1.3 | 4.2 | 3.1 | 157 | -0 |
| | Sodium 4-hydroxybutyrate | P(3HB-*co*-5,8%4HB) | 1.8 | 5.5 | 3.2 | 155 | -18 |
| | Sodium heptanoate | P(3HB-*co*-13%3HV) | 1.8 | 6.9 | 2.6 | 173 | -11 |

*3.2.1.4. Effect of nitrogen sources*

Yeast extract exhibited as suitable nitrogen source for growth and also PHA accumulation of *Yangia* sp. ND199 strain. Morever. $KNO_3$ was also potential nitrogen source for PHA production by this strain. Adequate high PHA accumulation but very low CDW obtained when various amonium were used as nitrogen sources (table 3.4).

*Table 3.4. Effect of nitrogen sources on growth and PHA accumulation of Yangia* sp. ND199 *strain*

| **Nitrogen source** | **CDW (g/L)** | **PHA content (%)** | **PHA concentration (g/L)** | **Residual cell mass (g/L)** |
|---|---|---|---|---|
| Yeast extract | 4.95 ± 0.16 | 67.7 ± 2.1 | 3.4 | 1.55 |
| Monosodium glutamate | 3.85 ± 0.22 | 47.8 ± 0.6 | 1.8 | 2.01 |
| $KNO_3$ | 4.23 ± 0.06 | 57.7 ± 1.3 | 2.4 | 1.83 |
| $NaNO_3$ | 3.16 ± 0.22 | 44.8 ± 2.7 | 1.4 | 1.74 |
| $(NH4)_2SO_4$ | 0.64 ± 0.11 | 43.3 ± 0.2 | 0.3 | 0.36 |
| $NH_4HCO_3$ | 1.48 ± 0.07 | 58.4 ± 2.3 | 0.9 | 0.62 |
| $NH_4Cl$ | 0.67 ± 0.05 | 45.7 ± 0.8 | 0.3 | 0.36 |

***3.2.2. Results of PHA production on bioreactor***

3.2.2.1. Kinetics of batch fermentation for PHA production of Yangia *sp. ND199*

The growth curve of *Yangia* sp. ND199 can be divided to three phases (figure 3.2). Intracellular PHA accumulation by this strain occurred at early in batch fermentation despite rich nutrient was available. High fructose concentration in culture medium had trigged the PHA accumulation of this strain even there were no nutrient limitations. It means that the strain *Yangia* sp. ND199 can be classified to the second PHA producing bacteria group which require no such nutrient limitations for polymer accumulation. The result on fig. 3.2 show low CDW of 8.2 g/L and low yield of PHA production with polymer content of 46 % and conversion yield of 0.22 g/g fructose.

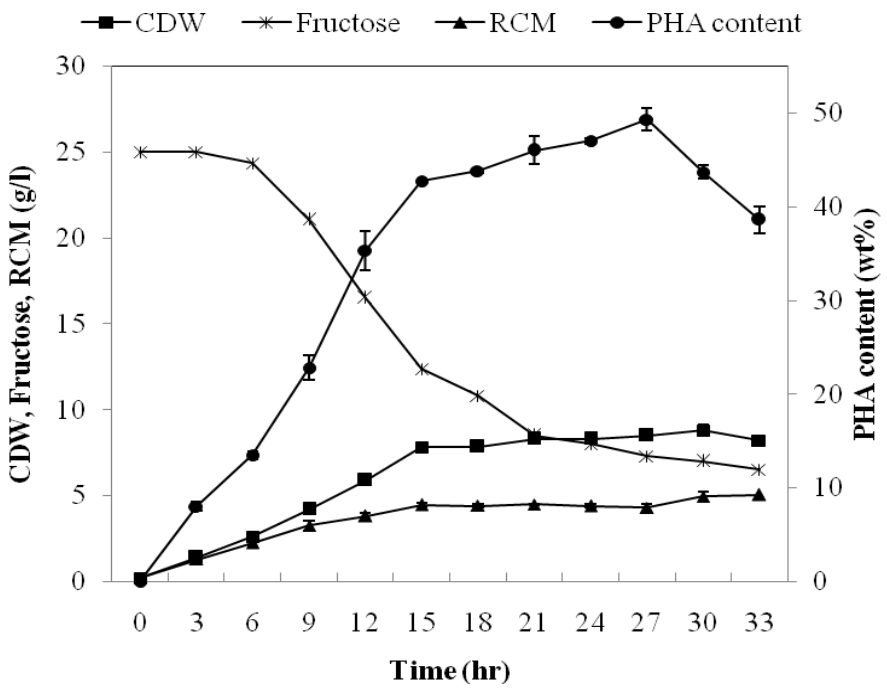



*Figure 3.2. Kinetics of batch fermentation for PHA production of Yangia* sp. ND199 *strain*

