-

Ricardo Jorge Henriques da Conceição

Bachelor Degree in Biomedical Engineering

Resource recovery from used water

Thesis submitted in partial fulfillment of the requirements for the degree of Master of Chemical and Biochemical Engineering

Supervisor: Willy Verstraete, Director, Avecom NV
Professor, Gent University

Co-supervisors: Maria da Ascensão Carvalho Fernandes Miranda Reis, Prof. Dr., Departamento de Química, FCT-UNL

March, 2015

Resource recovery from used water

‘My mother is the most beautiful woman I ever saw. All I am I owe to my mother. I attribute my success in life to the moral, intellectual and physical education I received from her.’
(George Washington)

I dedicate this work to my mother. Thank you for the encouragement!

Abstract

Single cell protein (SCP) defines the dried cells of microorganisms that can be used as a protein supplement both in animal feed or human food. SCP could represent an effective alternative to meet the high and growing demand of feed products.

This work aimed to develop a simple and economically feasible process to obtain SCP by adding value to an industrial waste stream. Spent sulfite liquor (SSL), pure glycerol and crude glycerol and two different types of wood oils were selected. However, the wood oils were early discharged (after characterization) as possible substrates due to their low chemical oxygen demand (COD) content and an unpleasant and undesirable smell. Thus, only SSL and pure and crude glycerol were tested.

Crude glycerol proved to be the most viable choice given the high COD content – - g O_2/L – and the low price per ton of COD – 200 €/ton COD. In continuous mode it was possible to achieve biomass concentrations of - g CDW/L (- g CDW/g COD) while efficiently removing - % of the COD of the influent stream. When tested in batch mode, biomass concentration reached a maximum of - g CDW/L (- g CDW/g COD) after - days of operation. After - days of operation, CDW per liter started to decrease which could indicate some sort of inhibition by fermentation by-products or other toxic compounds.

Considering a batch operation mode, it was possible to produce SCP (- %w/w of protein content) with a commercial price of - €/ton protein. Thus, SCP production was considered feasible since it was possible to obtain a product that can highly compete with other feed products, for example, fishmeal (market price: 1.940 €/ton protein).

Keywords: Single cell protein (SCP), bioreactors operation, COD removal efficiency, economic feasibility.

Resumo

Single cell protein (SCP) são suplementos proteicos obtidos a partir de microrganismos, após secagem, que podem ser usados como aditivo alimentar em ração animal ou para consumo humano. SCP podem representar uma alternativa eficiente para atender à elevada e crescente procura de produtos alimentares para animais.

Este trabalho teve como objetivo a projeção de um processo simples e economicamente viável para obtenção de SCP valorizando um efluente industrial. Licor de sulfito gasto, glicerol (puro e bruto) e dois tipos de óleos foram estudados. Contudo, após caracterização, os óleos foram descartados como possível substrato devido à baixa carência química de oxigénio (CQO) e ao seu odor desagradável e indesejável. Posto isto, apena o licor de sulfito e os gliceróis foram testados.

Devido ao elevado CQO (- g O_2/L) e ao baixo preço por tonelada de CQO (200 €/ton CQO), o glicerol bruto foi selecionado como substrato mais viável. Em contínuo foi possível obter densidades celulares de - g CDW/L (rendimento de - g CDW/g CQO), removendo - % do CQO do influente. Em *batch*, após - dias de operação, a densidade celular máxima obtida foi de - g CDW/L (- g CDW/g CQO). A densidade celular diminui após o segundo dia de operação devido, provavelmente, a inibição por subprodutos de fermentação ou outros compostos tóxicos.

Operando em *batch* foi possível produzir SCP (- % w/w de proteína) com um preço comercial de - €/tonelada de proteína. A produção de SCP foi considerada exequível considerando que se obteve um produto que poderá ser altamente competitivo com outros produtos alimentares para produção animal. Ração a partir de desperdícios de piscicultura, por exemplo, é atualmente vendida a 1.940 €/tonelada proteína).

Palavras-chave: *Single cell protein* (SCP), operação de bioreatores, eficiência de remoção de CQO, viabilidade económica.

Acknowledgements

I would like to take this moment to acknowledge everyone that took part in this adventure with me. It is rather amazing how I spent 7 months living abroad, with no financial aid, and when I returned to my home country I was richer. Not with money. Richer in knowledge. In experience. In memories. In friends.

Firstly, I would like to express my special appreciation and thanks to my advisor Prof. Willy Verstraete, Director of Avecom, who so well received me in Avecom. Thank you so much for your inspiration, guidance and constructive advices. Thank you for giving me the opportunity to finish my master studies in Avecom.

To Prof. Maria da Ascensão Reis, my thesis coordinator, who made the first contact with Prof. Verstraete. Thank you for your support and guidance throughout my master studies.

