UNIVERSITY OF MARIBOR
FACULTY OF CHEMISTRY AND CHEMICAL ENGINEERING

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BIOREFINERY: VOLARISATION OF PULP AND PAPER INDUSTRY SIDE – PRODUCTS FOR BIOPLASTIC PRODUCTION

BIORAFINERIJA: VOLARIZACIJA STRANSKIH PRODUKTOV INDUSTRIJE CELULOZE IN PAPIRJA ZA PROIZVODNJO BIOPLASTIKE

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BIORAFINERIJA: VOLARIZACIJA STRANSKIH PRODUKTOV INDUSTRIJE CELULOZE IN PAPRIRJA ZA PROIZVODNJO BIOPLASTIKE

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Izjavljam, da sem diplomsko delo izdelal sam, prispevki drugih so posebej označeni. Pregledal sem literaturo iz področja diplomskega dela po naslednjih elementih:

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SKLEP O DIPLOMSKEM DELU

Sebastjan Huš, študent-ka univerzitetnega študijskega programa KEMIJSKA TEHNOLOGIJA, lahko izdela diplomsko delo pri predmetu Biokemijska tehnika.

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BIOREFINERY: VOLARISATION OF PULP AND PAPER INDUSTRY SIDE – PRODUCTS FOR BIOPLASTIC PRODUCTION

Abstract

Polyhydroxyalkanoates (PHAs) are biodegradable biopolymers, which have been recognized as a good substitution for petroleum-based polymers. The main reason for slow replacing of the synthetic plastic is the production cost, which can be four times higher than the chemical synthesis. PHA production in activated sludge is a possible technology to decrease production costs, since no sterilization is needed and bacteria can adapt easily to the substrate in low cost feedstock. Until now, best results were obtained in a process called aerobic dynamic feeding (ADF) or also known as feast and famine cycle which is based on alternation of periods of excess carbon (feast) and periods of starvation (famine). Under ADF conditions microorganisms have to compete for survival and only the one with high storage capacity survive.

Hardwood spent sulfite liquor (HSSL) is a by-product of paper industry and is rich in carbohydrates and acetic acid. It can be used as a substrate for PHA production, where mainly acetic acid is used, and also for bioethanol production, if acetic acid is removed.

The aim of this work was production of polyhydroxyalkanoates from spent sulfite liquor by mixed microbial culture and also removal of acetic acid from SSL for bioethanol production with *P. stipitis*. The culture was selected in Sequenced batch reactor (SBR) under ADF conditions. The system was operating for 72 days and microorganisms were able to store 29% of PHA per cell dry weight. The microorganisms were able to uptake acetic acid and sugars and convert it to PHAs. The acetic acid was not removed, because microorganisms were fermenting sugars in acetic acid, or fungus present in the system were producing acetic acid.

Key words: Polyhidroxyalkanoates, mixed microbial culture, spent sulfite liquor, acetic acid.

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Used abbreviations

PHA	–	Polyhydroxyalkanoate
PHB	–	Polyhydroxybutyrate
HB / HV / HX	–	Hydroxybutyrate / Hydroxyvalerate / Hydroxyexanoate
ADF	–	Aerobic dynamic feeding
SBR	–	Sequencing batch reactor
SSL / THSL	–	Spent sulfite liquor / Thick hardwood sulfite liquor
PP	–	Polypropylene
PE	–	Polyethylene
PS	–	Polystyrene
PET	–	Polyethylene terephthalate
PVC	–	Polyvinyl chloride
PLA	–	Polylactic acid
HPLC	–	High pressure liquid chromatography
GC	–	Gas chromatography
FISH	–	Fluorescent in situ hybridization
HRT	–	hydraulic retention time
PAO	–	Polyphosphate accumulating organisms
GAO	–	Glycogen accumulating organisms
RNA	–	Ribonucleic acid
DNA	–	Deoxyribonucleic acid
CoA	–	Coenzyme A
ISA	–	Ionic strength adjustment solution
NAD(P)H	–	Nicotinamide adenine dinucleotide
PBS	–	Phosphate buffer solution
EU	–	European union
B2B	–	Bio-based to biodegradable

RAZŠIRJEN POVZETEK V SLOVENSKEM JEZIKU

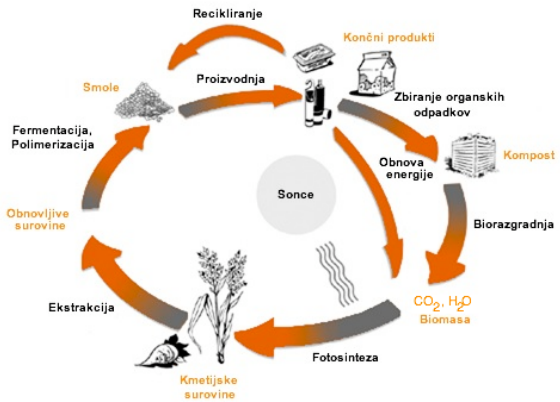
1 UVOD

1.1 BIOPLASTIKA

Pod pojem bioplastika lahko vključimo celotno družino raznovrstnih polimerov, ki so biorazgradljivi in narejeni iz obnovljivih virov.

Trg plastike predstavlja poleg energetskega in transportnega sektorja, največje področje uporabe nafte, s porabo več kot 200 milijonov ton plastike in 5% rastjo letno [1, 2]. Manj kot 20% odpadkov, proizvedenih iz kemično sintetizirane plastike je mogoče reciklirati ali sežgati, ostalih 80% pa se še vedno, po večini nenadzorovano, odlaga v okolju [3]. Sintetični polimeri, kot so polipropilen, polietilen, polivinil klorid in drugi, ki prevladujejo na svetovnem trgu, se štejejo za okolju škodljive, saj so nerazgradljivi [4].

Polimeri, ki se lahko proizvajajo iz obnovljivih virov in ki so biorazgradljivi, sledijo zaprtemu življenskemu ciklu B2B (Slika 1) [1].



Slika 1 – B2B življenski cikel [1]

Čeprav potreba po biorazgradljivi plastiki narašča in ima visok potencial rasti, je delež na trgu še vedno zelo nizek (okoli 1% celotnega trga). Glavni razlog za to je visoka cena bioplastike, ki je v primerjavi s plastiko proizvedeno iz fosilnih goriv, lahko kar 9 krat dražja [1]. Visoke proizvodne stroške je možno zmanjšati z uporabo poceni substratov in mešanih mikrobioloških kultur [5]. Čeprav je cena še vedno višja v primerjavi z nerazgradljivo plastiko, razgradljivost in s tem zmanjšanje onesnaževanja izničita to razliko [6].

Na tržišču so trenutno štiri glavne vrste bioplastike: polimeri na osnovi škroba, na osnovi celuloze, na osnovi polimlečne kisline in polihidroksialkanoati [7].

1.2 POLIHIDROKSIALKANOATI

Polihidroksialkanoati so termoplasti sintetizirani v bakterijah. Imajo zelo podobne lastnosti kot običajni polimeri, vendar so za razliko od njih biorazgradljivi in proizvedeni iz obnovljivih virov [8].

Odkril jih je francoski znanstvenik Lemoigne leta 1926 v bakteriji *Bacillus megaterium* [9]. Sintetizira in shrani, kot znotrajcelične rezerve, jih lahko več kot 250 različnih vrst bakterij [10-12]. Polihidroksialkanoati se med sabo razlikujejo v lastnostih gleda na njihovo sestavo, mikrostrukturo in molsko maso [13]. Njihove lastnosti so zelo podobne lastnostim nerazgradljive plastike, razlikujejo pa se v tem da so popolnoma biorazgradljivi. Razgradijo se s pomočjo encimov imenovanih depolimeraze. Pod aerobnimi pogoji se polihidroksialkanoati razgradijo v vodo in ogljikov dioksid, pod anaerobnimi pogoji pa v vodo in metan [14].

Polihidroksialkanoati lahko nadomestijo večino plastičnih izdelkov, baziranih na osnovi fosilnih goriv ravno zaradi podobnih lastnosti. Uporabijo se lahko za izdelke, kot so vrečke, plastenke, plasični jedilni pribor, ki se uporabljajo dnevno in predstavljajo velik del odpadkov. Uporabljajo se lahko pri pakiranju hrane, prav tako pa tudi za ostale vrste folij. Zaradi njihovih piezoelektričnih lastnosti se uporabljajo za izdelavo senzorjev za pritisk, merilnih instrumentov, mikrofonov,

slušalk, zvočnikov, ultrasoničnih detektorjev in tako naprej. V medicini se najpogosteje uporabljajo na kardiovaskularnem področju. Velik napredek je bil narejen tudi na področju tkivnega inženirstva in regeneracije prav tako pa tudi na področju zdravil z nadzorovanim sproščanjem. Polihidroksialkanoati se uporabljajo tudi v kmetijstvu kot biorazgradljivi filmi in za nadzorovano sproščanje insekticidov [15, 16].

1.3 MIKROORGANIZMI

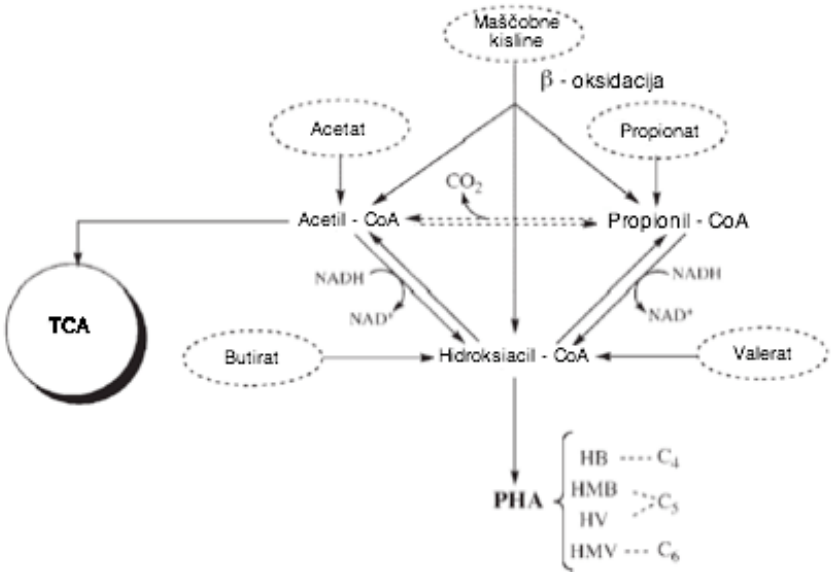
Polihidroksialkanoati so lahko proizvedeni s pomočjo čistih ali mešanih mikrobioloških kultur. Proizvodna trenutno temelji na uporabi čistih kultur, kot so *Ralstonia eutropha, Alcaligenes latus, Pseudomonas oleovorans* in drugih. To pa zaradi velike potrebe po kisiku med fermentacijo in potrebe po sterilizaciji, privede do visokih proizvodnjih stroškov [17, 18].

Visoke stroške je možno zmanjšati z uporabo mešanih mikrobioloških kultur, saj je mešane kulture v primerjavi s čistimi, možno dobiti veliko ceneje. Kot substrat lahko uporabimo cenene odpadne materiale, prav tako pa se izognemo tudi sterilizaciji. Mešane kulture so populacije mikroorganizmov neznane sestave. Organizmi so izbrani glede na operativne pogoje sistema in so tako kot čiste kulture, sposobni shraniti visoke kapacitete polihidroksialkanoatov [8, 17].

Najboljši rezultati pri proizvodnji polihidroksialkanoatov so bili dobljeni pod pogoji aerobnega dinamičnega hranjenja, kjer se izmenjujejo obdobja presežnega vira ogljika, z obdobjem stradanja. V takih pogojih morajo mikroorganizmi tekmovati za preživetje, kar privede do tega, da preživijo tisti organizmi, ki so sposobni skladiščiti največ rezerv - polihidroksialkanoatov, kar je ključno za proizvodnjo le teh [17].

Metabolizem aerobnega dinamičnega hranjenja je prikazan na sliki 2. Ocetna kislina se pretvori v acetil – CoA, ki se porabi pri ciklu trikarboksilne kisline za rast, za proizvodnjo NADPH ter proizvodnjo polihidroksialkanoatov. Iz dveh enot acetil

– CoA nastane acetoacetil – CoA, iz njega pa hidroksiacil – CoA, ki da na koncu željen monomer [17].



Slika 2 – Metabolizem aerobnega dinamičnega hranjenja [17]

1.4 LES

Portugalska je država z veliko gozdnih površin in ima zato zelo močno in dobro razvito papirno industrijo in industrijo celuloze. Od iznajdbe izdelovanja papirja, so se za ta namen uporabljali različni materiali, vendar je med njimi prevladal prav les. Sestavljen je iz celuloze (40-50%), hemiceluloze (15-30%), lignina (20-30%) in ostalih snovi, ki pa so prisotne v malih količinah. Vrednosti posameznih komponent se razlikujejo glede na vrsto lesa in tipa celične stene [19].

1.5 ODPADNA SULFITNA TEKOČINA

Odpadna sulfitna tekočina je stranski produkt sulfitnega postopka predelave lesa, ki se uporablja za raztapljanje lignina z uporabo alkalijskih soli žveplene kisline (Na^+, NH^{3+}, Mg^{2+}, K^+ ali Ca^{2+}) [20].

Z izparevanjem dobimo koncentrirano ali zgoščeno odpadno sulfitno tekočino. Obe se uporabljata pri formulaciji polimerov, pri proizvodnji etanola, fenolov, lepil, enoceličnih proteinov in drugih. Sta zelo cenen vir ogljika in imata zelo velik potencial pri zamenjavi fosilnih virov, vendar je potrebno pred uporabo odstraniti vse kontaminante, ki zavirajo proces fermentacije [21-23].

2 MATERIALI IN METODE

2.1 MATERIALI

Mešane kulture, uporabljene v tej študiji, so bile dobljene iz čistilne naprave Aveiro Sever, Portugalska.

Proces proizvodnje polihidroksialkanoatov je potekal v SBR (sequencing batch reactor) reaktorju. Med samim procesom smo beležili spremembo temperature in pH. Celoten cikel v SBR reaktorju je potekal sprva 12 h, kasneje pa smo ga podaljšali na 24 ur, ker ogljik ni bil popolnoma porabljen. Cikel je bil sestavljen iz 30 minut praznenja reaktorja na polovičen volumen, 15 minut mirovanja in 15 minut polnenja reaktorja. Mešalo in prezračevanje v reaktorju sta bila vključena ves čas.

Odpadni sulfitni tekočini, dobljeni iz podjetja Caima, smo pred uporabo najprej znižali pH, nato smo jo prezračevali in nato še filtrirali z 0,2 µm filtri.