3.2.2.2. PHA production using fed batch fermentation

*a) Fed batch fermentation using fructose*

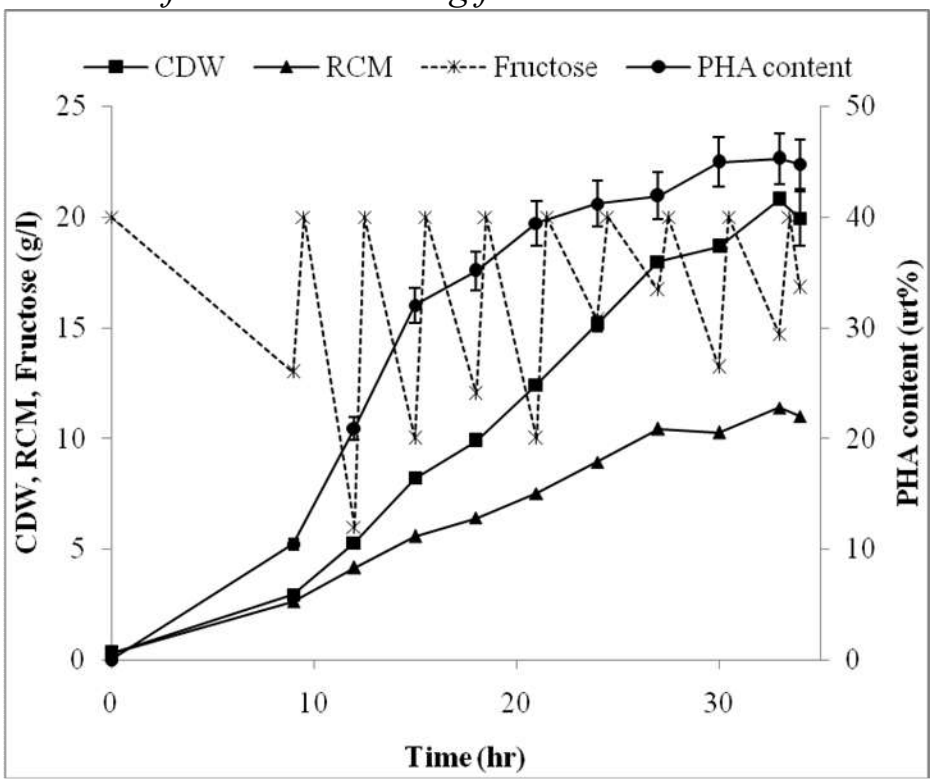



*Figure 3.3. Fed batch fermentation with 20 g/L fructose for PHA production*

*The first strategy*, the result of CDW, PHA content and CRM

showed in figure 3.3. The addition of fed solutions to medium helps to improve DCW. But PHA accumulation occurred at early of cultivation that lead PHA content just of 45 %wt and low CDW of 20.8 g/L. Thus change fructose concentration in medium may help to improve CDW and PHA accumulation.

*The second strategy,* CDW was increased and reached maximum values of 49 g/L after 36 hr of cultivation. But PHA accumulation was still occurred during early of fermentation. So PHA content was not reached high at the end of cultivation. Conversion yield of 0.33 g/g fructose while the productivity value of 0.73 g/L/h.

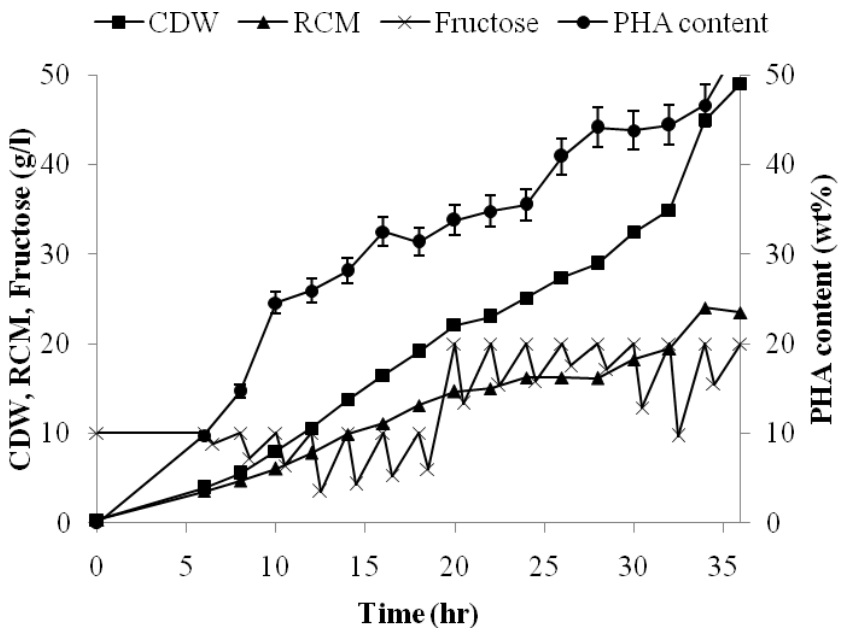



*Figure 3.4. Fed batch fermentation with two phase fructose concentration for PHA production*

*The third strategy,* during first stage the PHA synthesis was inhibited and only 10 %wt of PHA was accumulated when fructose concentration was low while requirement nutrient was provided. CDW was increased and achieved maximum value of 83,9 g/L after 48 hr of cultivation. Morever, maximum PHA content and productivity values were reached of 67.5 %wt and 1 g/L/h after 54 hr of cultivation. The results obtained from this experiment are comparable with those of the highest reported so far for PHA production by halophilic bacteria.

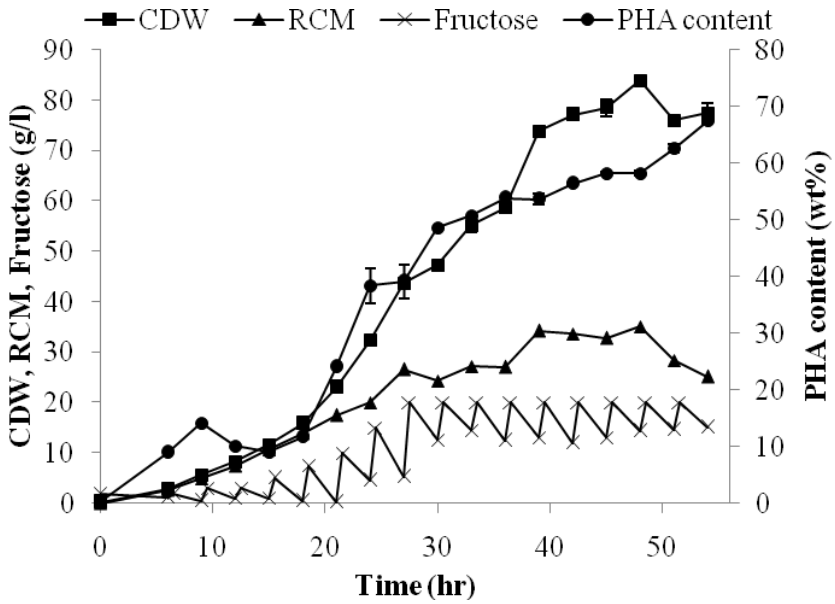
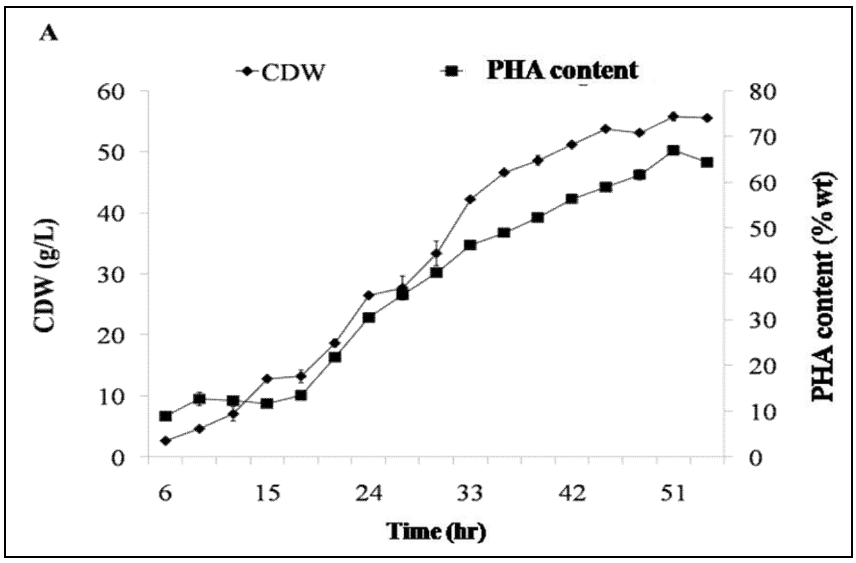