To all my colleagues at Avecom, thank you for being so helpful and encouraging. A special thank you to engineers Silvio Matassa and Stef Vervaet for their guidance during my internship. A deepest acknowledgment to engineers Filipe Silva and Vanda Branco for being so helpful, even before I arrived Avecom. Thank you to the other interns, especially Irene González Barahona, for being a great colleague and friend.

A big and warm thank you to my parents who always encouraged me to take part in this adventure. Thank you for your support, education and financial aid. Thank you for everything! To the other family members, thank you for your love and support especially during my university years.

My heartfelt gratitude to Ana. More than believing in me, thank you for believing in us. The past months we proved that a 9 years relationship can survive the distance. Thank you for showing me that it is possible to love you even more. I appreciate your patience and your support during my stay in Gent.

To my friends, my other family: Fábio, Gil, Inês, João, Lénia, Mauro, Milton, Rafaela, Raquel and Rute. Thank you for your support and friendship. A special thank you to Inês and Rafaela for their big companionship, mainly during the past two years. Thank you Inês for the brainstorm during this thesis.

Thank you to all the other students I have met during my stay in Gent and shared their experience with me. A special thank you to my *bratu* Blaž: thank you for all the moments we shared and for your support. This adventure would not be the same without you! Also, a big and warm thank you to the amazing Portuguese people (*Seixos*) I have met: Afonso, Cátia, Diana, Isabel, João, Liliana, Maria Inês, Mariana, Patrícia, Raul, Sara, Thibaut, and Tiago. Thank you for all the memories and for your support.

Obrigado! Thank you! Dank je wel!

Table of contents

List of figures

List of tables

List of terminology:

Abbreviations, Units and others

€ - Euro, monetary unit

CDW – Cell dry weight

CFU – Colony forming unit

CG – Crude glycerol

cm – centimeter, unit of length

COD –Chemical oxygen demand

CSTR – Continuous stirred-tank reactor

d – day, unit of time

FAO – Food and agriculture organization

h – Hour, unit of time

HRT – Hydraulic retention time

L – Liter, unit of volume

mL – milliliter, unit of volume

NA – Not available

ºC – Celsius degree, unit of temperature

OLR – Organic loading rate

OPEX – Operational expenditure

PG – Pure Glycerol

sCOD – Soluble chemical oxygen demand

SRT – Sludge retention time

SSF – Solid state fermentation

SSL – Spent sulfite liquor

tCOD – Total chemical oxygen demand

Ton – tonne, unit of mass

TSS – Total suspended solids

VSS – Volatile suspended solids

WO-1 – Wood oil 1

WO-2 – Wood oil 2

Chapter 1

Introduction

World resources for feed production, and thus food production, by conventional methods are hard-pressed to satisfy the needs of an increasing population. *Agriculture alone is unlikely to cover the additional demand for feed protein*. (Hamdan & Senez, 2009).

Population growth and development are increasing the demand, both quantitatively and qualitatively, of animal protein. This is expected to continue as real income grows, particularly in emerging economies, changing the nutritional habits of the population (Food and Agriculture Organization, 2004). With the increasing demand for animal protein supply, the demand for feed, mainly cereals and seed-oil meals, will also increase. The increasing demand for feed are a cause of great concern since the same grains can compete directly, or in the use of land, with grains for human consumption (FAO, 2004; Feed International, 2012). According to the International Grain Council (2014), the 746 million tons of grains used as feed in the crop year of 2010/2011 will increase to a staggering 834 million tons in 2015/2016 due to the increased protein demand and consumption.

Recently, for economic and healthy concerns, an increasing consumption of aquaculture products has been observed and, consequently, an increasing demand for feed for farmed fish and crustaceans. In fact, over the last 30 years aquaculture production was the segment of world food production that grew more rapidly, with the highest growth rate (approximately 6 % per year) (FAO 2012; Feed International, 2012). In 2008, 29 million tons of feed were produced globally for aquaculture production and the forecasts for 2015 are 51 million tons of feed (Feed International, 012). Aquaculture production alone is projected to increase by 90% between 2010 and 2050, according to the International Feed Industry Federation (2013). Hence, there is the need to find alternative, profitable and sustainable feed sources to support the substantial growth in protein

production from livestock and aquaculture. Protein is the key building block for feed formulation systems thus the main focus for feed improvement (FAO, 2004).

Protein production via microbiological synthesis could be an effective alternative for feed production in comparison with conventional agricultural technologies (e.g. soybean cultivation). Microbiological production uses resources more efficiently, requires less arable land, does not depend on climate and does not pollute the environment with pesticides. Proteinaceous biomass, usually called single cell protein (SCP), already outperform plant proteins and are comparable to traditional animal proteins Daramwal & Gaur, 2004; Jay, Loessner, Golden., 2005).