Sestava uporabljenega medija je prikazana v tabeli 1. Da bi preprečili možne reakcije, smo fosfatne soli pripravili ločeno od magnezijeve soli. Za preprečitev nitrifikacije smo dodali 2 g Tioureje.

Tabela 1 – Sestava medija

SESTAVINE	KONCENTRACIJA (g/l)
KH_2PO_4	0,016
K_2HPO_4	0,064
$MgSO_4.7H_2O$	0,16
$CaSO_4.2H_2O$	0,08
$FeCl_3$	0,04
$Na_2MoO_4.2H_2O$	0,008

2.2 METODE

2.2.1 Analitske metode

- Visokotlačno tekočinsko kromatografijo smo uporabili za določanje koncentracije sladkorjev in ocetne kisline v vzorcih. Vzorce smo filtrirali s pomočjo 0,2 µm filtrov pri 8000 obratov/min in jih injicirali v kromatograf. Kot topilo smo uporabili 0,01 N žvepleno kislino s pretokom 0,4 ml/min pri sobni temperaturi.

- Z določitvijo suhe teže biomase smo določili maso snovi brez vsebnosti vode. To smo storili tako, da smo vzorce filtrirali skozi prej stehtane filtre, jih 24 ur pustili v pečici pri 105 °C in jih nato ohlajene stehtali. Dobljena razlika v masi je suha teža biomase.

- Amoniak smo v vzorcih določali z uporabo elektrodo Thermo Orion 9512. 1 ml vzorca smo dodali 20 µl raztopine ISA, vstavili elektrodo in po 5 minutah odčitali rezultat. Kalibracijsko krivuljo smo dobili z uporabo standardov amonijevega klorida.

- Spektrofotometrično metodo smo uporabili za določevanje koncentracije polihidroksialkanoatov v vzorcu. Peletom v epruvetah smo dodali 1 ml vode in 2 ml 2 M HCl in vse skupaj segrevali v vodni kopeli 4 ure na 100 °C. Raztopino smo pustili, da se je usedla. Tekočo fazo smo odlili stran, usedlini pa dodali 5 ml kloroforma in pustili čez noč na stresalniku pri 150 obratih/min. 2 ml raztopine smo nato odpipetirali v drugo epruveto, posušili z N_2 in dodali 5 ml H_2SO_4 ter 2 uri segrevali v vodni kopeli na 100 °C. Po ohladitvi na sobno temperaturo smo z UV spektrofotometrom pri valovni dolžini 235 nm izmerili absorbance vzorcev in s tem določili koncentracijo polihidroksialkanoatov.

2.2.2 Mikroskopske metode

- Nile blue je tehnika histološkega barvanja pigmenta in se v povezavi s fluorescentno mikroskopijo uporablja za detekcijo mikroorganizmov, ki skladiščijo polihidroksialkanojske kisline. Granule polihidroksialkanoatov imajo pod mikroskopom fluorescentno rdečo barvo.

  Ependorfko z vzorcem in kapljico barvila Blue nile smo postavili v peč za 10 minut na 55 °C in jo nato centrifugirali. Tekočo fazo smo odlili in dodali 1 ml 0,9% natrijevega klorida. Analiza je potekala z uporabo epifluorescentne mikroskopije.

3 REZULTATI IN DISKUSIJA

Cilj te diplomske naloge je bila proizvodnja polihidroksialkanoatov z mešanimi mikrobnimi kulturami in stranskim produktom papirne industrije, odpadno sulfitno tekočino, kot virom ogljika.

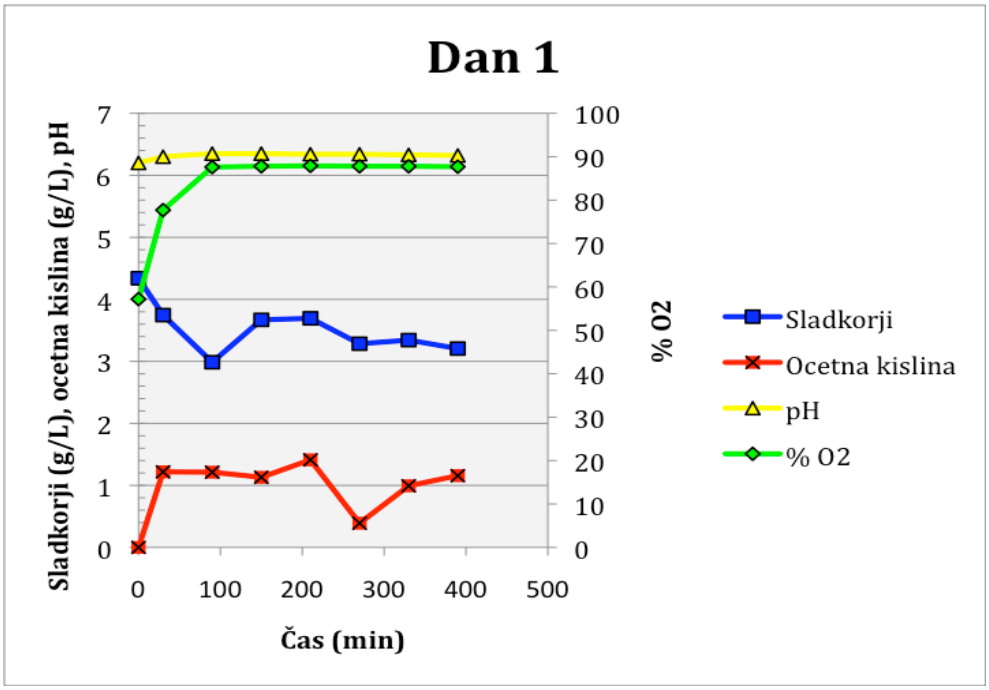
Celoten sistem je pod pogoji aerobnega dinamičnega hranjenja v SBR reaktorju deloval 72 dni, vendar smo lahko uporabili rezultate le za prvih 30 dni, saj so se v tem času v sistemu iz spor, prisotnih v odpadni sulfitni tekočini razvile glive, kar je preprečilo, da bi kulture še naprej shranjevale polihidroksialkanoate.

Preučevali smo vpliv različnih parametrov, kot so poraba ocetne kisline in sladkorjev, spreminjanje koncentracije amoniaka in koncentracije biomase, na proizvodnjo polihidroksialkanoatov. Prav tako pa smo spremljali tudi pH in koncentracijo kisika v sistemu.

Pri določevanju amoniaka dobljeni rezultati niso imeli nobenega smisla, saj se je koncentracija amoniaka skozi cikel povečevala oz. se je spreminjala alternirajoče, kar je v nasprotju z rezultati iz razpoložljive literature, kjer je koncentracija amoniaka padala. Razlog za to bi lahko bil v tem, da so bili

analizirani vzorci premalo razredčeni, torej so vsebovali preveč biomase, zato elektroda in membrana nista delovali pravilno.

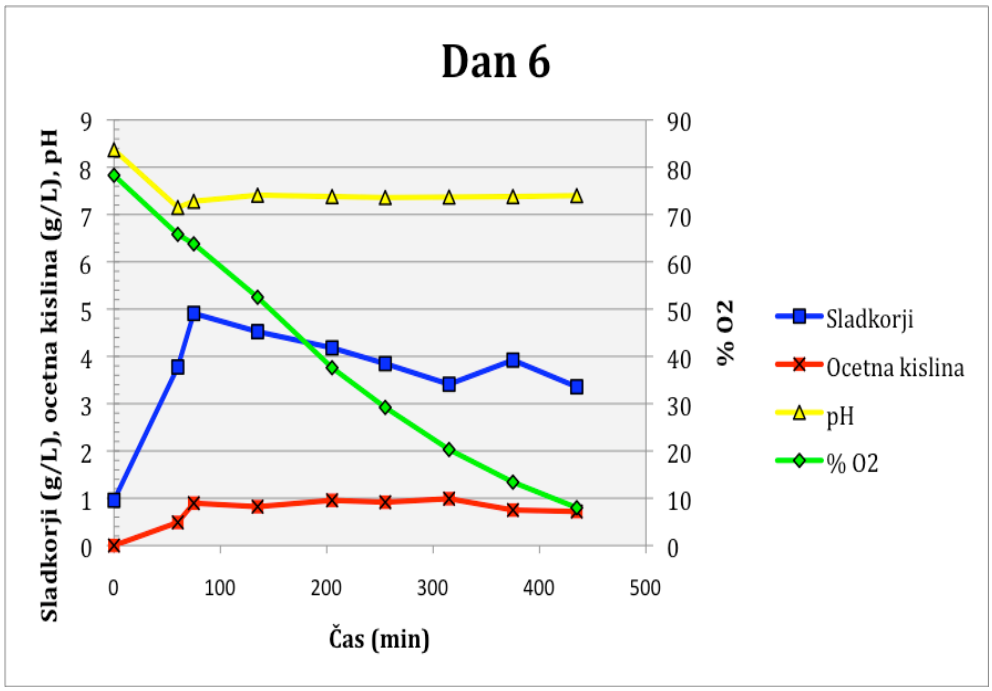
Kot je razvidno iz slike 4, se koncentracije ocetne kisline in sladkorjev prvi dan niso zniževale ampak so se spreminjale alternirajoče, kar pomeni, da vir ogljika ni bil v celoti porabljen.



Slika 4 – Proizvodnja polihidroksialkanoatov prvi dan

To potrjuje tudi vrednost kisika, ki je skozi celoten cikel visoka. To nam je onemogočilo jasno opredelitev faze obilja in faze lakote. pH vrednost v sistemu je bila skozi celoten cikel konstantna. Da bi dosegli popolno porabo vira ogljika, smo dolžino cikla podaljšali iz 12 ur na 24 ur.

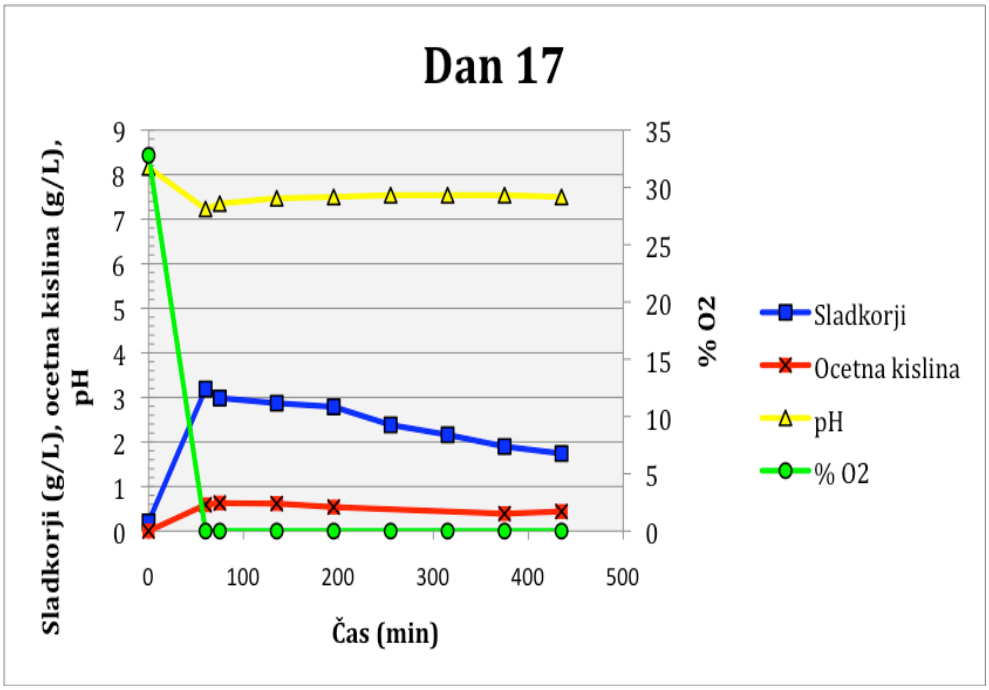
S podaljšanjem dolžine cikla, se je kot pričakovano, povečala poraba ocetne kisline.



Slika 5 – Proizvodnja polihidroksialkanoatov šesti dan

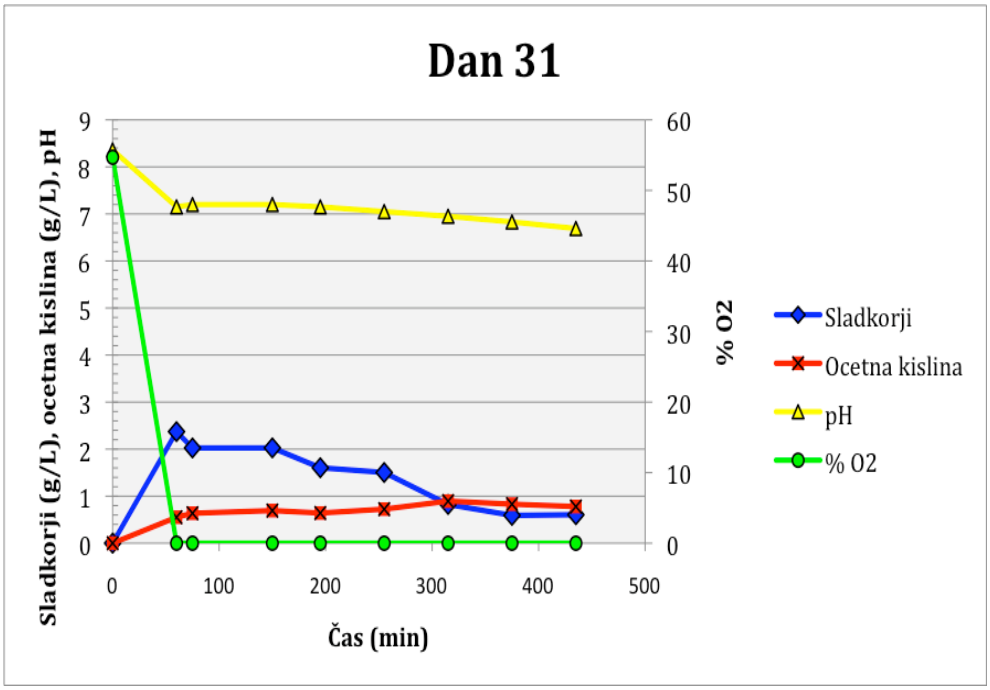
Prav tako pa je iz slike 5 razvidna tudi poraba sladkorjev oz. ksiloze. Porabo ogljika potrjuje tudi zmanjšanje koncentracije kisika v sistemu. pH vrednost se je prvih 60 min zmanjšala iz 8 na 7 in nato ostala konstantna.

Kot vidimo na sliki 6, je v naslednjih dneh še naprej razvidna poraba ocetne kisline, prav tako pa tudi poraba ksiloze. Porabo ogljika potrjuje zmanjšanje koncentracije kisika v sistemu. Takšen rezultat ni bil pričakovan, saj je v razpoložljivi literaturi pod pogoji aerobnega dinamičnega hranjenja, kot vir ogljika, omenjena samo poraba hlapnih maščobnih kislin, kot je ocetna kislina, ne pa tudi poraba ksiloze. pH vrednost je v prvih 60 min padla iz 8,4 na 7,2 in je nato ostala konstantna skozi celoten cikel.