*Figure 3.5. Fed batch fermentation with step by step increasing of fructose concentration*

*b) Fed batch fermentation using HFCS as carbon source*

With the same strategy fermentation, kinetics of growth and PHA accumulation of *Yangia* sp. ND199 are similar as on pure fructose. PHA content of 66.3 %wt was obtained at the end of cultivation. Although high conversion yield of 0.38 g/g HFCS, but lower productivity (0.73 g/L/h) and CDW (55.8 g/L) comparing to those on pure fructose.



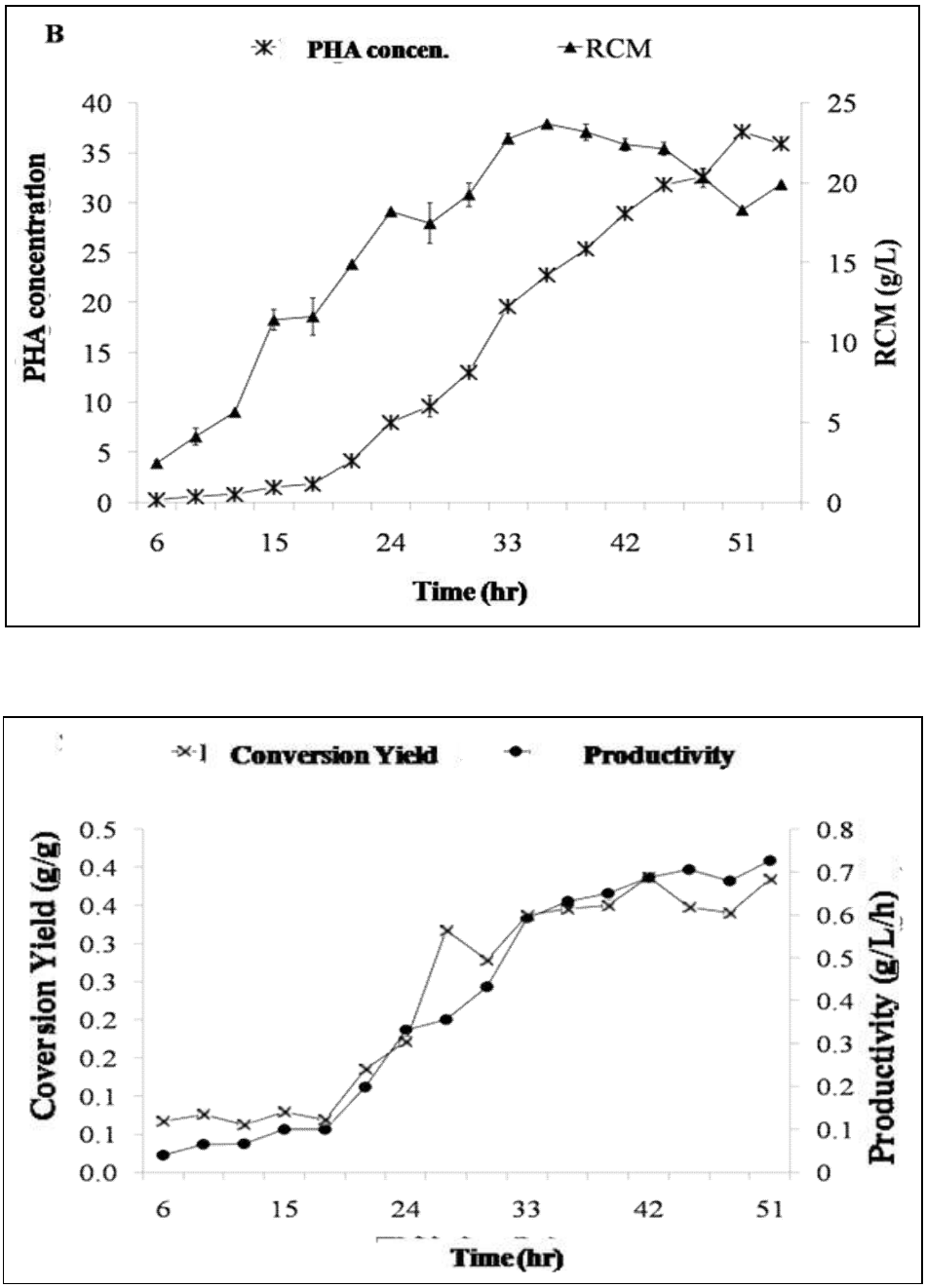



*Figure 3.6. Fed batch fermentation using HFS for PHA production by Yangia* sp. ND199

*c) Fed batch fermentation using molasses as carbon source*

Using similar strategy, we get results following: maximum CDW of 54.8 g/L and PHA content of 39.8 %wt after 54 hr of cultivation.