1.1 Avecom NV

This thesis was carried out at Avecom NV located in Gent, Belgium, within a six months period internship.

Avecom NV was founded in 1995 as a spin-off of the Laboratory of Microbial Technology (LabMET) of Prof. Dr. Ir. Willy Verstraete (Ghent University). Avecom NV is now a small and medium-sized enterprise with focus on steering and optimizing microbial and environmental processes. It has expertise in research, development and tailor-made solutions for particular problems related to microbial waste water treatment and soil remediation. The company offers its own product portfolio as well as a wide spectrum of lab- and pilot-scale feasibility studies for valorization of organic side streams, waste water treatment, anaerobic digesters and soil remediation. Avecom NV has a broad know-how in biological concepts thanks to a profound collaboration with different universities, institutes and industrial partners.

1.2 Goals of the internship

The goal of this 6 months work was to gain know-how on the operation of lab-scale bioreactors, namely for production of protein-enriched biomass, and on several analysis methods typically associated with biotechnological processes. In this scope, the goal was to add value to a waste stream in order to develop a new product that Avecom NV could add to their already vast portfolio.

The waste stream to be used was selected according to the bioconversion efficiency of the waste into proteinaceous biomass as well as economic factors (e.g. operation costs and price of final product). The product obtained should be market-competitive.

In order to have a more accurate follow-up of the bioreactors there was also interest in implementing new analytical procedures in Avecom NV.

1.3 Thesis organization

This thesis is organized into 6 chapters with 1 appendix. Chapter 1 provides the introduction, an overview of the company and the goals of the internship. In Chapter 2 is presented a literature review about the current food and feed consumption as well as a review about single cell protein. Chapter 3 presents the materials and methods of this work. In Chapter 4 is presented a standard operation procedure developed during the internship, regarding the determination of carbohydrates in aqueous solutions. Chapter 5 contains the results of the study and respective discussion of results. Finally, Chapter 4 is the conclusion chapter and you can also find some future perspectives of this study.

Chapter 2

Literature review

2.1 World feed panorama

The growing worldwide demand for food of animal origin has been caused by continuing population growth combined with income growth, urbanization and changes in lifestyles and food preferences. According to the Population Division of the Department of Economic and Social Affairs of United Nations (2013), between 2000 and 2011 the world population grew 5,4 % from 6,1 to 7 billion. Within the next 14 years, population is projected to increase by more than 1 billion reaching 8,2 billion in 2015 and further increase to 9,6 billion in 2050. Since population growth and development are correlated with a larger protein consumption, demand for protein, mainly from animal origin, is also increasing (FAO, 2014; Hamdan & Serez, 2009). Between 2000 and 2011, demand for food of animal origin rose from 226 to 291 million tons, representing 42 kg meat per capita per year, and this growing trend is expected to continue in the following years (FAO,

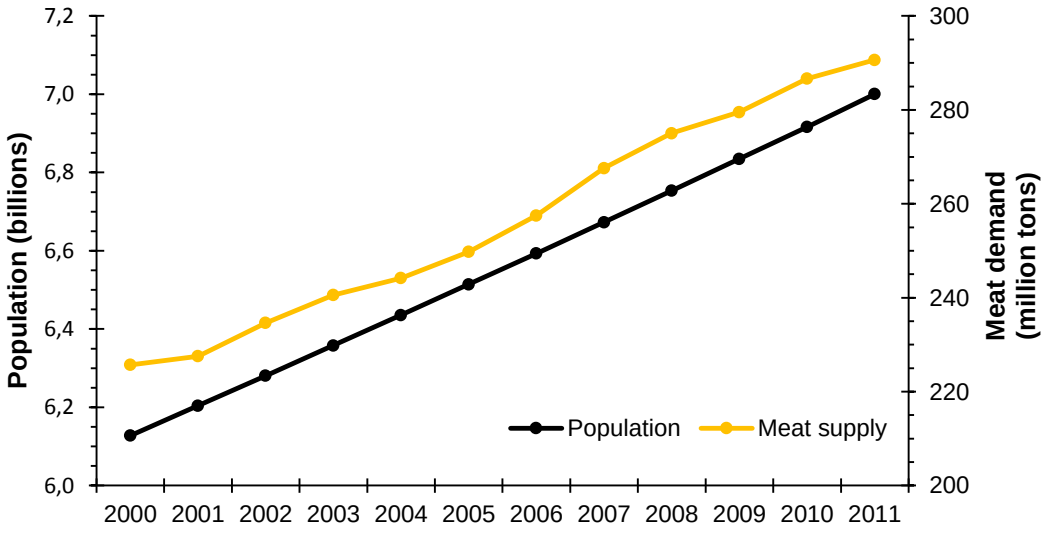

Figure 2.1 – Population, in billion, and meat demand, in million tons, between 2000 and 2011

2014). The graphic of Figure 2.1 shows the population growth between 2000 and 2011 and meat consumption in the same period.