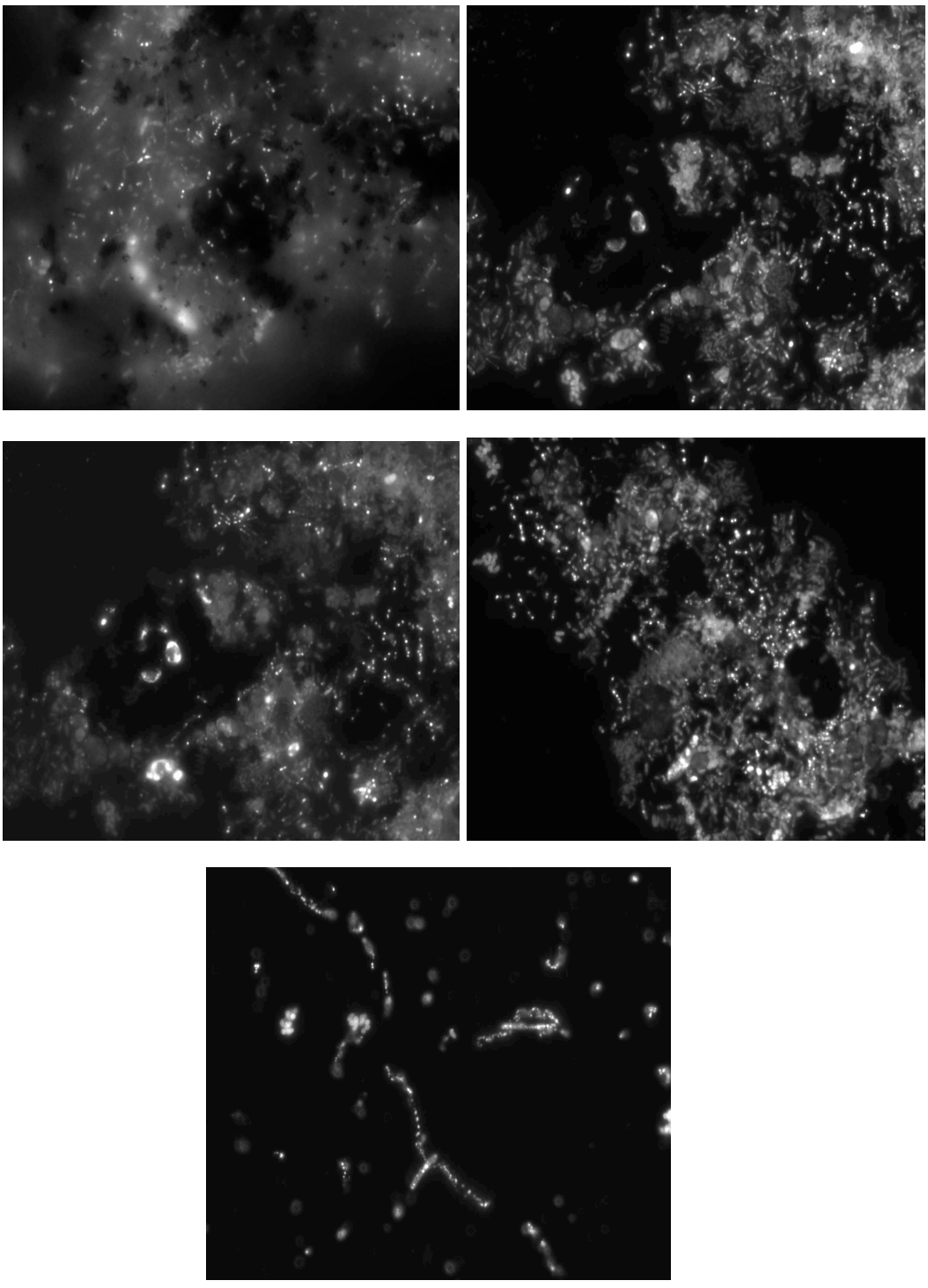
Slika 6 – Proizvodnja polihidroksialkanoatov sedemnajsti dan

Enaintrideseti dan je poraba sladkorjev močno narasla, poraba ocetne kisline pa je začela upadati. Razlog za to je lahko v tem, da so mešane mikrobiološke kulture spreminjale ksilozo v ocetno kislino in je tako koncentracija ocetne kisline v sistemu s časom naraščala. Lahko pa je tudi posledica fermentacije ocetne kisline nekaterih organizmov. Zmanjšanje koncentracije kisika je lahko tako razlog za porabo ogljika ali pa se je kisik porabil pri fermentaciji. pH vrednosti so se zopet znižale v prvih 60 min, prav tako pa so začele padati proti koncu cikla (slika 7).



Slika 7 – Proizvodnja polihidroksialkanoatov enaintrideseti dan

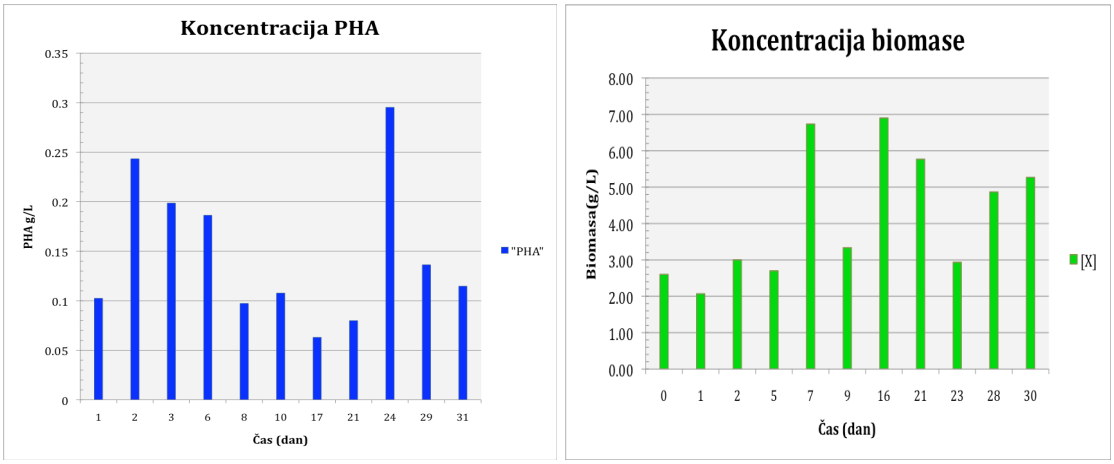
Slike 8, dobljene z uporabo Nile blue tehnike na vzorcih vzetih 22. dan proizvodnje, kažejo, da so bili v sistemu poleg polihidroksalkanoatov (svetle granule) prisotni tudi drugi organizmi, večinoma glive, kar je najverjetneje razlog za visoke koncentracije ocetne kisline v sistemu.



Slika 8 – Slike granul polihidroksialkanoatov, dobljene z uporabo Nile blue tehnike

Najverjetneje so se glive razvile iz spor, prisotnih v odpadni sulfitni tekočini. Lahko rečemo, da je to velik problem pri uporabi cenenih materialov, kot substrata za rast mikroorganizmov, saj so ti materiali odpadki ali industrijski stranski proizvodi, ki so lahko kontaminirani. Če so pogoji ugodni za rast takšnih spor, lahko s časom v sistemu ti organizmi prevladajo. Prav to se je zgodilo v našem primeru po tridesetem dnevu, saj se je v reaktorju na površini pojavila plesen.

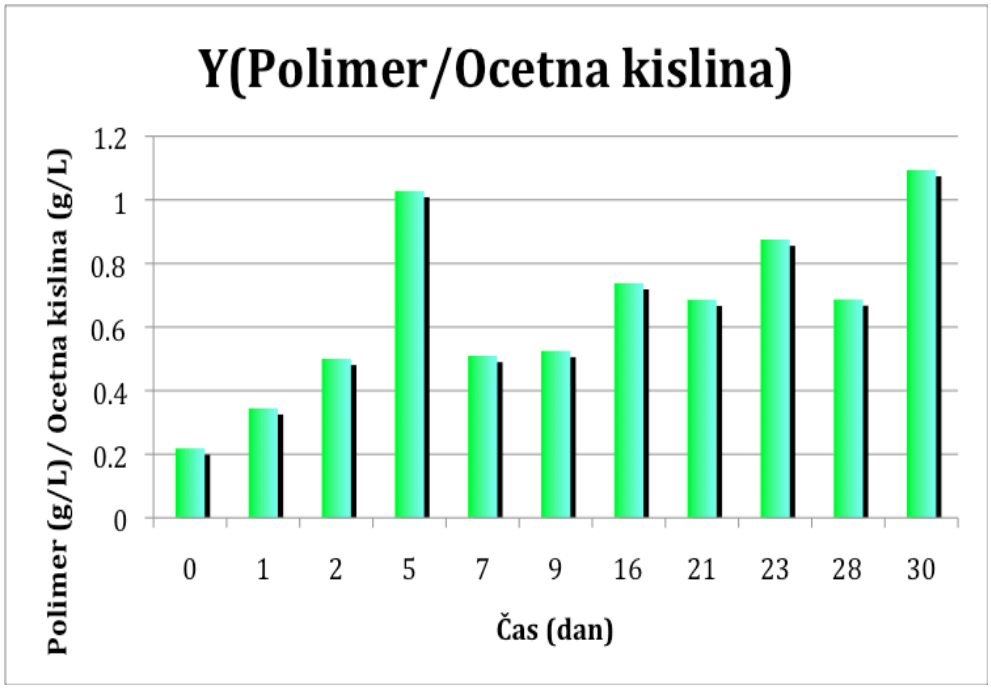
Iz slike 9 je razvidno, da poteka v sistemu tekmovanje med rastjo mikroorganizmov in proizvodnjo polihidroksialkanoatov, saj ko je bila koncentracija biomase visoka, je bila koncentracija polihidroksialkanoatov nizka in obratno. To potrjuje trditve iz razpoložljive literature, ki pravijo da v SBR reaktorju pod pogoji aerobnega dinamičnega hranjenja, poteka tekmovanje med proizvodnjo polihidroksialkanoatov in rastjo biomase.



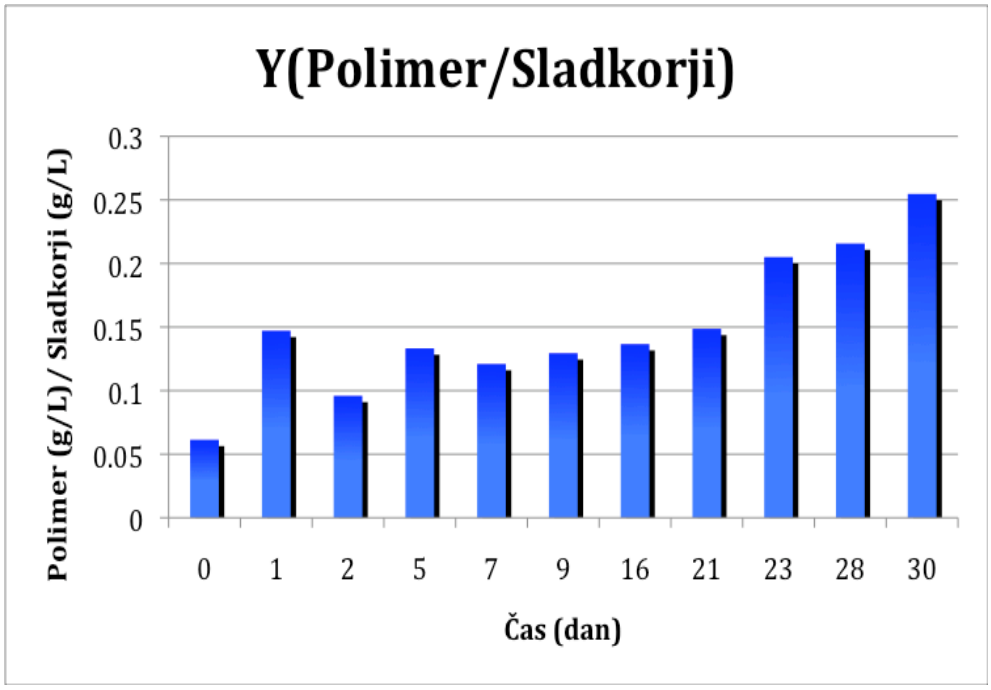
Slika 9 – Tekmovanje med Proizvodnjo polihidroksialkanoatov in rastjo biomase

Kljub zelo kratkemu času delovanja sistema in nestabilnosti mešanih mikrobioloških kultur, je bila najvišja dobljena koncentracija proizvedenih polihidroksialkanoatov štiriindvajseti dan 29 %, kar potrjuje predpostavko, da je proizvodnja polihidroksialkanoatov z uporabo mešanih kultur in odpadne sulfitne tekočine možna.

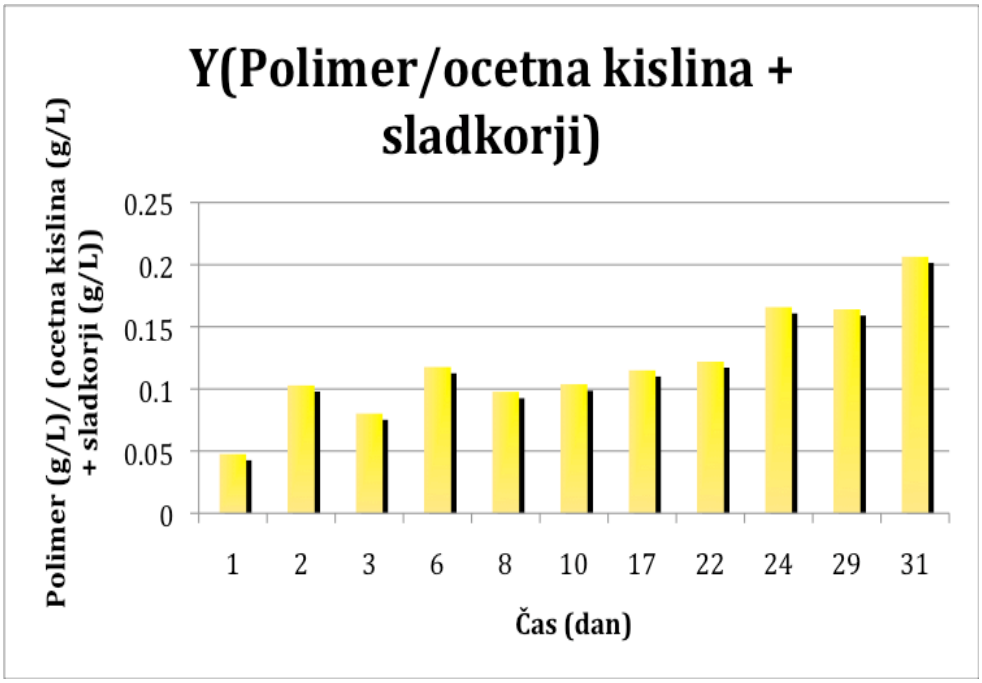
Če pobližje pogledamo rezultate proizvodnje polihidroksialkanoatov glede na vir ogljika (slike 10, 11, 12), vidimo, da k visoki koncentraciji proizvedenih polihidroksialkanoatov ne prispeva samo ocetna kislina, ampak mikroorganizmi za proizvodnjo uporabijo tudi ostale sladkorje prisotne v substratu.