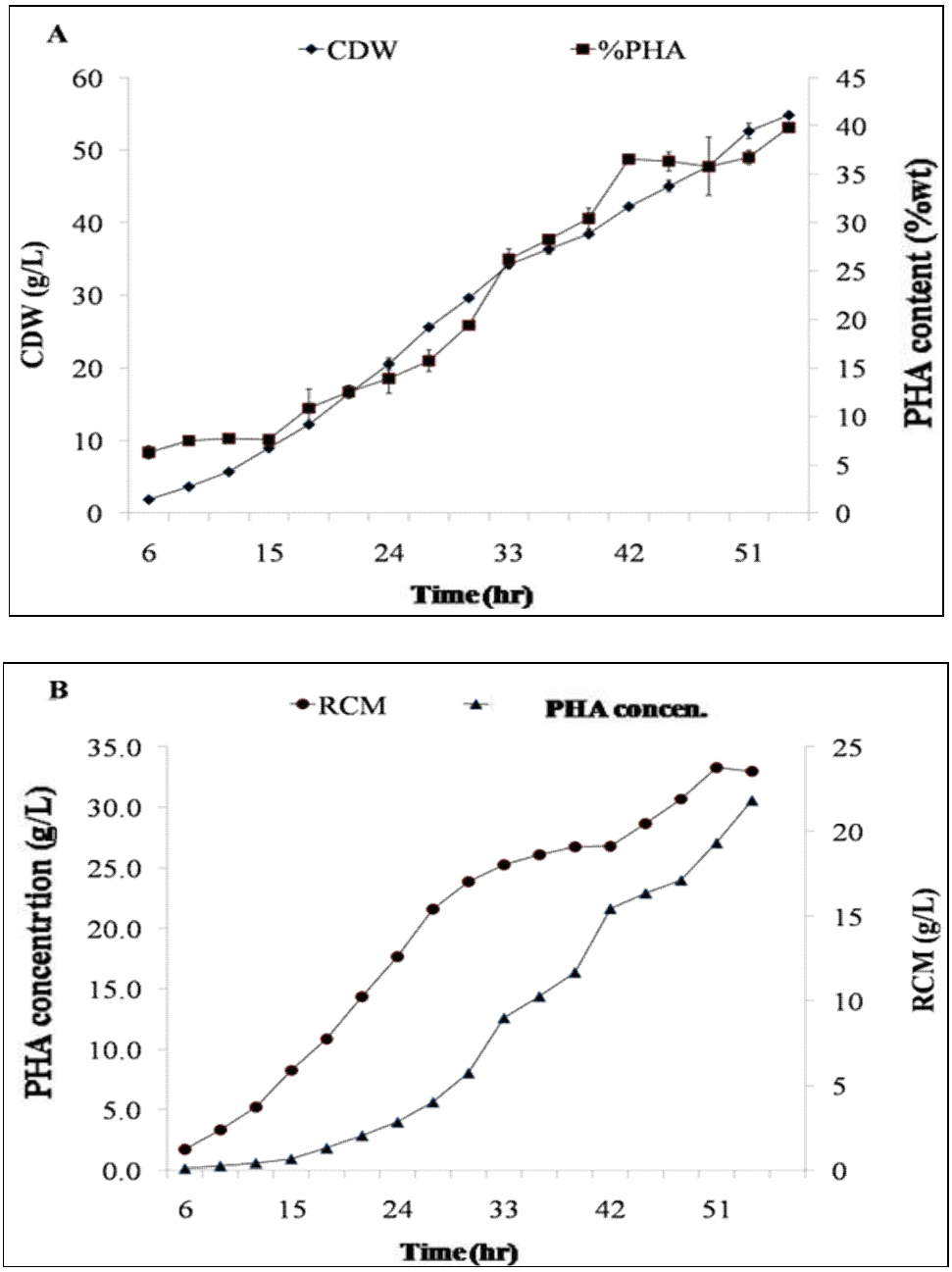



*Figure 3.7. Fed batch fermentation using molasses for PHA production by Yangia* sp. ND199

Using two-phase fed batch fermentation with molasses in phase 1 and glucose in phase 2 as carbon sources, results were improved. Although lower CDW of 50 g/L was obtained comparing to previous fermentation, but PHA content of 52.9 %wt and productivity of 0.48 g/L/h were higher (fig. 3.8-A). The PHA content increased and reached 54.3 %wt at 54 hr of cultivation (at 33 hr of second phase), but RCM value was steady during second phase (fig. 3.8-B).