The growing trend of meat demand shown in Figure 2.1, projected to continue in the next years, will severely impact both livestock and crop production systems.

As the demand for meat is climbing, demand over the last few years has been largely met by the worldwide growth in intensive livestock production, particularly poultry, pigs and aquaculture products (FAO, 2006). The growth in this three sectors is due to a shifting towards animals that convert grain into protein more efficiently, representing lower cost products. Considering the four major meat sources (cattle, pigs, poultry and aquaculture), aquaculture is the livestock sector with the highest efficiency grain-into-protein, followed by poultry and pigs respectively, while cattle is the sector with the lowest efficiency of grain conversion (Alexandratos & Bruinsma, 2012; FAO 2014). Cattle requires roughly 7 kilograms of grain to produce 1 kilogram gain in live weight while most species of farmed fish require less than 2 kilograms of grain. Pigs and poultry production require 4 and 2 kilograms, respectively, per kilogram of weight gain (Brown, 2006).

Hence, given the highly efficient conversion of feed into protein, aquaculture production expanded at a compound annual growth rate of 6,2 % within the period 2000-2011 (FAO, 2014), being the sector with the highest growth rate (see Figure 2.2). In the same period, the growth rate of poultry, pigs and cattle was 3,7%, 2,5% and 0,9%, respectively. As shown in Figure 2.2, aquaculture already overtook cattle production, becoming the third most demanded animal protein source.

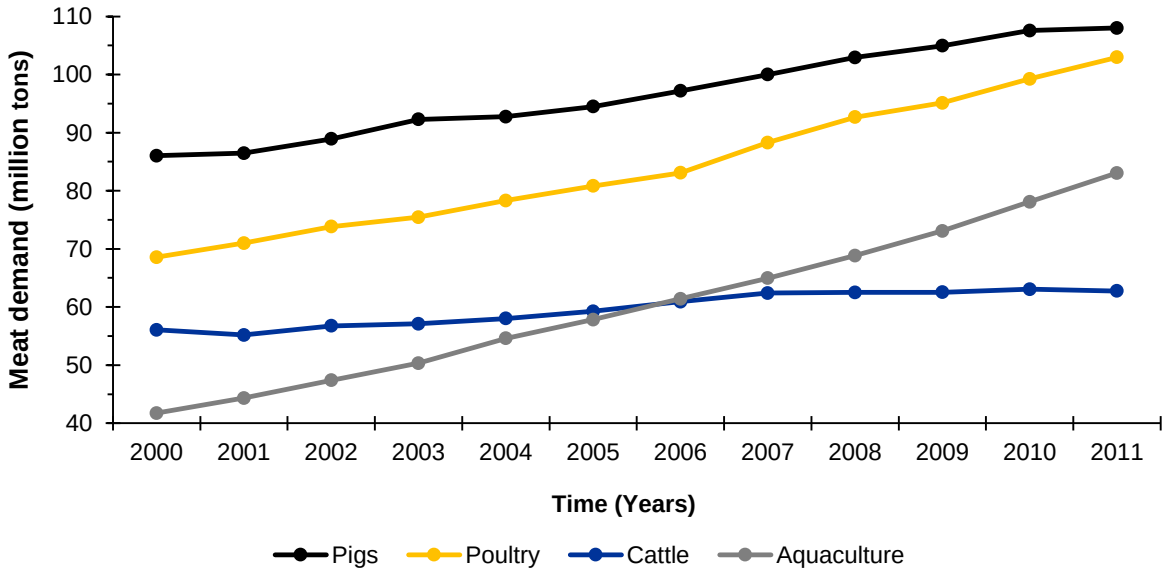

Figure 2.2 – Worldwide demand for livestock (pigs, poultry and cattle) and aquaculture, in million tons, from 2000 to 2011

Although feed production for aquaculture does not currently represent a great amount, given the increasing consumption of aquaculture products, thus increasing demand for feed, special attention should be given to this sector. Production of feed for the aquaculture sector is expanding at a rate of 6-11 % per year, with no signs of peaking, being the most rapidly expanding market

of animal feeds production (Rust, Barrows, Hardy, Lazur, Naughten, Silverstein, 2011; Tacon, Hasan, Metian, 2011). Commercial aquaculture feed sector has grown from 7,6 million tons in* 1995 to 35,3 million tons in 2010 and is projected to reach 71 million tons by 2020 (Feed International, 2012; Tacon *et al.*, 2011). In contrast to commercial aquaculture feeds, farm-made aquaculture feeds, non-commercial, a rough estimate made by Tacon (2008) forecasted a production between 18,7 and 30,7 million tons in 2006.