Slika 10 – Proizvodnja polihidroksialkanoatov glede na porabo ocetne kisline



Slika 11 – Proizvodnja polihidroksialkanoatov glede na porabo sladkorja



Slika 12 – Proizvodnja polihidroksialkanoatov glede na porabo ogljika

THESIS IN ENGLISH LANGUAGE

1 INTRODUCTION

1.1 BIOPLASTICS

1.1.1 Definition

Bioplastics are a whole family of high-diversified polymers. Both bio-based polymers (produced from renewable biological sources) and polymers which meet the criteria defined for the biodegradability and/or compostability of plastic products (the latter is defined by EU regulation EN 13432/EN 14995) can be designated as bioplastics. An example of bioplastics are polyhydroxyalkanoates (PHAs), which are both bio-based (made from 100% renewable resources) and fully biodegradable [1].

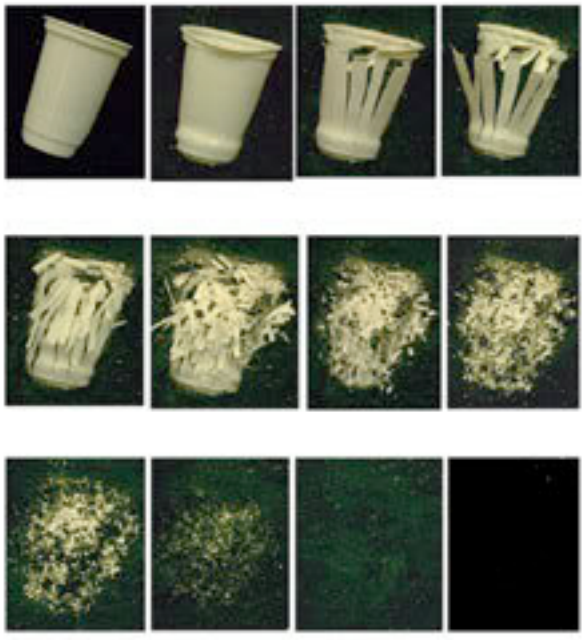


Figure 1 - Degradation of bioplastic [2]

1.1.2 Environmental and economical relevance of bioplastics

The plastics market represents the largest field of application for crude oil outside the energy and transport sectors by consumption of over 200 million tons of plastics yearly all over the world and a growth of 5% annually [1, 2]. The use of chemically synthesized plastics produces millions of tones of waste every year. Only less than 20% can be recycled or burned, most still going to landfills or marine environments [3]. The use of synthetic polymers that dominate the world's current plastic market (PP, PE, PS, PET and PVC) is considered environmentally harmful since they are non-biodegradable. Elimination of these non-biodegradable plastics causes significant problems because their production depends on limited fossil fuels, the availability of landfills is limited, and incineration is bound to the release of greenhouse gases and toxic compounds [4].

Polymers which can be produced from renewable source and which are additionally biodegradable follow a closed loop bio-based to biodegradable (B2B) life cycle [1].

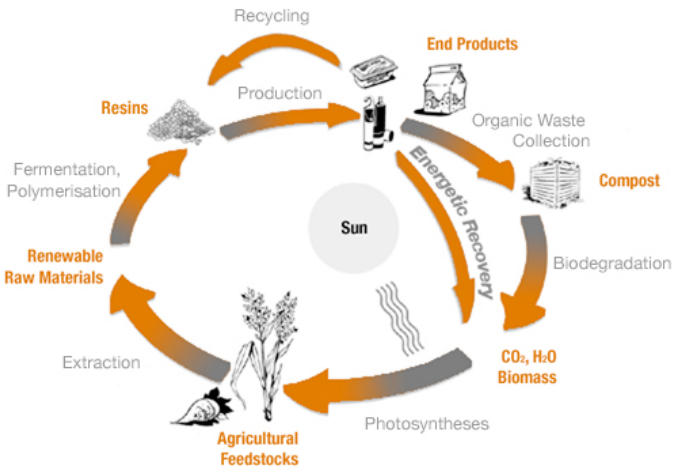


Figure 2 - B2B life cycle [1]

Although the need for biodegradable plastics is increasing faster and faster and has a very high growth potential, the bioplastic market share is still under one percent of the total plastics market (consumption estimate of approx. 50,000 t in Europe in 2008) [1].

The high price of bioplastic (9 €/kg for PHAs vs. 1 €/kg for synthetic plastics [24]), which depends on the substrate cost, yield, and on the downstream process used for polymer extraction [25], was the main reason for slow replacing of the synthetic plastics.

It is possible to reduce high costs of PHAs to 4 €/kg by using low-cost substrates and mixed cultures [5]. Although the price is still 4 times higher, comparing to synthetic plastics, it can be neglected when we consider the problem of pollution with non-degradable plastics [6].

1.1.3 Types of Bioplastics

Bioplastics are characterized by the vast diversity of polymer types and possible applications (Table 1). There are currently three main bio-based polymer types on the market: starch based polymers, cellulose based polymers and polylactic acid (PLA) based polymers. Polyhydroxyalkanoates (PHAs) can be included as an emerging fourth class. Apart from these four main groups, other bioplastics available on the market are mostly mixtures or blends containing synthetic components [7].

Table 1 - Overwiev of currently most important groups and types of bio-based polymers [7]

No.	Bio-based polymer (group)	Type of polymer	Structure/Production method
1.	Starch polymers	Polysaccharides	Modified natural polymer
2.	Polylactic acid (PLA)	Polyester	Bio-based monomer (lactic acid) by fermentation, followed by polymerization
3.	Other polyesters from bio-based intermediates	Polyester	
	Polytrimethyleneterephthalate (PTT)		Bio-based 1,3-propanediol by fermentation plus petrochemical terephthalic acid (or DMT)
	Polybutyleneterephthalate (PBT)		Bio-based 1,4-butanediol by fermentation plus petrochemical terephthalic acid
	Polybutylene succinate (PBS)		Bio-based succinic acid by fermentation plus petrochemical terephthalic acid (or DMT)
4.	Polyhydroxyalkanoates (PHAs)	Polyester	Direct production of polymer by fermentation or in a crop (usually genetic engineering in both cases)
5.	Polyurethanes (PURs)	Polyurethanes	Bio-based polyol by fermentation or chemical purification plus petrochemical isocyanate
6.	Nylon	Polyamide	
	Nylon 6		Bio-based caprolactam by fermentation
	Nylon 66		Bio-based adipic acid by fermentation
	Nylon 69		Bio-based monomer obtained from a conventional chemical transformation from oleic acid via azelaic (di)acid
7.	Cellulose polymers	Polysaccharides	Modified natural polymer
			Bacterial cellulose by fermentation

1.2 POLYHYDROXYALKANOATES

Polyhydroxyalkanoates (PHAs) are thermoplastics synthesized by bacteria. They have similar material properties as conventional polymers, such as polypropylene and polyethylene. In addition PHAs are biodegradable, biocompatible and produced from renewable resources (sugars and fatty acids). PHAs have a significant number of possible industrial applications [8].

1.2.1 Chemical structure

PHAs were discovered in 1926 by French scientist Lemoigne in *Bacillus megaterium* [9]. They can be synthesized and accumulated by more than 250 different bacterias [10-12] as a carbon and energy storage materials known as intracellular granules, whose size and number vary and depend on the type of synthesized bacteria. They are produced under nutrient-limiting conditions in the presence of excess carbon source [10, 26, 27]. The stored PHAs can be degraded by intracellular depolymerases and metabolized as carbon and energy source as soon as the supply of the limiting nutrient is restored [26]. The majority of PHAs has the same composition. They consist of 100 to 3000 hxdroxy fatty acid monomers ranging from C_3 to C_{14} carbon atoms [28, 29]. PHA are subdivided into three groups, short-chain (PHA_{SC}), medium-chain (PHA_{MC}), and long-chain (PHA_{LC}). PHA_{SC} contain three to five atoms in their carbon chain (C_3–C_5), PHA_{MC} contain from C_6 to C_{14} atoms and PHA_{LC} over C_{14} atoms. The best known and studied among these polymers is polyhydroxybutyrate (PHB) which was the first one commercially manufactured [30, 31].

1.2.2 Properties

PHA differ in their properties depending on chemical composition, microstructure and molecular weight distribution [13]. Physical, mechanical, thermal and other properties are shown in Table 2.

Table 2 – PHA properties [13]

PHYSICAL PROPERTIES	RELEVANCE FOR PLASTIC APPLICATIONS	PHA			OTHER BIO-BASED PLASTICS	FOSSIL BASED POLYOLEFINS					
		P(3HB)	P(HB-co-HV)	P(HB-co-HHx)	PLA	HDPE	LDPE	PS	PP	PET	PVC
Molecular weight, M_w	Strength Processability	$1 \times 10^4 – 3 \times 10^6$			$1 \times 10^5 – 3 \times 10^5$	$1 \times 10^5 – 5 \times 10^5$	9×10^4				
Melt flow rate (g/10min)	Processability	5 - 25	15 - 25	0.1 - 100	3 – 6	0.1 – 3.5	0.2 – 3.5				
Density (g/cm^3)		1.20 – 1.26	1.2 – 1.4	1.07 – 1.25	1.25	0.94 – 0.97	0.92 – 0.93	1.05	0.95	1.37 – 1.46	1.39
Crystalinity (%)	Transparency Stiffness Brittleness	Semi-crystalline (40 – 80 %)			Often amorphous	Semi – crystalline					
		55 - 80	40 - 50	Lower than P(3HB)							
MECHANICAL PROPERTIES											
Tensile strength at yield (MPa)	Resistance to permanent deformation	15 - 45	25 - 30	10 - 20	53; 70	25 – 32	15 - 20	42 - 60	12 - 43	55 - 75	50 - 80
Elongation to break (%)	Flexibility	2 - 10	3; 20 - 30	10 – 25	10/100*; 2.4	600 - 900	600	2 - 4	150 - 400	50 - 150	20 - 40
Young's modulus (GPa)	Elasticity / Stiffness	0.9 – 3.5	1.2 – 1.8; 3.2	Several orders of magnitude	0.35 – 0.45; 3.6	0.7 – 1.2	0.15 – 0.45	3 – 3.5	0.6 – 1.2	2.8 – 3.1	2.9 – 3.3
THERMAL PROPERTIES											
Melting temperature, T_m (°C)	Processability	175 - 180	155 - 170	Lower than P(3HB)	85; 120/170	120 - 130	105 - 115	240	170	265	100 - 260
Glass transition temperature, T_g (°C)	T bellow which presents rigid structure	4	Lower than P(3HB)		55 – 65		-30	95	0	69 - 75	82
In use temperature range (°C)		-30 to 120	Similar range than P(3HB)		< 60	0 to 100					
OTHER PROPERTIES											
Water resistance		Yes			No	Yes					
O_2 permeability		Very low (2 x lower than PET, 40 x than PE)			High	Low					
Water vapor permeability		Lower than other bio-based plastics but higher than most polyolefins			High	Low					
Biodegradability		Yes			No	No					

PHAs are available in molecular weights ranging from thousand up to three million [7, 25]. Chain length affects on properties as hydrophobicity, the melting and glass transition temperatures and level of crystallinity. PHAs have a semi-crystalline structure, with crystallinity lying in the range between 40 and 80% [32]. The density depends on the type of PHAs and varies between 1.07 and 1.40 g/cm^3. PHAs have good thermoplastic properties (melting point of PHB close to 180 °C

and glass transition temperature around 4 °C [33]) and can be processed as classic thermoplasts. PHB has a wide use in-use temperature range from -30 °C to 120 °C but is fairly stiff and brittle [7]. Mechanical properties are close to those of polypropylene (PP) except the elongation to break, which is 2 – 10% for PHB and 400% for PP. PHAs are water resistant which makes them different from other bio-based plastics. Also water vapor permeability is lower compared to other bio-based plastics but higher compared to most polyolefins. The oxygen permeability is very low (2x lower than polyethylene terephthalate (PET) and 40x than polyethylene (PE)), which makes PHAs a suitable material for use in packaging oxygen-sensitive products [7].

Some properties of PHAs copolymers are different compared to properties of PHAs. Copolymers with hydroxyvalerate (HV) and hydroxyexanoate (HXx) have lower crystallinity, decreased stiffness and brittleness but flexibility, tensile strength, toughness and melt viscosity are higher compared to PHB. Medium chain length PHAs such as P(3HB-co-3HHx) are elastomere and have much lower melting point and glass transition temperature compared to PHB and are result of chain defects [7].

According to producers of PHAs: Telles, Biomer, PHB Industrial ans Metabolix, biodegradation of PHAs depends on temperature, moisture level and pH in the environment and the composition, cristallinity and surface area of the polymer. PHAs are full biodegradable in anaerobic and aerobic conditions and also at slower marine environments. PHAs are under aerobic condition degraded to carbon dioxide and water and under anaerobic conditions to water and methane [14]. PHAs can be degraded intracellular or extracellular with enzymes called depolymerase and are used as carbon source. Both processes happen in nature when conditions are suitable [34].

1.2.3 PHA Applications

1.2.3.1 Industrial applications

PHAs have many industrial applications and can replace most of fosil-based plastics because of their similar properties. They can be used for daily used things,

such as plastic bags, bottles, plates and plastic cups. They can be used as cover materials to make surfaces water-resistant. Very low oxygen permeability makes them a good material used in food packaging and also as all kinds of foils, films and diaphragms. Because of their piezoelectric properties they are used as pressure sensors for keyboards, stretch and acceleration measuring instruments, for material testing, shock wave sensors, lighters, microphones, ultrasonic detectors, sound pressure measuring instruments, headphones, loudspeakers, for ultrasonic therapy and atomization of liquids. A German company Biomer, produces PHB on a large scale and uses it as combs, pens and bullets. Because copolymers have different properties, there are so much more options for applications. P(3HB-3HHx), developed by Tsinghua University is used to make binders, flexible packaging, thermoformed articles, synthetic paper and medical devices [15, 16].