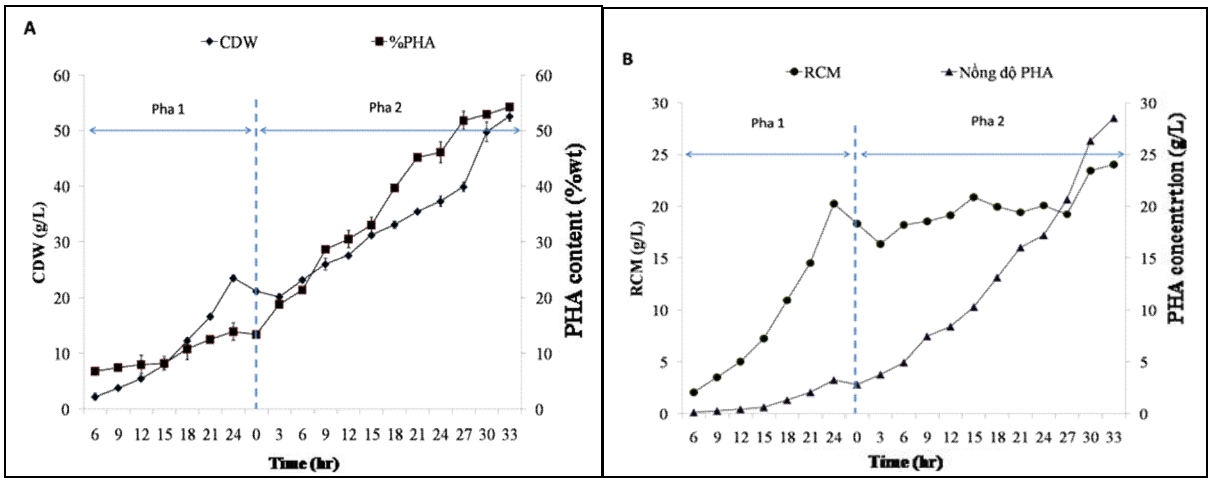



*Figure 3.8. Two phase fed batch fermentation*

### *3.3. Extration and revovery of PHA from wet biomass of* Yangia *sp. ND199*

#### *3.3.1. Effect of temperature and NaOH concentration on purified yield*

The results were described in table 3.5. The purity of PHA samples increased when NaOH concentration and temperature increase. However the recovery yield decreased when NaOH concentration more than 0.15 M at 50 $^{o}$C.

The influence of various NaOH concentrations on PHA quality have examined. The results showed slight decrease of $M_w$ and $M_n$ values, but almost no difference in thermal properties of the treated PHA samples.

Processing condition with 0.125M NaOH and 50 $^{o}$C was chosen for further studies.

*Table 3.5. Effect of temperature and NaOH concentration on purity yield*

| **NaOH concen. (M)** | **30 $^{o}$C** | | **40 $^{o}$C** | | **50 $^{o}$C** | |
|---|---|---|---|---|---|---|
| | ***Purity*** *(%wt)* | ***Recovery Yield*** *(%)* | ***Purity*** *(%wt)* | ***Recovery Yield*** *(%)* | ***Purity*** *(%wt)* | ***Recovery Yield*** *(%)* |
| Control | 70 ± 2.3 | 100 | 70 ± 2.3 | 100 | 70 ± 2.3 | 100 |
| 0.05 | 69 ± 0.73 | 96.6 ± 0.9 | 69± 0.52 | 95.7 ± 0.9 | 70 ± 3.9 | 97.3 ± 1.2 |
| 0.075 | 74 ± 1.5 | 96.2 ± 3.5 | 78 ± 0.45 | 100 ± 0.55 | 75 ± 1.87 | 96.7 ± 2.7 |
| 0.1 | 76 ± 0.72 | 98.1 ± 1.2 | 79 ± 1.4 | 95.7 ± 2.8 | 79 ± 0.27 | 95.4 ± 0.9 |
| 0.125 | 79 ± 0.45 | 98.2 ± 1.6 | 82 ± 1.35 | 98.0 ± 0.4 | 84 ± 1.85 | 98.3 ± 2.5 |
| 0.15 | 79 ±0.44 | 93.7 ± 2.2 | 84 ± 0.79 | 97.2 ± 1.9 | 82 ± 0.07 | 91.8 ± 0.4 |
| 0.2 | 82 ± 1.12 | 94.2 ± 1.3 | 85 ± 0.4 | 95.2 ± 0.7 | 84 ± 0.26 | 91.9 ± 2.9 |

#### *3.3.2. Effect of time on purified yield*

The PHA purity increased to 91.9 %wt and recovery yield of more than 97 % were obtained when treatment time was extended to 300 min.

Morever, GPC and DSC analysis showed no difference of thermal properties between treated samples and control.

*Table 3.6. Effect of time on purified yield*

| **Time** (min) | **Purity** (%wt) | **Recovery yield** (%) | $M_w$ ($10^6$ Da) | $M_n$ ($10^5$ Da) | **DPI** ($M_w/M_n$) | **Thermal properties** | |
|---|---|---|---|---|---|---|---|
| | | | | | | ***Tm*** ***(°C)*** | ***Tg*** ***(°C)*** |
| 0 | 72.2 ± 1.96 | 100 | 1.86 | 4.79 | 3.9 | 173.21 | 3.59 |
| 30 | 76.3 ± 0.1 | 96.7 ± 2.66 | 1.14 | 3.02 | 3.8 | 171.11 | 5.42 |
| 60 | 81.2 ± 0.48 | 96.2 ± 3.14 | 0.95 | 4.4 | 2.15 | 170.03 | 5.18 |
| 90 | 84.6 ± 0.5 | 97.6 ± 3.0 | 0.92 | 3.87 | 2.4 | 172.14 | 4.36 |
| 120 | 87.6 ± 1.1 | 98.8 ± 2.12 | 0.86 | 3.7 | 2.3 | 172.51 | 5.34 |
| 180 | 87.5 ± 0.21 | 98.3 ± 1.4 | 0.84 | 3.85 | 2.18 | 172.44 | 6.46 |
| 240 | 90.7 ± 0.01 | 98.2 ± 1.53 | 0.72 | 3.23 | 2.23 | 167.24 | 6.73 |
| 300 | 91.9 ± 0.22 | 97.7 ± 2.76 | 0.68 | 3.07 | 2.21 | 169.15 | 8.62 |

### *3.3.3. Effect of cell mass on putified yield*

As described in table 3.15, the purity obtained from 89.3 %wt to 90.6 %wt with recovery yields are above of 96 %. No significant difference of purified yield when increasing of biomass.