Feed for aquaculture can be obtained from three different sources: animal nutrient sources (aquatic or land animal protein meals and lipids), plant nutrient sources and microbial nutrient sources. Although animal and plant nutrient sources play the major role in terms of aquaculture diet, they have several disadvantages. Plant sources main concerns are the high occupation of arable land (arable land for feed crops is increasing mainly due to deforestation which has severe environmental impacts) (Alexandratos & Bruinsma, 2012; FAO, 2006) and competition between cereals and oil-seeds for human consumption and feed purposes; besides that, plant sources are highly dependent upon the climate and have other environmental implications (e.g. continuing soil degradation and loss of fertility) (FAO, 2004). Soybean meal, the most common plant source of protein, accounted for 25% of the total aquafeed production in 2008 (Feed International, 2012).

As for the animal sources, considering land animals, they are directly and highly dependent on plant sources; besides, land animal sources are also undesirable considering the severe environmental impacts of massive livestock production (e.g. land degradation, high emissions of greenhouse gases and high water usage) (FAO, 2006). Considering aquatic animals the major concern is the currently highly restrictive policies of fish capture that are slowing down the use of wild fish as feed for both aquaculture and livestock; the competition factor (human consumption and feed purposes), already mentioned for plant sources, is also a major concern (FAO, 2014; Rust *et al*., 2011). Animal sources have an advantage towards plant sources since sometimes plant feedstuffs have more indigestible organic matter (carbohydrates and fibers) that result in higher amount of wastes (Naylor, Hardy, Bureau, Chiu, Elliott, Farrel, 2009).

There is then the need to identify and develop sustainable alternatives to both agricultural and animal nutrient sources that have limited requirements of land, freshwater, nutrients and energy (Diana, Egna, Chopin, Peterson, Cao, Pomeroy *et al*., 2013). Microbial nutrient sources (algae, thraustochytrids, yeasts, fungi and bacteria) are a promising substitute of conventional nutrient sources – animal and plant. Currently, there is only yeast-derived products (brewer's yeast and extracted fermented products), in commercial quantities, available for feed purposes (Naylor *et al.*, 2009; Tacon *et al*., 2011). Single Cell Protein (SCP) are currently seen as one of the major and most promising alternatives to plant and animal proteins. The main reason is the high growth rate and efficiency of microorganisms to produce protein compared to traditional sources (animal and plant) (Jay, Loessner, Golden, 2005).

Despite the potential of SCP to be used as animal feed has been recognized for years, SCP never fully replaced fish meal at production scale (Zee, Logan, Terry, Spear, 2015). The potential to substitute, mainly, plant-based proteins into aquafeeds is high but depends on their relative prices, availability, digestibility and palatability for individual species. To be a viable alternative

the first criteria that should be addressed is the competitive pricing. Since feed costs typically account for 40 to 60 % of production costs of aquaculture products is then imperative to use good quality feeds at reasonable prices to ensure profitability (Hasan & New, 2013; Naylor *et al*., 2009). Other properties should also be present, such as high protein content, nutritional suitability (e.g. favorable amino acid profile), high digestibility and pleasant odor and palatability. Additionally, the product should be ease to handle, ship and store (Naylor *et al*., 2009).

2.2 Single cell protein (SCP)

The term *Single Cell Protein* (SCP) was used for the first time by C. Wilson, professor in the Massachussetts Institute of Technology, in 1966, so the product could be seen more favorably by the target market which could have been reluctant to the idea of a *microbial protein* or *bacterial protein* (Ware, 1977). SCP is the name given to dried cells of microorganisms (algae, fungi, yeast and bacteria), used as protein supplement in animal feed or human food (Najafpour, 2015; Ware, 2007). The term should not be understood only as protein source since it refers to the whole microbial biomass which includes proteins, carbohydrates, lipids, nucleic acids, vitamins, minerals and other cell constituents.

2.2.1 Historical Perspective

Before the term SCP was first introduced in the 60's, a pioneer research project conducted by M. Delbrück (Institut für Gärungsgewerbe, Germany), in 1910, had already showed the value of adding yeast as a feeding supplement for animals. This study would be useful years later in the Word War I, when Germany faced a shortage of grain and replaced as much as half of its imported protein sources by yeast, obtained via batch aerobic fermentation of beet and cane molasses (Nasseri *et al.*, 2011; Rose & Harrison, 1993). In 1919, S. Sak (Denmark) and F.F. Hayduck (Germany) patented a fed-batch process – *Zulaufverfahren* – in which the sugar supply solution (molasses) was incrementally added to an aerated suspension of yeast instead of adding yeast to diluted sugar solution (Rose & Harrison, 1993; Ugalde & Castrillo, 2002). Though the interest in yeast technology declined post-war period, regained interest in 1936 with the Word War II when several types of yeasts were used as supplement for both human food and animal feed (Ugalde & Castrillo, 2002).