1.2.3.2 Medical applications

In medicine PHAs are used as cardiovascular products such as pericardial patch for closing pericardium after heart surgery. Non-woven patches can be used as scaffolds in the augmentation of pulmonary artery. Cardiovascular PHA absorbable stents could replace metallic stents and prevent excessive growth of the blood vessel wall. Damaged vessels in arterial or venous system can be replaced with synthetic vascular grafts. Big progress was also made in tissue engineering and regeneration. PHAs are used for heart valves. Barrier membranes are used to create new periodontal ligaments and help periodontal and other wounds to heal faster. PHAs and their copolymers showed promising results in drug delivery (implants, tablets and intravenous carriers) for their biodegradability, biocompatibility and degradation by surface erosion. They can be used for bone tissue engineering to generate new bones or to replace the damaged ones because of properties as mechanical strength, which is similar to that of human bones. Barrier membranes can also be used. Another fields of use are in pro-drugs, nerve repair and urology. Sutures, dusting powders and dressings are used in applications for wound management. There are many applications of PHAs in medicine and with the growing production of PHAs and their investigations the number of applications is still growing [15, 16].

1.2.3.3 Agricultural applications

Like in medicine, PHAs can also be used in agriculture in many ways. They can be used as mulch films, which are biodegradable. Another application is in the controlled release of insecticides. In this case Insecticides are integrated into polymer and are released at a rate depending on the activity on the surface. They can be used for horticulture and vegetable gardening products. Used in bacterial inoculants, they can increase nitrogen fixation in plants [15].

1.3 MICROORGANISMS

1.3.1 Pure cultures and recombinant strains

PHAs can be produced by pure cultures and also by mixed cultures. Industrially, PHAs are produced by pure cultures such as *Ralstonia eutropha*, *Alcaligenes latus*, *Pseudomonas oleovorans, Burkholderia sacchari* and others [17]. To make production less expensive, researchers developed recombinant strains by cloning PHA syntheses from microorganisms. These strains have very promising properties such as rapid growth, high cell density, ability to use inexpensive substrates and simple polymer purification, which simplify production and make it less expensive. It has been reported that production of PHAs by microorganisms with high storage capacity can reach up to 90% of cell dry weight [18]. Although PHAs production by pure cultures gives great results, it also has some disadvantages like high oxygen demand during fermentation and need for equipment sterilization, which mean high price. By using mixed cultures, these problems can be avoided.

1.3.2 Mixed cultures

Because of the possibility to use mixed cultures for PHAs production as alternative to more expensive production by pure cultures, the interest is increasing more and more. Mixed cultures are microbial populations of unknown composition. Like pure cultures, they are able to store quite high capacity of PHAs and are selected by the operational conditions directed by the biological system. They can adapt continuously to changes in substrate and there is no need for sterilization [8, 17].

PHAs production in mixed cultures was mostly studied in relation to wastewater treatment. The system, which removes phosphorus, has alternating anaerobic-aerobic conditions. In the system, there are two kinds of organism present: the polyphosphate accumulating organisms (PAO's) and the glycogen accumulating organisms (GAO's). PAO's synthesize PHAs under anaerobic conditions from external carbon sources and glycogen, and consume the PHA in the presence of oxygen or nitrate for cell maintenance, growth, and glycogen replenishment, but

they have to compete with GAO's for the carbon substrate [17]. Both, PAO's and GAO's are able to store PHAs, but not so high capacities (20% of its dry weight) compared to production by pure cultures. Researchers proposed a microaerophilic-aerobic process, where 62% PHA was stored [17]. In the first step (microaerophilic), oxygen limitation prevents the growth of microorganisms, while carbon is used for PHA production. In the second step (fully aerobic), PHAs are used as carbon and energy sources for growth and maintenance stable [35]. Until now, best results gave a process called "aerobic dynamic feeding" (ADF) or also known as "feast and famine cycle". With this process it is possible to store up to 65% of PHA. This process can be a good alternative for PHAs production by pure cultures because it is simpler and less expensive [17].

1.3.3 Aerobic dynamic feeding

Also know as feast and famine cycle, is a process where PHA is stored by activated sludge under aerobic conditions. It is based on the alternation of periods of excess carbon (feast) and periods of starvation (famine), which generates so called unbalanced growth. Under these conditions microorganisms have to compete for survival [6, 17]. In the system with long periods of starvation only microorganisms with high storage capacities survive, which is good for PHAs production. During the feast cycle microorganisms can use substrate for accumulation of intracellular reserves (PHAs) or for growth. Researchers assume that accumulation occurs when growth is limited by external factors such as a lack of nutrients or internal factors such as an insufficient amount of RNA or enzymes required for growth, which is most generally accepted explanation for the storage phenomena in the feast and famine process. During famine cycle, where microorganisms are starving, the amount of intracellular components (RNA and enzymes) needed for growth is decreasing and stored polymer can be used as energy and carbon source. When these cycles continue, after starvation, microorganisms have again excess of external carbon source and can take it up quickly. It is expected that microorganisms will start to grow but it is not like that. The growth rate does not increase proportionally with the substrate uptake rate. As mentioned before, the main reason for this is that the amount of enzymes required

for storage is lower than amount of RNA and enzymes needed for growth. Under these conditions it is possible to store up to 84% [36] of PHAs, which is really close to storage capacities of pure cultures (80-90%) [17].

ADF metabolism of PHAs production in activated sludge with acetic acid (acetate) as the carbon substrate is shown in Figure 3. From acetate produced acetyl-CoA (two carbons) is partially channeled to the tricarboxylic acid cycle (TCA) for growth, NAD(P)H production and for PHA production. Two units of acetyl-CoA are than condensed to produce acetoacetyl-CoA. Acetoacetyl-CoA is reduced to hydroxyacyl-CoA, which in the end gives desired monomer [17].

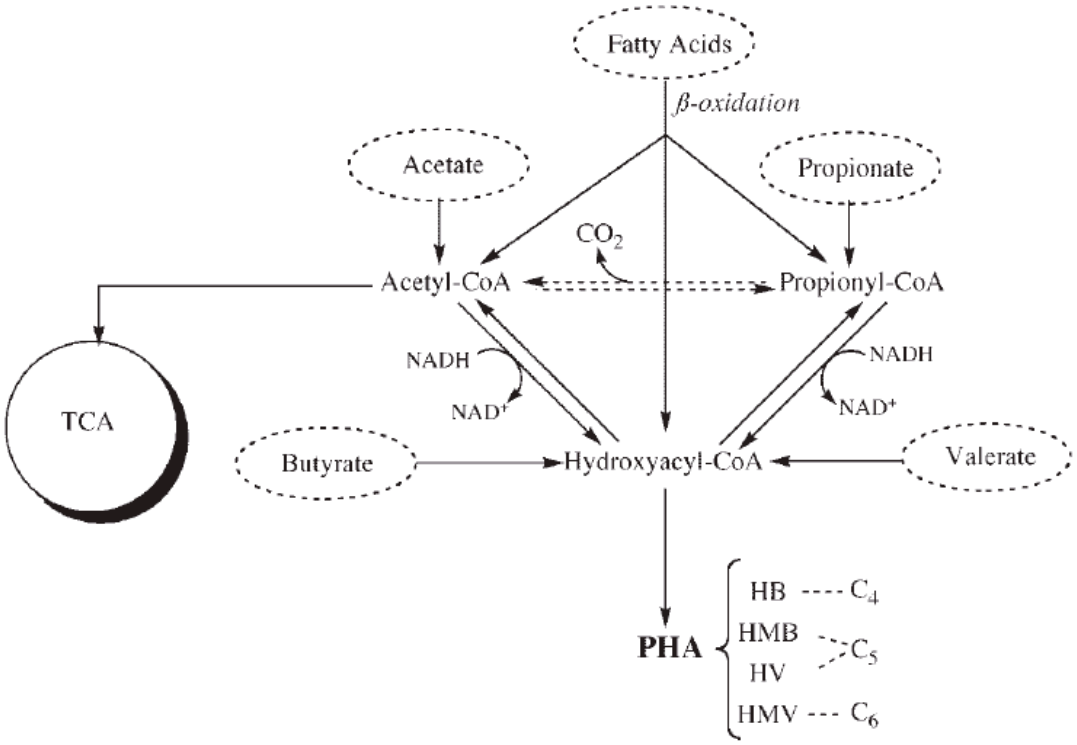


Figure 3 – Schematic representation of PHA production from different fatty acids [17]

1.4 WOOD

Portugal is a country with strong pulp and paper industry. The manufacture of pulp and paper involves many different and varied operations. Since the invention of papermaking, many different fibres have been used for its manufacture, such as fibres of flax and mulberry, the stalks of bamboo and other grasses, cotton and linen rags and straw, various leaf fibres, cottonseed hair, wool, asbestos and the woody fibres of trees. However, wood continues to be the primary raw material from which paper pulp is made [19].

1.4.1 Chemical Structure

Wood is mainly composed of cellulose (40-50%), hemicelluloses (15-30%) and lignin (20-30%). Other substances of low molecular weight are present in small quantities. The relative amounts of wood components vary with the type of wood and the type of cell wall [21].

Table 3 - Main components of wood [37]

COMPONENTS	SOFTWOOD (%)	HARDWOOD (%)
Cellulose	45-50	40-50
Lignin	25-35	18-25
Extractives	3-8	1-5
Hemicellulose (Galacto)glucomannans Xylans	20-25 5-10	2-5 15-30
Ash	0.2-0.5	0.4-0.8

1.5 SULFITE SPENT LIQUORS

Sulphite spent liquor (SSL) is a side product from acidic sulfite wood pulping (Figure 4). After concentrating by evaporation, SSL is normally burned for the base (excepting the calcium base) and the energy recovering [20].

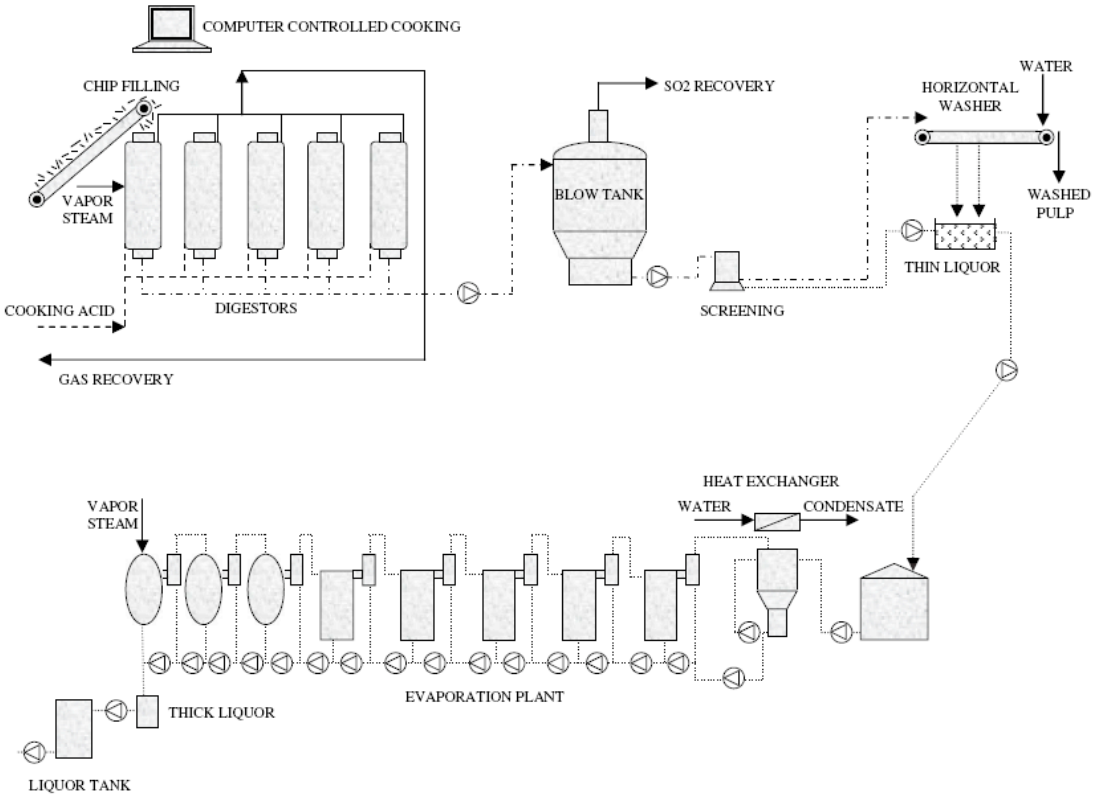


Figure 4 - Sketch of the wood digestion, screening and evaporation in the pulp and paper plant [38]

Concentrated SSL is known also as the thick liquor (THSL). SSL and THSL are recognized as valuable by-products for the production of phenolics, polymer formulations, adhesives, ethanol, single cell proteins, and others [22, 23, 39]. The use of SSL and THSL has quite high potential by decreasing the dependence from fossil resources and improving the economic sustainability of pulp mills. Application of SSL and THSL depend on their chemical composition, which is basically determined by wood origin involved in the pulping process [21]. The sulphonated lignin (lignosulphonates) and sugars are the major SSL components,

which structure and chemical composition varies notably among softwoods and hardwoods.

Table 4 - Main substance groups in sulfite spent liquors [21]

COMPONENT		SOFTWOOD (KG/TON PULP)	HARDWOOD (KG/TON PULP)
Lignosulfonates		480	370
Carbohydrates		280	375
	Arabinose	10	10
	Xylose	60	340
	Mannose	120	10
	Galactose	50	10
	Glucose	40	5
Aldonic acids		50	95
Acetic acid		40	100
Extractives		40	40
Other compounds		40	60

The industrial use of the carbohydrates present in spent sulfite liquors is mainly limited to fermentation processes. The most common product is ethanol fermented from hexoses by yeasts (*Saccharomyces cerevisiae*) [21]. *Pichia stipitis* has also been used to produce ethanol from pentoses (mainly xylose) [40]. Because some contaminants, such as sulfur dioxide, inhibit the growth of the yeast, they must be removed from the liquor prior to the fermentation.

2 METHODS AND MATERIALS

2.1 MATERIALS

2.1.1 Culture

The mixed culture used in the experiment was collected from wastewater treatment plant Aveiro North.

2.1.2 Bioreactor

For PHAs production a sequencing batch reactor (SBR) was used. The total cycle length of SBR was first 12 h and later changed to 24 h because the carbon source was not completely consumed. During the 12 h or 24 h cycle, reactor mixing and the aeration were permanently connected. The feeding period started 15 minutes after each new cycle beginning and finished 15 minutes later. Withdrawing of half of the total volume of the bioreactor occurred in the last 30 minutes of each cycle. In both cycles the hydraulic retention time (HRT) and the sludge retention time (SRT) were the same. The biomass concentration in the bioreactor was kept at 2 – 3 g/L. Pumps for feeding and withdrawing were controlled with timers. Dissolved oxygen values were measured and controlled with Oxygen meter Transmitter M300 (Mettler-Toledo Thornton, Inc). The system worked without pH and temperature regulation, but their values were monitored.