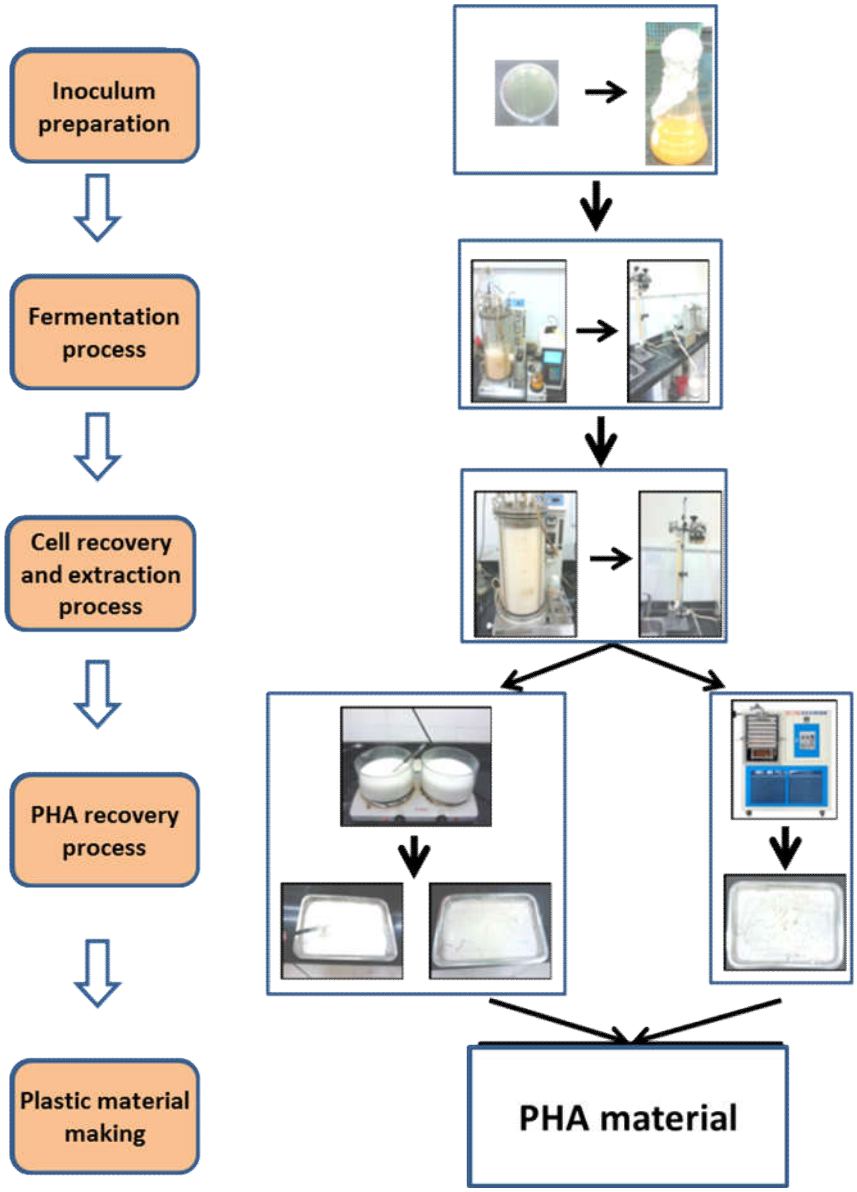
*Table 3.7. Effect of biomass on purified yield*

| **Bimass on CDW (g/L)** | **Purity (%)** | **Recovery yield (%)** |
|---|---|---|
| 50 | 89.3 ± 1.0 | 97.2 ± 1.5 |
| 70 | 90.6 ± 2.8 | 96.6 ± 1.3 |
| 90 | 89.8 ± 0.6 | 97.1 ± 1.05 |

*(Note: PHA content of all oridinal samples were* 71.15 ± 1.5 %wt)

### *3.4. PHA making process construction at laboratory scale*

The process was constructed by using all results above. It contains 5 step:




*Figuure 3.9. PHA production profile at lab scale*

### *3.4.1. Inoculum preparation*

The inoculums are prepared on nutrient medium in 15 hr for cell density value is 7±0.5 at wavelength 600 nm.

### *3.4.2. Fermentation*

The fermentations are operated by using fed batch cultivation technique with fructose concentration increasing. Fermentation time is 54 hr.

### *3.4.3. Biomass recovery and PHA extraction*

The cell biomass are recovered by using untrafiltration method with 0.2 µm catridge.

PHA extraction are operated using treatment condition containing 0.125 M NaOH at 50 $^{o}$C in 300 mins.

### *3.4.4. PHA recovery and purification*

PHA products are recovered by using heat treat ment or lyophilyzation methods.

### *3.4.5. PHA based-bioplastic resins formation*

PHA materials are extrusted at 180 $^{o}$C to form plastic granules

### *3.5. Biodegradable of PHA material from* **Yangia** *sp. ND199*

As showed on figure 3.17 and 3.18, all PHA film samples have lost weight after 4 weeks of experiment. The lost weight of buried samples were higher than those of surface experiments. No significant difference of lost weight was determined beween different thichnees samples. However, the lost weight of chloroform extracted PHA were higher than NaOH recovered.

GPC analysis demonstrated strongly decreasing of $M_w$ of chloroform extracted PHA more than NaOH recovered samples. Morever, broken structure and bacterial appearance on the film surface were determined by using scan electron microscopy (SEM) analysis.