Post-war years rose awareness to face and tackle the problems of humanity and a number of international organizations emerged in this scope, under the leadership of the United Nations. Regarding the United Nations, was created the Food and Agricultural Organization (FAO) which highlighted worldwide malnutrition and hunger problems in 1960 by showing that *25% of the world population had a deficiency of protein intake in their diet*. The population growth predictions combined with a prospect that the agricultural production would fail to meet the increasing food requirements of the growing society increased interest in fermentation processes and products

(Hamdan & Senez, 2009; Ugalde & Castrillo, 2002). Relatively low selling price of SCP and abundant substrates (e.g. by-products such as cheese whey, molasses, methanol, hydrocarbon substrates and spent sulfite liquor), with low prices, steered design towards large scale production and low product cost. In the 60's, almost 250 million tons of yeast were being product worldwide in order to compensate agricultural shortages.

Although, SCP ended up by being outmarketed in the late 80's due to an increased agricultural output lead by improvements in plant breeding and crop production and the approval of an open agricultural product trade agreement. With these developments, the price of the major agricultural crops (e.g. soybeans, maize, rice and wheat) did not show the forecasted increase. The decrease observed in the market price of protein of plant origin effectively conditioned the expansion of the promising SCP market and several industrial processes for SCP production were discontinued (Ugalde & Castrillo, 2002). Despite that, in 1985 Quorn™, a SCP-based company, received unrestricted clearance to start marketing its products and, as for 2007, Quorn™ products were currently the only SCP produced exclusively to human consumption (Glazer & Nikaido, 2007; Quorn, 2014).

Nowadays, SCP technology is still an attraction as part of an integrated food system for animal production (poultry, veal and fish) as well as human consumption (e.g. meat substitutes, texture providing agents and flavor enhancers) (Ugalde & Castrillo, 2002).

2.2.2 SCP sources

Bacteria, yeast, fungi and algae are the main sources of microbial protein that can be used as SCP (Anupama & Ravindra, 2000). Microorganisms are an excellent source of SCP due to their rapid growth, ability to grow on inexpensive waste materials and high yield of carbon source-to-protein (in grams of protein per kilogram of waste). Thus, the requisites for a microorganism to be suitable for SCP production are high protein content, high growth rate (high productivity), non-pathogenic characteristics, cellular resistance against medium fluctuations and should be able to utilize complex mixture of carbon sources, with particular interest to waste materials (Cooney & Tannenbaum, 2012; Daramwal & Gaur, 2004; Nasseri *et al.*, 2011). The average composition of SCP, considering the four main sources, is shown in Table 2.1.

Table 2.1 – Composition of SCP (protein, fat, ash and nucleic acid content), in % dry weight, according to the four main sources of SCP: algae, bacteria, fungi and yeasts (Najafpour, 2015).

	Composition (% dry weight)			
	Protein	**Fat**	**Ash**	**Nucleic acid**
Algae	40 – 60	7 – 20	8 – 10	3 – 8
Bacteria	50 – 65	2 – 6	5 – 10	8 – 12
Fungi	30 – 45	2 – 8	9 – 14	7 – 10
Yeasts	45 – 55	1 – 3	3 – 7	8 – 12

Bacteria and yeast are particularly interesting due to the shorter doubling time (5-15 minutes) when compared to algae and fungi (2-4 hours) (Najafpour, 2015). Other advantages of yeasts include their high lysine content, ability to grow at acidic pH and high acceptability by the population (Nasseri *et al.*, 2011). As example of species of yeasts used as SCP are *Pichia*, *Candida*, *Saccharomyces*, *Kluyveromyces* and *Koloechera* (Jay *et al.*, 2005; Nasseri *et al.*, 2011).

Bacteria have the highest protein content, considering the four major SCP sources, and protein with good quality, mainly due to a higher amount of lysine and sulfur-bridge amino acids (Daramwal & Gaur, 2004). However, the high content of nucleic acids and the general public resistance to bacterial products may represent a disadvantage of SCP from bacteria, if considering food purposes. *Cellulomo*nas, *Pseudomonas*, *Methylococcus*, *Bacillus*, *Rhodopseudomonas*, *Lactobacillus*, *Methanomonas* and others, are amongst the genera of bacteria used for SCP production (Anupama & Ravindra, 2000; Daramwal & Gaur, 2004).