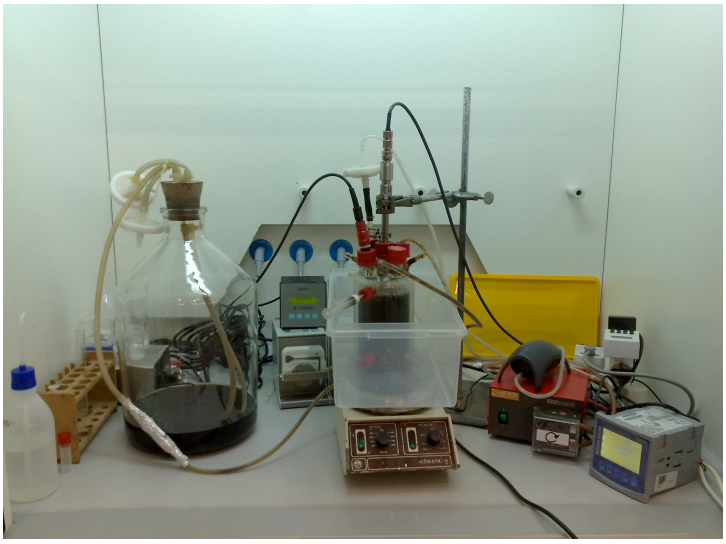


Figure 5 - Bioreactor

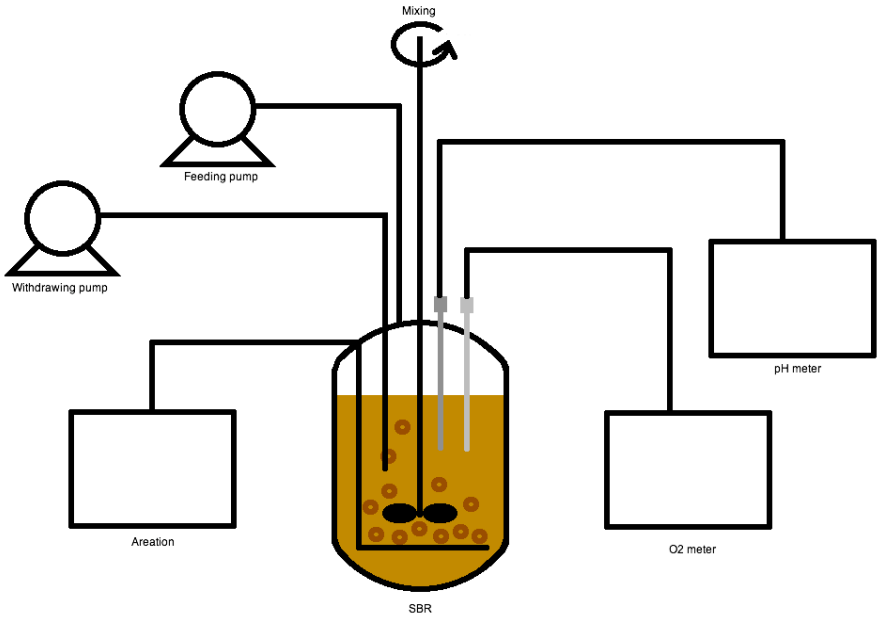


Figure 6 – Bioreactor scheme

2.1.3 Culture medium

2.1.3.1 HSSL pre-treatment

Hardwood spent sulfite liquor from acidic sulfite pulping of *Eucalyptus globulus* was supplied by Caima - Indústria de Celulose SA (Constância, Portugal). Pre-

evaporated HSSL was collected from seventh evaporator. This liquor has a very high pH, inhibiting at microorganisms growth. To lower pH (pH=7), NH_4OH was added due to the fact that the NH_4^+ ion is an important source of nitrogen for growth of microorganisms. After the addition of base the liquor was left to settle down overnight for hydroxides deposition and was than aerated (2 h/L). Aeration is performed for the precipitation of some oxidized compounds present in the liquor, which inhibit microbial growth. The liquor was centrifuged at 2000 rpm for 1 hour and after that filtrated twice. First with fiberglass membrane with pore diameter of 1 μm (Filtrac. Herzberg, Ahlstrom) and than in sterile conditions with 0.2 μm (Cellulose nitrate filter, Sartorius).

2.1.3.2 Medium composition

Culture medium composition is listed in Table 5. Phosphate salts were prepared apart in a small Erlenmeyer, to avoid an irreversible precipitate with magnesium salts. To inhibit nitrification, 2 g of Thiourea were added in the same Erlenmeyer. The flask with magnesium salts dissolved in 8.6 L of distilled water and small Erlenmeyer with phosphate salts dissolved in 200 ml of distilled water were sterilized in Autoclave and supplemented with 1.2 L of pre-treated liquor.

Table 5 - Culture medium

COMPOUNDS	CONCENTRATION (g/L)
KH_2PO_4	0.016
K_2HPO_4	0.064
$MgSO_4.7H_2O$	0.16
$CaSO_4.2H_2O$	0.08
$FeCl_3$	0.04
$Na_2MoO_4.2H_2O$	0.008

2.2 METHODS

2.2.1 Analytical techniques

2.2.1.1 High - performance liquid chromatography (HPLC)

HPLC was performed in order to determine the concentration of acetic acid, xylose and glucose. Samples were filtered using a membrane of 0.2 micrometer (Whatman) at 8000 rpm and injected in a liquid chromatography column equipped with an ion-exchange column packed with Eurokat ® of 10 mm, connected to a pump Gilson 307 and detector Gilson. The eluent was H_2SO_4 0.01 N at a flow of 0.4 mL/min at room temperature.

The calibration curves for the analyzed compounds (glucose, xylose and acetic acid) are attached in Annex 1.

2.2.1.2 Cell dry weight

To determine the biomass dry weight 3 ml of sample was filtrated using dried and weighted filters (Cellulose nitrate filter, Sartorius) with vacuum filtration. The membranes were placed in the oven at 105 °C for 24 hours. After cooling down they were weighted again and with the difference between their masses the dry weight of the biomass was determined in g/L.

2.2.1.3 Ammonia determination

Ammonia was determined using an ammonia gas sensing combination electrode Thermo Orion 9512. After mixing 1 ml of sample with 20 µl of ISA (Ionic Strength Adjuster, composed of NaOH 5 M, 0.05 M disodium EDTA, 10% methanol with color indicator solution), the electrode was introduced into the solution and 5 min later the result was taken. A calibration curve was obtained using ammonium chloride standards.

2.2.1.4 Spectrophotometric method

Determination of the amount of PHB was performed chemically. The pellets were suspended in 1 ml water. To the suspension 2 ml of 2 M HCl was added and

heated at 100 ºC for 4 h in a water bath. The solution was than left to precipitate and the liquid phase was poured away. Five milliliters of chloroform were added to the resulting precipitate. The tubes were left overnight at 28 °C on a shaker at 150 r/min. Then 2 milliliters of solution was dried in the hotte with N_2. Five milliliters of 2 M sulphuric acid was added. The tubes were heated at 100 ºC in a water bath for 2 hours. After cooling to 25 ºC, the amount of PHB was determined on a UV Spectrophotometer, wavelength 235 nm [41].

2.2.2 Microscopic techniques

2.2.2.1 Nile Blue

Nile Blue is a dye widely used in biology and histology. It can either be used to detect polyhydroxyalkanoic acid-accumulating microorganisms or to detect polyhydroxyalkanoic acids in microorganisms through the conjunction with fluorescence microscopy. PHA granules display a strong red fluorescence when stained with this dye.

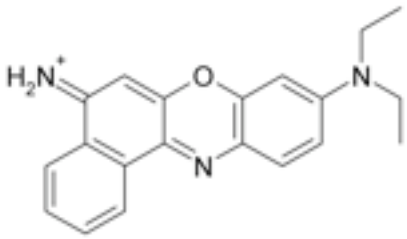


Figure 7 - Structural formula of Nile Blue [42]

A drop of Nile Blue was put in eppendorf containing a sample of the culture medium. Than it was placed in the oven at 55 °C for 10 minutes, centrifuged, discarded the supernatant and placed in 1 ml of sodium chloride 0.9%. The observation was made using epifluorescence microscopy.

3 RESULTS AND DISCUSSIONS

The objective of this work was to select a mixed microbial culture, able to produce PHAs from SSL, a by-product of pulp industry. The microbiological production of PHAs from SSL was performed in a SBR working under ADF conditions. The SBR was running for 72 days, however in this work only the results of the first 30 days were presented, since the system was contaminated with fungus consequently its PHAs storing capacity was reduced. During the period of operation acetate, sugars and ammonium concentration, cell dry weight and PHAs produced were quantified in order to analyze the microorganisms behavior.

Results from ammonium determination didn't make any sense because they were opposite compared results available in literature [43]. The ammonium concentration was increasing during the cycle in SBR or it was alternating but it should decrease. It is possible that samples that we measured were not suitable for the electrode and this kind of measuring, because they contained too much biomass.

At the beginning of the experiment (Figure 8) we see, that values of acetate and sugars were alternating and not decreasing, which means that the carbon source was not completely consumed by the microorganisms.

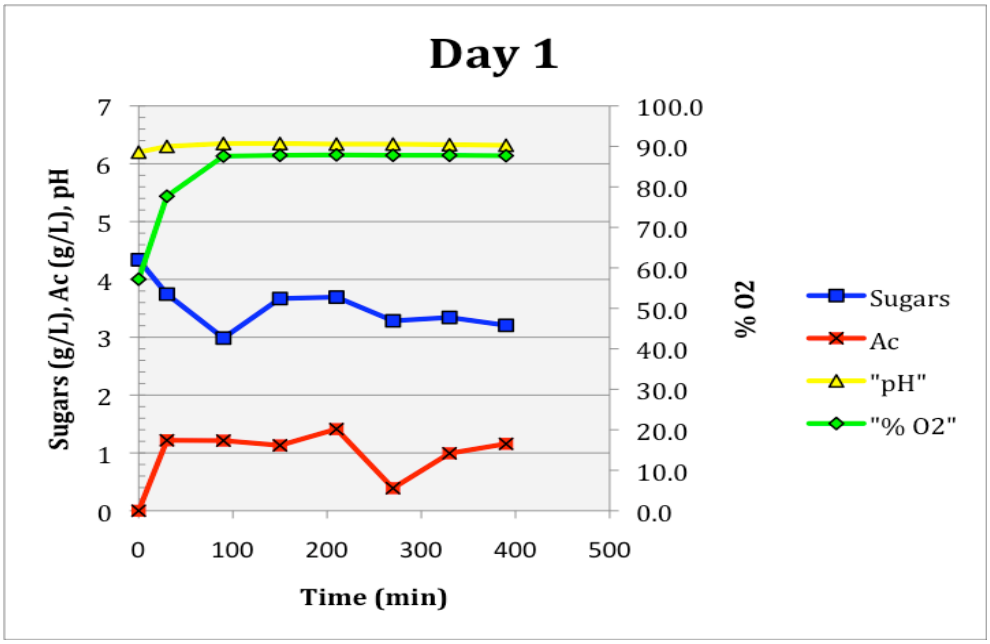


Figure 8 – PHAs production at day 1

This fact was confirmed by the oxygen values that remained high along the cycle. Consequently it was not possible to define clearly feast and famine phases. To achieve the consumption of carbon source, the total cycle length of the SBR was prolonged from 12h to 24h. The pH values were constant the whole cycle.

After changing the length of cycle to 24h, acetic acid started to be consumed as expected but also some xylose was consumed (Figure 9).

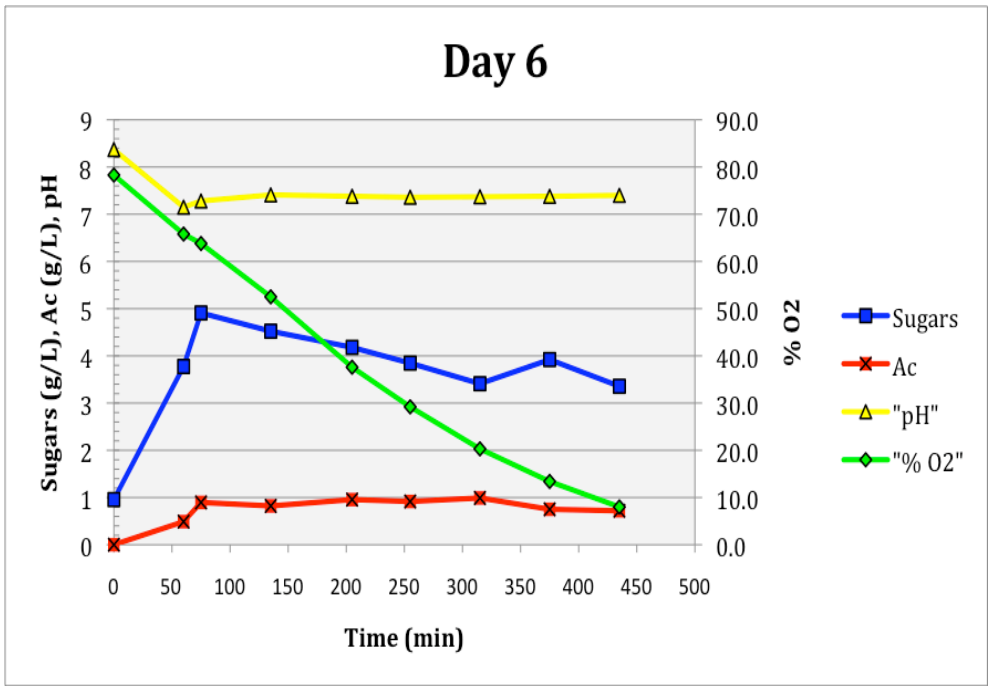


Figure 9 – PHAs production at day 6

Also the oxygen value decreased, confirming the carbon uptake. The pH value decreased the first 60 min of the cycle and was than constant.

In the following days, the microbial culture kept consuming the acetic acid but also consuming the xylose. The decreasing oxygen values are confirming the carbon uptake. This result was not expected since in literature mixed cultures under ADF conditions are supposed to consume volatile fatty acids as acetic acid but the consumption of xylose was never reported. The pH value decreased the first 60 min of the cycle and was than constant (Figure 10).