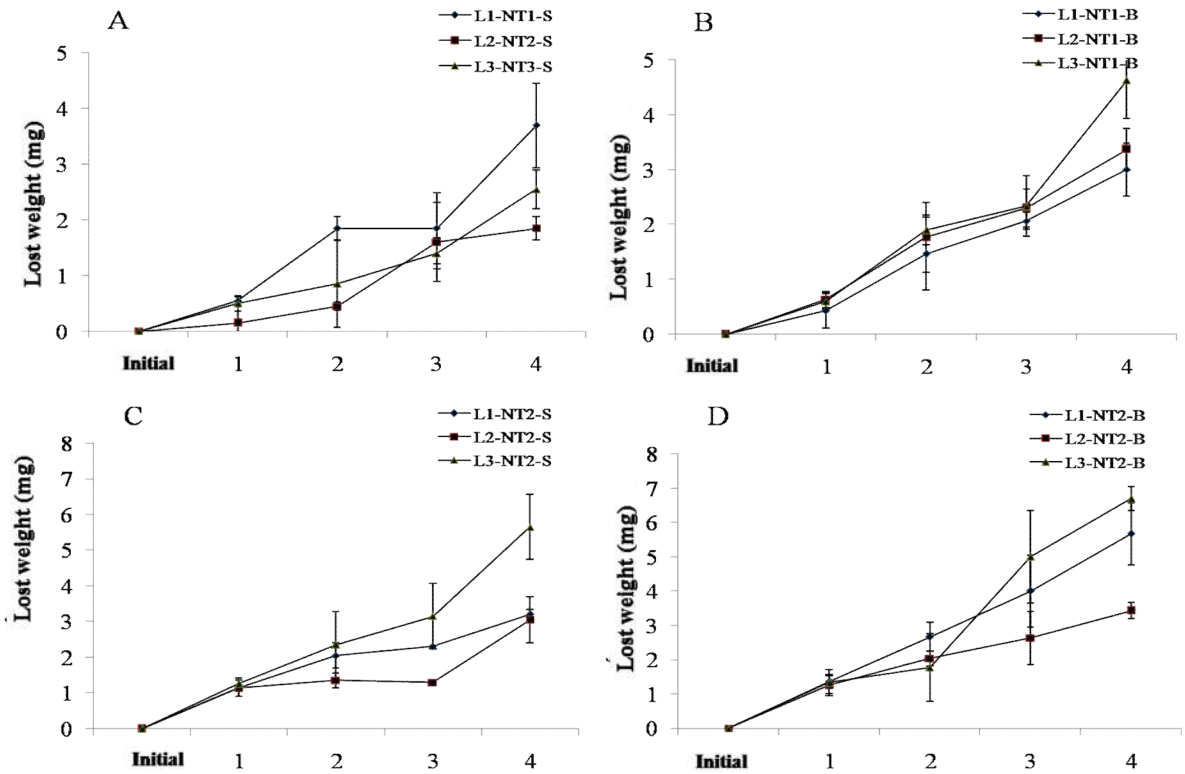



*Figure 3.10. The lost weight of $CHCl_3$ extracted PHA film samples during experimental time (week)*
*(A)-surface NT1, (B)-buried NT1, (C)-surface NT2, (D) buried NT2*

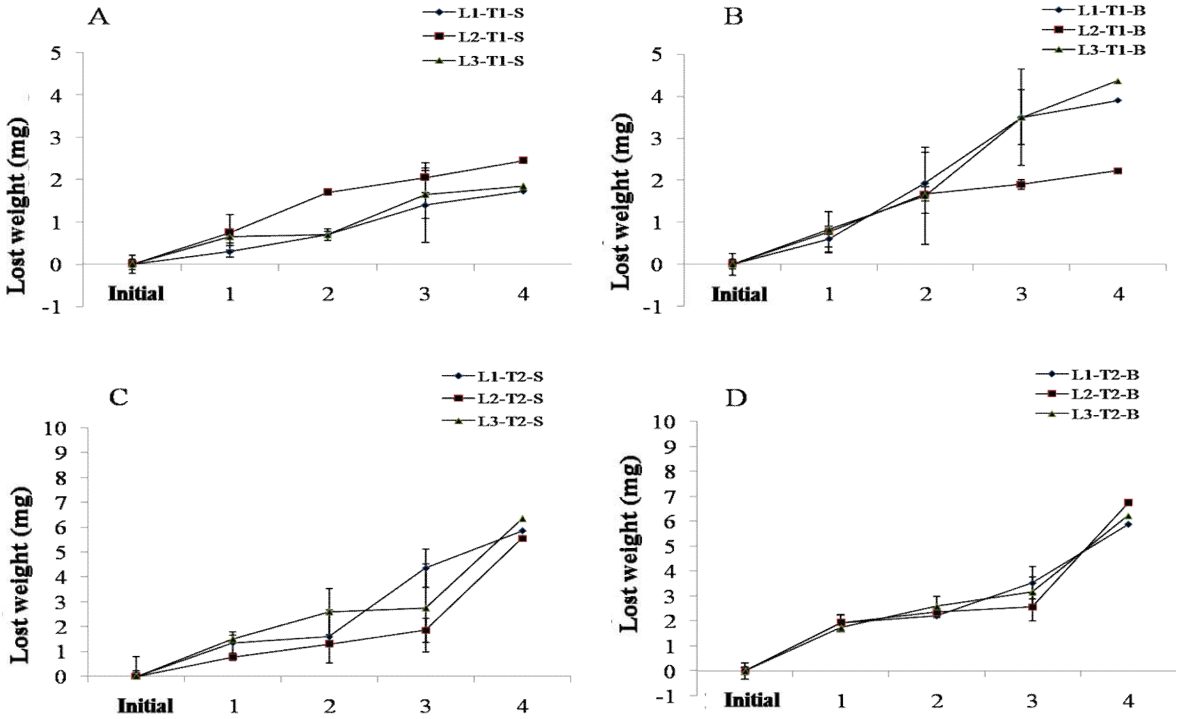



*Figure 3.11. The weight lost of NaOH recovered PHA film during experimental time (week)*
*(A)-surface T1, (B)-buried T1, (C)-surface T2, (D) buried T2*