Algae are the source of SCP with the lowest nucleic acid content and they also have high protein content. Although, the high production costs and technical difficulties associated with the cultivation, mainly due to the need of light and high surfaces to grow as well as a difficult harvesting process, are big disadvantages when considering cultivation of algae for SCP production. Production costs can be reduced by placing production plants of such cells in areas where sunlight is available most of the year although, this characteristic makes the process of producing SCP climate dependent which is undesirable. *Chlorella* and *Spirulina* are the most reported genera of algae used for SCP production (Daramwal & Gaur, 2004).

Fungi are the source of SCP with the lowest protein content. Additionally, they have slower growth rates, when compared to yeasts and bacteria, and low content of sulfur-bridge amino acids (Daramwal & Gaur, 2004; Nasseri *et al.*, 2011). However, some fungal species have the ability to bioconvert lignocellulosic wastes (Anupama & Ravindra, 2000). *Aspergillus*, *Penicillium*, *Rhizopus*, *Agaricus*, *Chaetomium*, *Fusarium* and *Phanerochaete* are amongst the several fungal species reported for SCP production (Daramwal & Gaur, 2004).

2.2.3 Substrates used for SCP production

The interesting characteristic inherent to SCP production is the flexibility and suitability of microorganisms to convert waste materials into protein-enriched biomass (Najafpour, 2015). Cost-effectiveness production of SCP is directly linked to the substrate used and the bioconversion efficiency into biomass (Daramwal & Gaur, 2004). Hence, there is a great interest in using waste material as substrate for SCP production since their use as substrate contributes for a cheaper production cost as well as control the accumulation of wastes (Daramwal & Gaur, 2004; Najafpour, 2015). Several waste materials have been reported for SCP production including petroleum-based hydrocarbons, methane, methanol, starch, bagasse, manure and animal wastes, wheat bran and straw and other agricultural and industrial wastes (Anupama & Ravindra, 2000; Najafpour, 2015).

Although there were already several industrial processes running on high energy sources (e.g. petroleum based hydrocarbons and methanol) there are several inherent disadvantages

such as price of substrates highly dependent of oil prices, carbohydrates remainders and political and economic decisions (Najafpour, 2015; Trinci, 1992). For instance, in the USA the price of methanol-derived SCP is 2 to 5 times higher than fish meal, making it an uninteresting alternative. Due to the highly competitive soya price and the presence of carcinogens in petroleum-based SCP is no documented company producing petrochemical-derived proteins (Nasseri *et al.*, 2011).

Waste materials as substrates is probably the only way to make a large-scale protein production unit profitable. Waste materials should be selected according to the local availability to avoid extra costs resulting from transportation of substrates over long distances. Constant supply and large volumes of substrate should also be ensured (Daramwal & Gamur, 2004). Lignocellulosic substrates can also represent an extra production cost since it is usually necessary a pretreatment step to release the fermentable sugars (Nasseri *et al.*, 2011). Despite the promising performance of waste materials as substrate for SCP production, selection of domestic wastewaters should be avoided. Zee *et al.* (2014) reported the contamination of SCP products with heavy metals and faecal pathogens due to the processing of domestic sewage.

2.2.4 SCP nutritional value and use limitations

The nutritional value of SCP is based on its composition (protein, fats/lipids, ash, nucleic acids, carbohydrates and vitamins) and is linked to the selected microorganism, the substrate and the growth conditions.

The most attractive characteristic of SCP is their high protein content and balanced amount of enzymes, minerals and vitamins. As drawbacks, mainly when considered for human consumption, there are the rigid cell wall (mainly in algal products), the high content of nucleic acids, allergies, and gastrointestinal effect (Jamal, Alam, Salleh, 2008; Najafpour, 2015). If considering SCP for food purposes, nucleic acids content should be reduced below 2% either by chemical or enzymatic procedures (Nasseri *et al.*, 2011); for the same purpose, the cell wall should also be removed since

Considering the four major SCP sources, bacterial cells produce biomass with the highest protein content while algae products have the highest lipid content (see 2.1.2 SCP sources, Table 2.1) (Najafpour, 2015). As previously said, amino acids and vitamins are well balanced in all SCP products. Vitamins of the B-complex are present in all SCP sources (Anupama & Ravindra, 2000; Jay *et al.*, 2005). Essential amino acids content in SCP from bacteria, yeast and algae is higher than that of plant proteins and essentially similar to animal protein (e.g. casein), as shown in Table 2.2 (Volova & Barashkov, 2010).

Optimal concentration of lysine, an important amino acid, found in SCP and not in major plant proteins (e.g. wheat) indicates that SCP can successfully replace plant proteins and are comparable to animal proteins (Daramwal & Gaur, 2004; Jay *et al.*, 2005). Amino acids content found in SCP is also comparable with FAO guidelines (Anupama & Ravindra, 2000). When it comes to digestibility, SCP slightly yield animal proteins but surpass plant proteins (Jay et al., 2005; Volova & Barashkov, 2010).