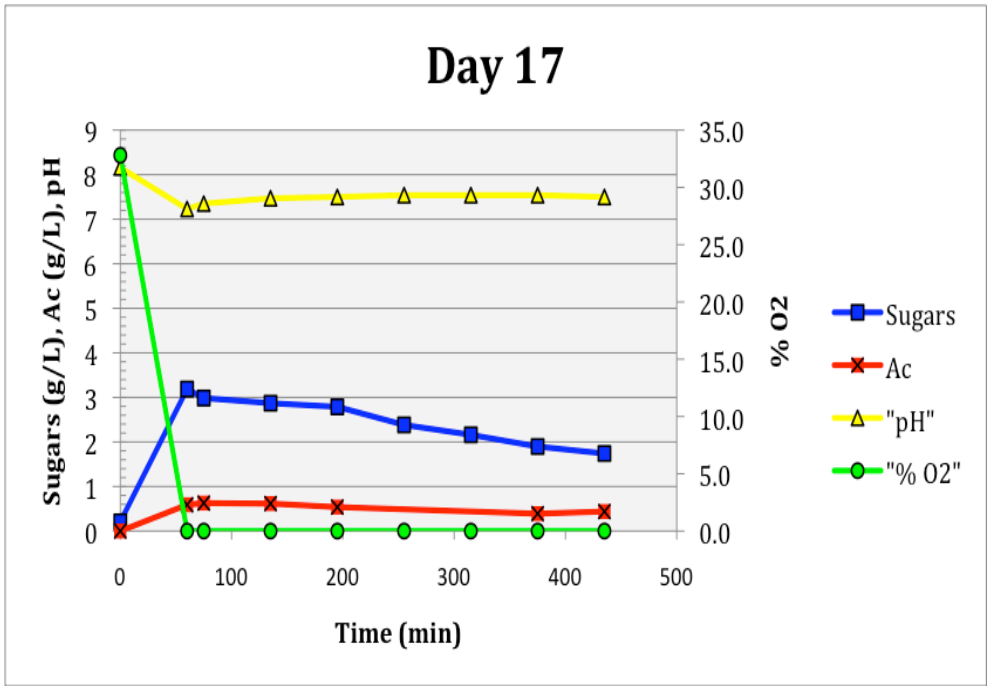


Figure 10 – PHAs production at day 17

On 31st day of experiment sugars started to be consumed in a much higher extend than acetic acid.

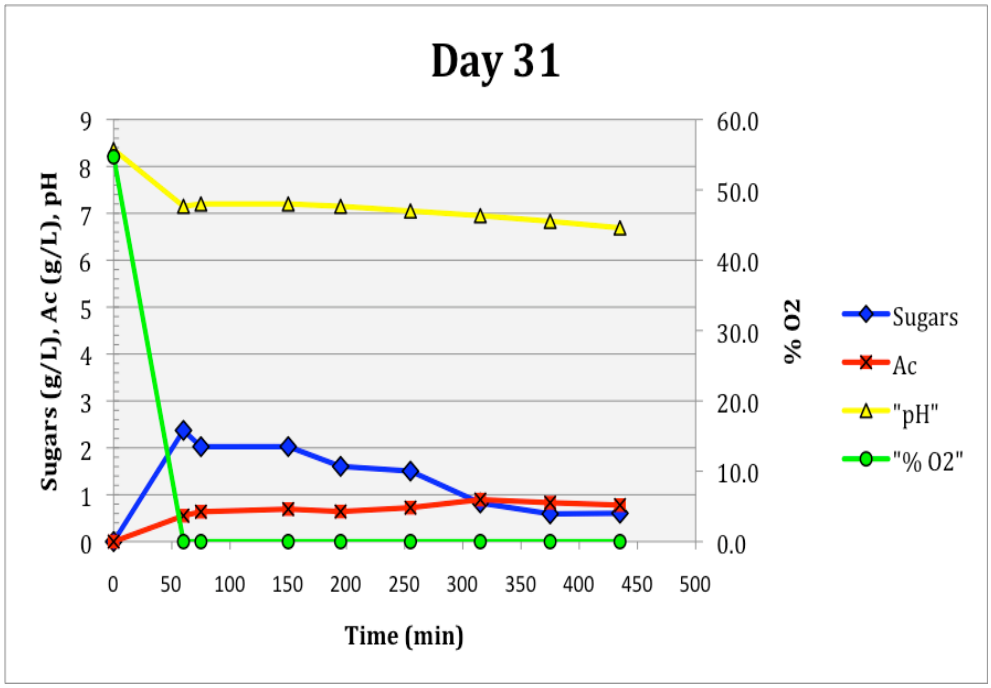


Figure 11 – PHAs production at day 31

Moreover the acetic acid started to increase along the cycle instead of decreasing. It seemed that the mixed culture started to convert xylose into acetic acid. The microbial consortia probably became dominant not in PHA-storing organisms but in fermentative bacteria (Figure 11).

The decreasing oxygen values could be result of carbon uptake or of the fermentation. The pH values decreased with time.

Nile blue staining performed in samples taken in day 22 showed the microbial colonies, which were composed by PHA-storing organisms (brighter signals) but also by other microorganisms that were no evolved in PHA storage (no signal). Some pale and diffuse positive signals were also possible to observe under the microscope not as a consequence of the PHA storage but related to lipidic structures present in some organisms (Figure 12). Most of these organisms are fungus, that were not present in the system inoculum. Probably these fungus came from spores present in the HSSL, since a non sterile real complex substrate was supplied to the system. This is a problem that can arise from the use of real complex substrates. Most of these substrates are wastes or industrial by-products that can be contaminated with spores that would germinate when the substrate is used for feeding a microbial culture. Moreover if the conditions are favorable to the contaminant, it will grow and dominate the original culture, as it seemed that happened in the SBR described in this work. The fungus observed in day 22 become dominant in the system after day 30. This was confirmed by the formation of mold on the reactor liquid surface.

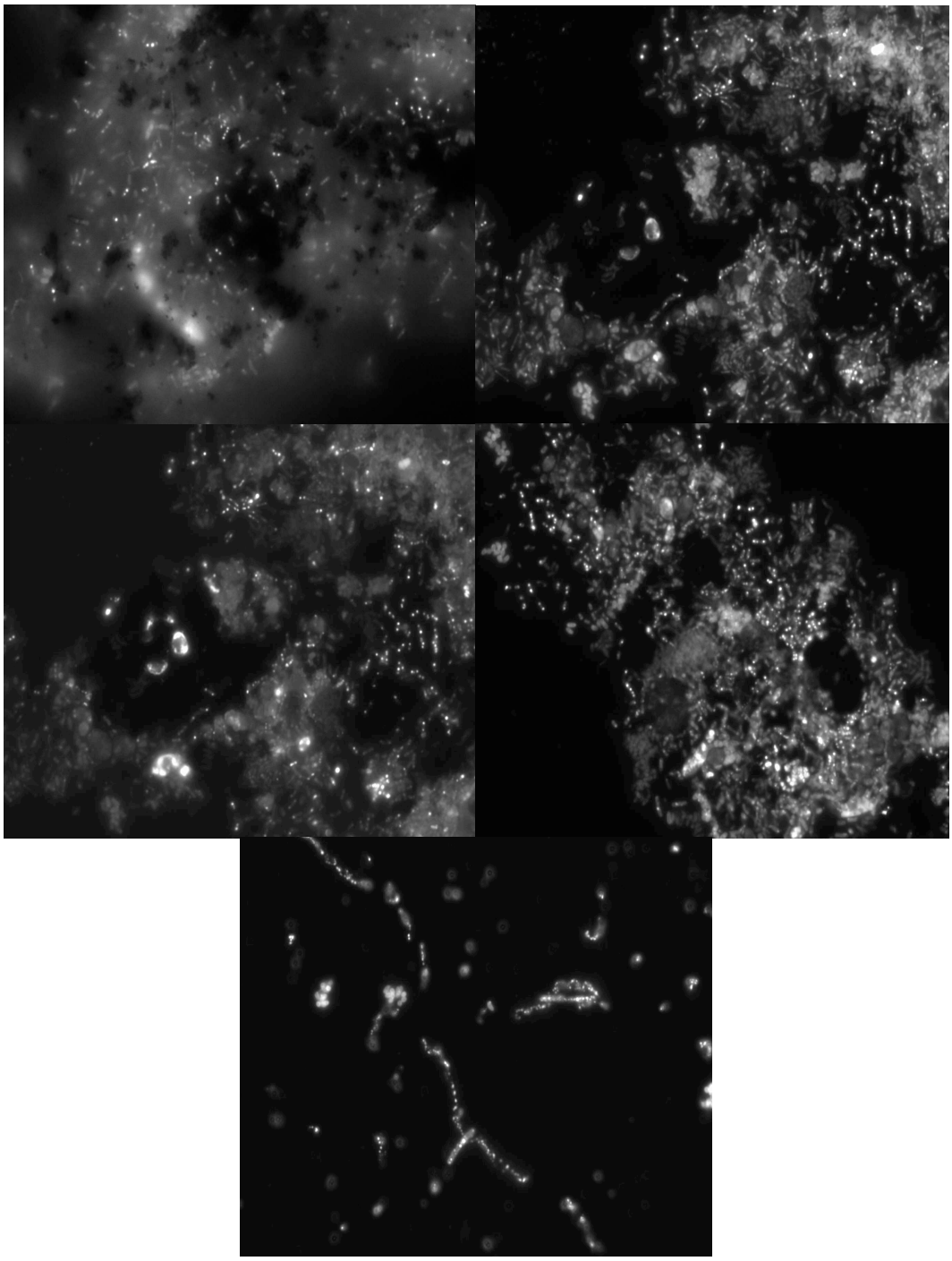


Figure 12 – Nile Blue staining displaying PHA granules and fungus

Along the period of operation of the SBR (30 days) the biomass and PHA produced where monitorized (Figure 13). There is no clear tendency for both parameters. The biomass concentration varied between 2 and 6 g/L and PHA

concentration was in the range from 0.1 to 0.3 g/L. Despite the values oscillation it is possible to observe that PHA and biomass had a opposite behavior: when the value of biomass concentration increased, the value of PHAs concentration decreased and vice-versa. As expected under ADF conditions, PHA storage competes with biomass growth [6].

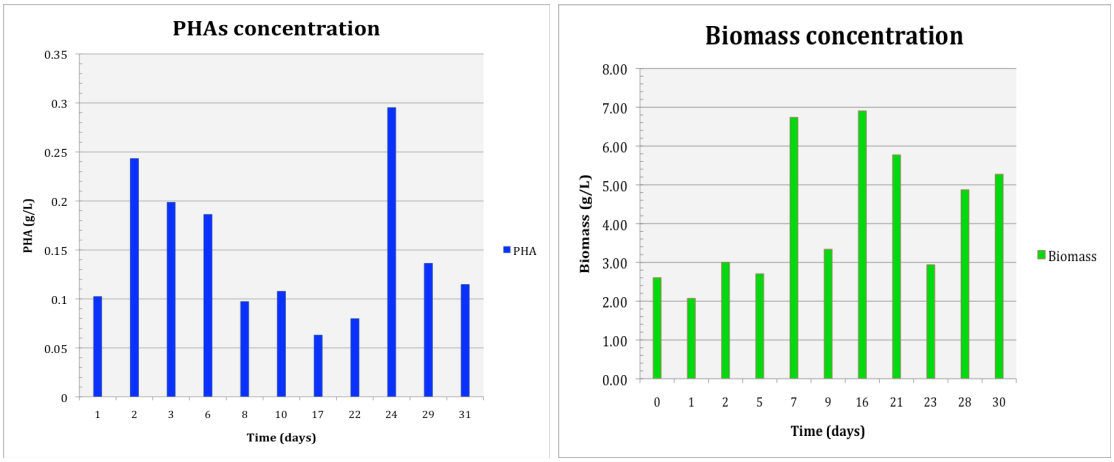


Figure 13 – Competition between PHAs production and biomass growth

Despite of very short period of operation and the instability of the microbial culture, the system present on day 23 a maximum PHA cell content of 29% of PHA. Results for PHA production from both, renewable sources and by mixed cultures, are not available. But if we compare our results to results of PHA production by mixed cultures or PHA production from renewable sources, our results are comparable to results from available literature [17, 18].

If we look closer to yield polymer/acetic acid, yield polymer/sugars and yield polymer/acetic acid + sugars charts, we can see that the quite high amount of PHAs stored in microorganisms is not the reason only because microorganisms used acetic acid for PHAs production but also because they used others sugars present in SSL (Figure 14, 15, 16).