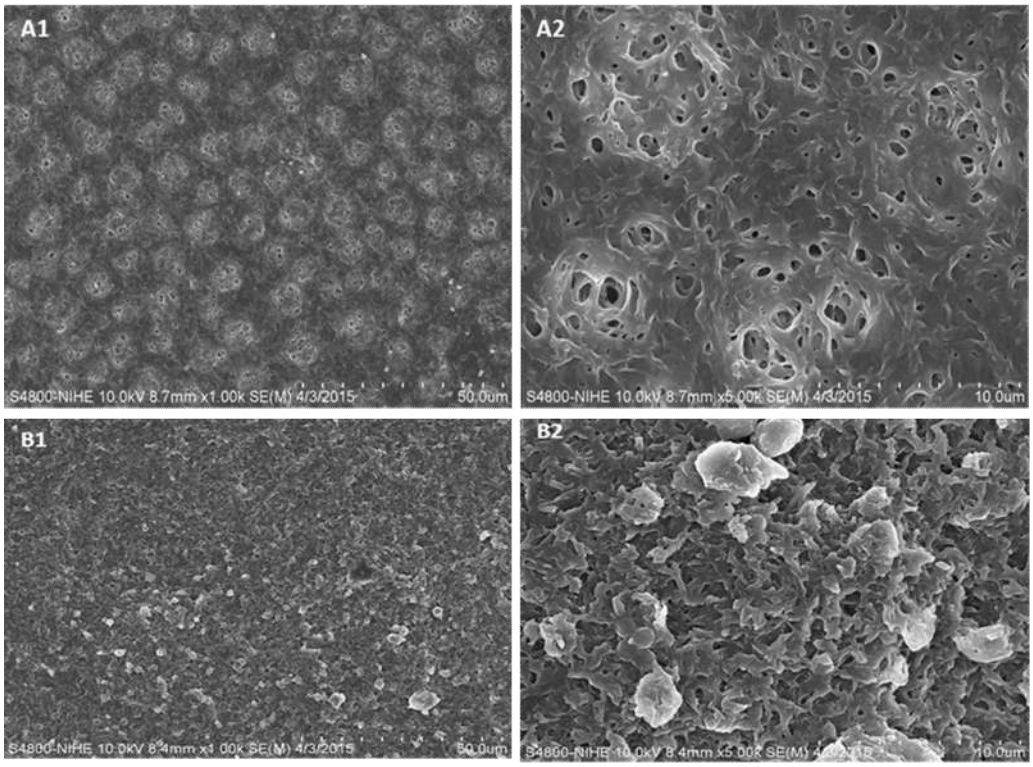



*Figure 3.12. Structure of PHA films on SEM analysis*
*(A1, A2) – NT1 control; (B1, B2) –L3-NT1-B sample*

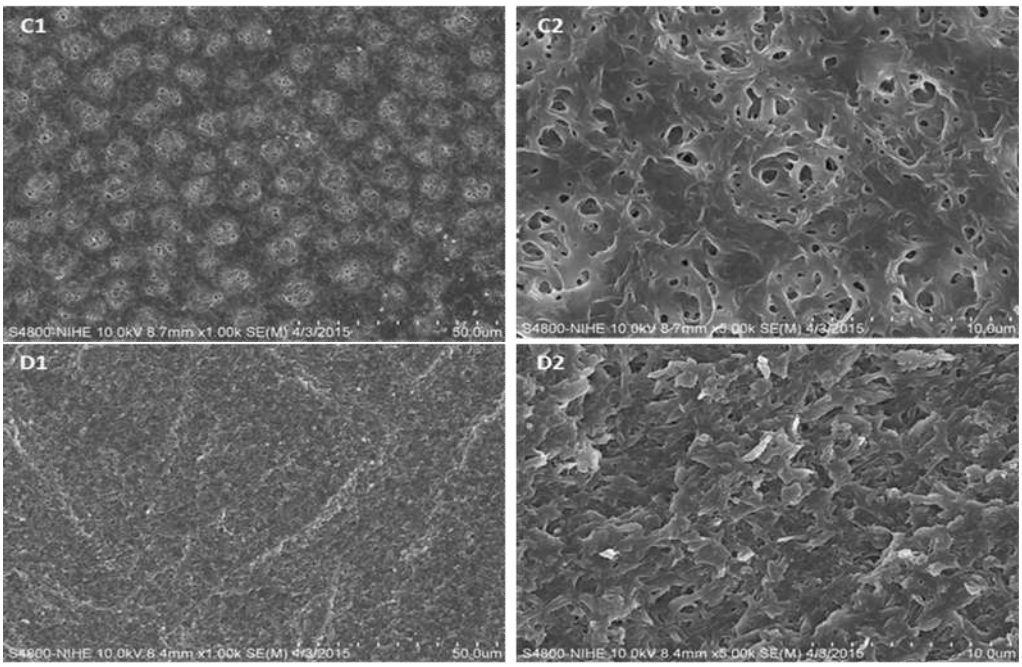



*Figure 3.13. Structure of PHA films on SEM analysis (C1, C2) –T1 control; (D1, D2) – L3-T1-B*

## CONCLUSIONS AND RECOMMENDATIONS

*1. CONCLUSIONS*

1.1. Our results showed that 50 of 869 isolated bacterial strains exhibited positive of PHA accumulation. Ten highest PHA producing strains were chosen for more studies, they are both negative and positive Gram bacteria. Eight bacterial strains isolated from mangrove soil were identified and classified into *Bacillus* (3 strains) and *Yangia* (5 strains) genus based 16S rDNA sequences and physio-chemical analysis. Nucleotide sequences of 16S rDNA of 8 strains were reported on GenBank with code numbers: JQ691602, JQ691603, JQ691604, JQ691605, JQ691606, JQ691607, JQ691608, JQ691609.

1.2. The strain *Yangia* sp. ND199 able to grow and accumulate various PHAs from different carbon sources. Fructose and yeast extract were most suitable C and N souces for growth and PHA accumulation of this strain. In addition, *Yangia* sp. ND199 could accumulate polymer without nutrient limitation requirement. In order to improve PHA production yield by *Yangia* sp. ND199, fed batch fermentation technique with different carbon feeding strategies was applied. Highest CDW of 78 g/L, PHA content of 67.5 % and PHA productivity of 1 g/L/h were

obtained after 54 h of cultivation.

1.3. Chemical method was used for PHA recovery from *Yangia* sp. ND199 cells. The highest PHA purity of 92 %wt and recovery yield of above 95 % were obtained after 300 min treatment with 0.125 M NaOH at 50 $^{o}$C. Physical and chemical properties of PHA from *Yangia* sp. ND199 have also been determined.

1.4. PHA product from *Yangia* sp. ND199 exhibited well biodegradable in buried condition (30 $^{o}$C and >80 % of humidity). The weight loss of PHA film samples were range from 15 % to 25 % after 4 weeks, it depended on the thickness and extraction method.

*2. RECOMMENDATIONS*

The results obtained here have demonstrated the potential of *Yangia* sp. ND199 as a candidate for PHA production in industrial scale. However, some more studies need to be done in the future:

1. Optimizing culture conditions for PHA production by *Yangia* sp. ND199 in large scale.

2. Purification and application of PHA material in different fields, particularly in medical application.

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