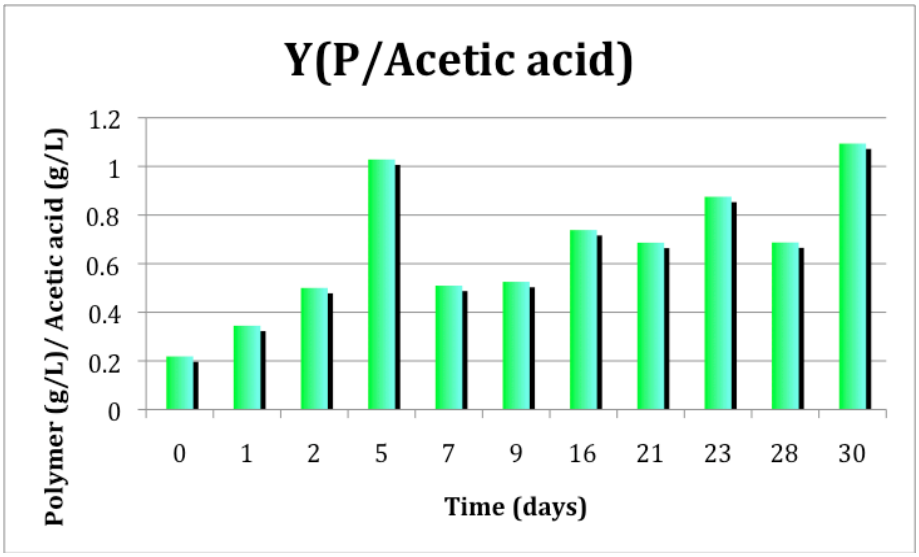


Figure 14 - Production of polymer depending on consumption of acetic acid

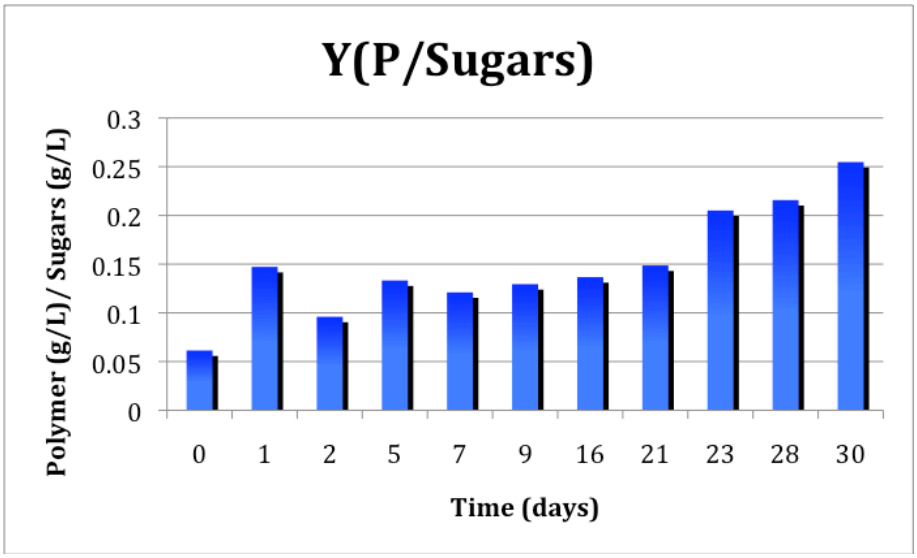


Figure 15 - Production of polymer depending on consumption of sugars

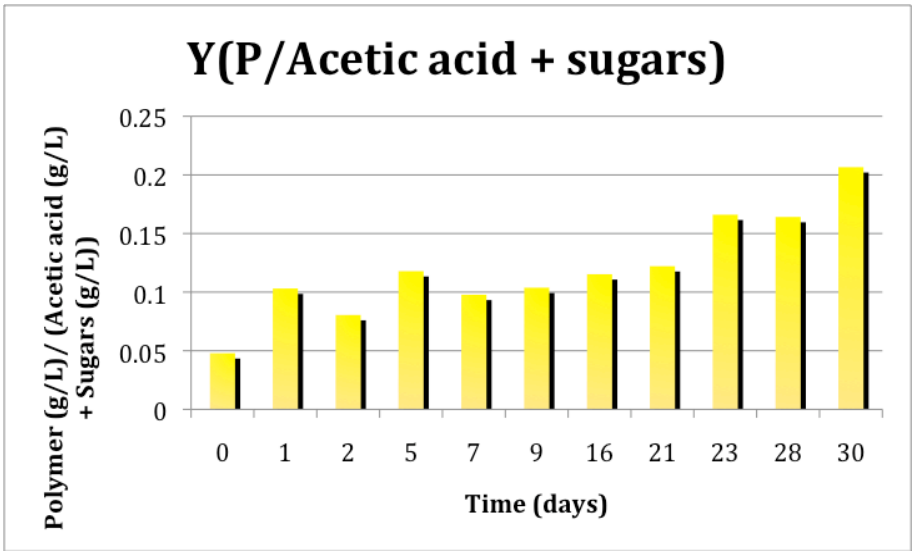


Figure 16 – Production of polymer depending on consumption of acetic acid and sugars

4 CONCLUSIONS

Results presented in this work showed that is possible to produce PHAs from a SSL using mixed cultures. Although the production achieved in this work was not so high in comparison with PHA production using simple substrates, this result can be considered very promising. It is noteworthy that a low-selected mixed culture was able to store 29% of PHA. With the optimization of process parameters and reactor conditions it is probably possible to store much higher amount of PHAs. The use of a cheap raw material as SSL can contribute to the decreasing of production costs of PHB.

Not only acetic acid contributes to PHAs production but also carbohydrates present in SSL. During PHAs production acetic acid is normally consumed and removed from SSL so it can be also used for bioethanol production by *P. stipitis*.

In the future fungus contamination should be avoided by more strict aseptic conditions during pretreatment. The use of filters with smaller pores could remove fungus spores and prevent later contaminations.

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6 ANNEXES

Annex 1 - Calibration curves for glucose, xylose and acetic acid

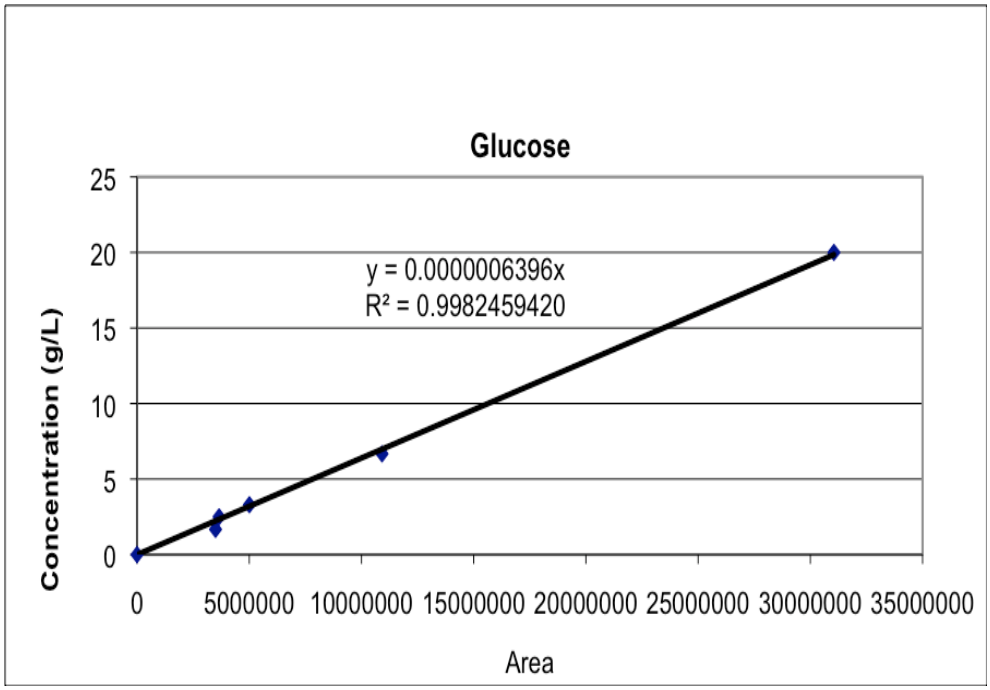

Figure 17 - Calibration curve for glucose

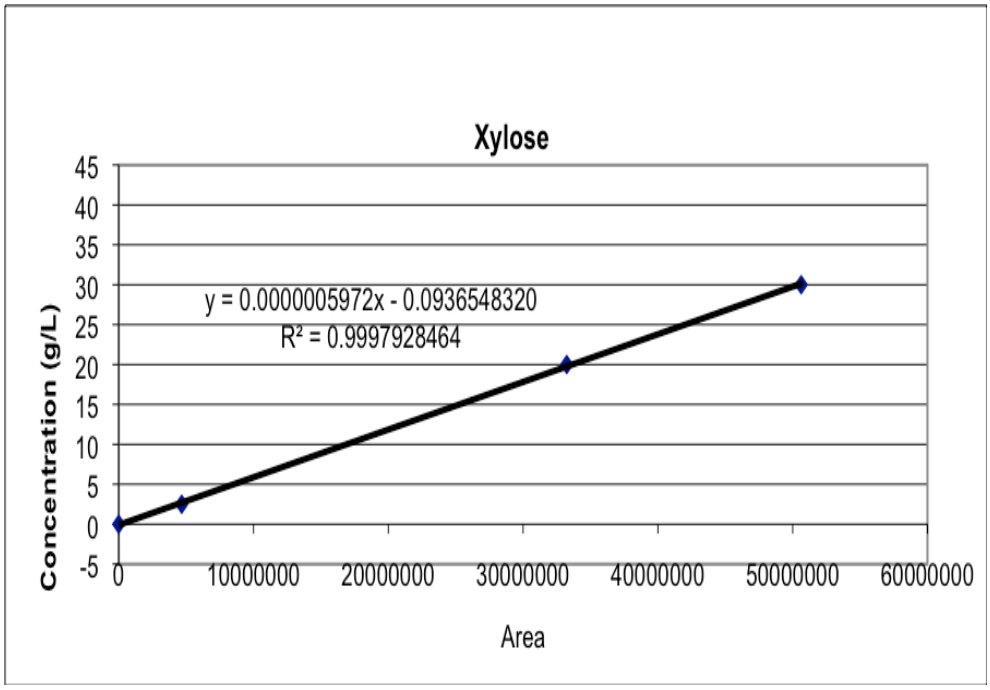

Figure 18 - Calibration curve for xylose

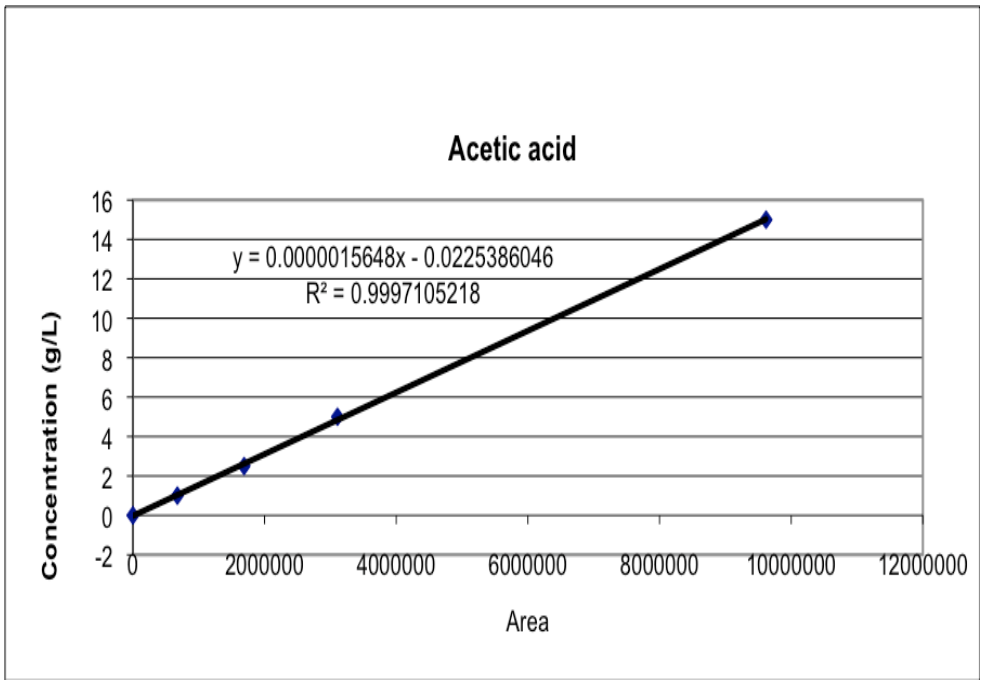

Figure 19 - Calibration curve for acetic acid

Annex 2 - Calibration curve for ammonium determination

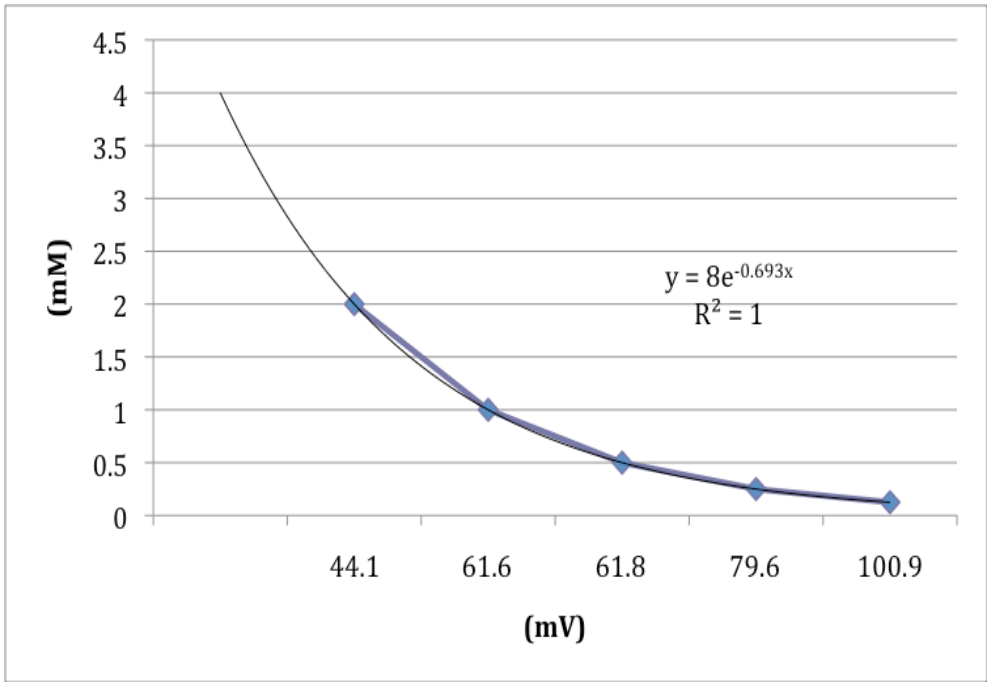

Figure 20 - Calibration curve for ammonium determination

Annex 3 - Calibration curve for PHAs determination by scpectrophotometric method

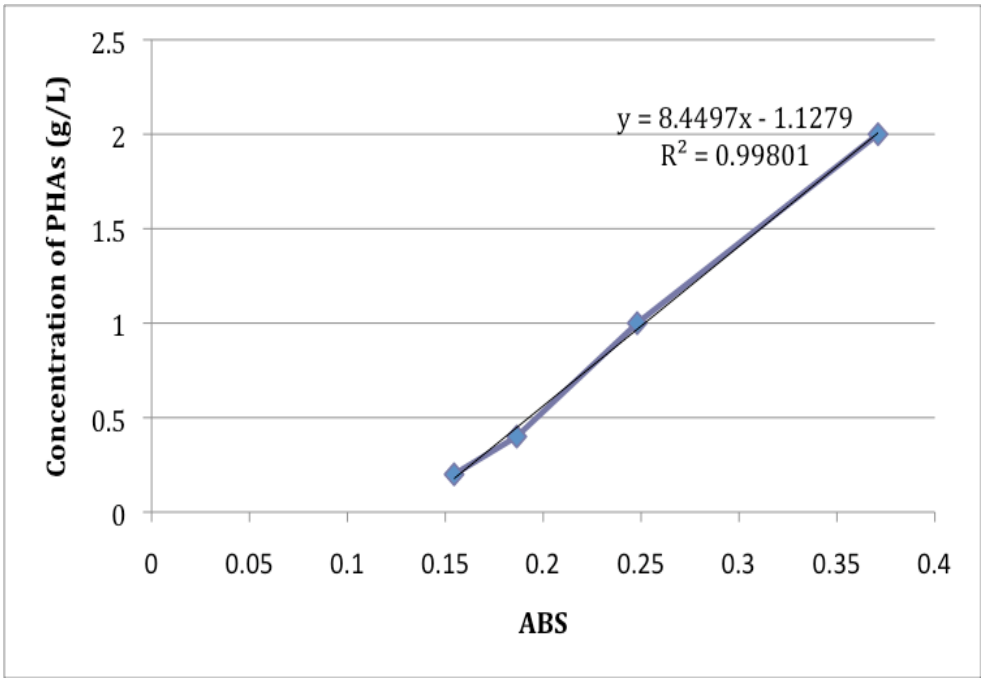

Figure 21 - Calibration curve for PHAs determination by spectrophotometric method