Table 2.2 – Amino acid composition, in % dry weight, of SCP from bacteria, yeast and algae and amino acid composition of a standard animal protein – casein (Volova & Barashkov, 2010).

	Amino acid composition (%dry weight)			
	Bacteria	**Yeast**	**Algae**	**Casein (animal protein)**
Lysine	8,61	7,02	5,98	7,33
Histidine	2,48	1,96	1,81	2,20
Arginine	8,00	7,30	7,74	3,19
Asparagine	9,57	10,08	9,49	7,11
Threonine	4,52	5,29	4,88	4,22
Serine	3,47	4,02	4,86	5,72
Glutamic	11,17	12,56	13,12	22,20
Proline	3,46	4,58	5,74	10,41
Glycine	5,47	6,05	6,34	1,88
Alanine	8,80	9,07	9,18	2,96
Cystine	-	0,56	1,37	0,42
Valine	7,13	6,38	5,41	5,72
Methionine	2,69	2,63	2,16	2,47
Isoleucine	4,58	4,47	3,55	4,10
Leucine	8,52	8,60	8,91	9,39
Tyrosine	3,26	3,62	3,13	4,75
Phenylalanine	3,96	4,42	4,41	4,62
Tryptophan	1,24	1,40	1,58	1,32

Hence, nutritive value of SCP is higher than plant proteins and can be used as a substitute or a fortifier of this protein source (Daramwal & Gaur, 2004). The majority of SCP products are found to have a comparable amino acid profile with fish meal (Tacon, Metian, Hasan, 2009; Zee *et al.*, 2015).

Although, when grown on substrates like hydrocarbons and wastes contaminated with heavy metals, several toxins and other undesirable compounds can accumulate which decreases the nutritive value of SCP (Anupama & Ravindra, 2000).

2.2.5 SCP production process

SCP are usually produced by two types of fermentation processes: submerged fermentation and solid state fermentation (SSF), as shown in Figure 2.3.

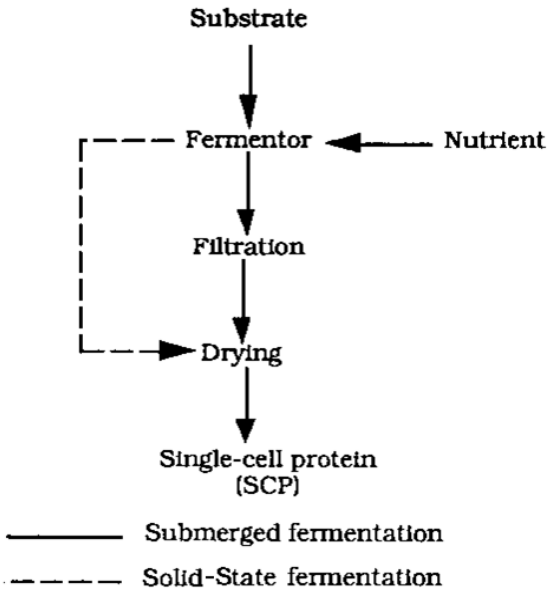

Figure 2.3 – Representative diagram of the two main fermentation processes to obtain SCP: submerged fermentation and solid-state fermentation (SSF).

(Adapted from FAO, n.d.)

In a submerged fermentation process, the substrate to be used is liquid and is held in the bioreactor which is operated continuously. The product is filtered or centrifuged and finally dried, originating the SCP. As for the SSF, is generally used with solid substrates and are more water efficient and have lower operating costs, when compared with submerged fermentation (Chen, 2013; Mitchell, Berovič, Krieger, 2006). SSF can run efficiently with a water content of 60 % (or in the range 12 – 80% depending on the process); on the contrary, submerged fermentation requires a water content of around 95%. SSF needs no centrifugation/filtration to harvest the microorganisms which is related to the reduced operation costs. In Table 2.3 is shown a comparison between both fermentation processes. Although, SSF has several advantages, their use is more suitable for fermentation of lignocellulosic wastes, where simultaneous saccharification and fermentation are desirable. It is also more suitable for SCP production from fungi since higher biomass concentrations are obtained (Mitchell *et al.*, 2006).

Table 2.3 – Comparison between the two fermentation processes usually used for SCP production: submerged fermentation and solid state fermentation (SSF) (Adapted from Chen and Xu, 2004).

Solid-state fermentation	Submerged fermentation
Low water content	Water is the main component of the culture
Microorganisms absorb nutrients from the wet solid substrate; a nutrient concentration gradient exists	Microorganisms absorb nutrients from the mixed liquor; there is no concentration gradient
Inoculation size is large (> 10%)	Inoculation size is small (< 10%)
Required oxygen is from the gas phase; low energy consumption	Oxygen is provided as dissolved oxygen; higher energy consumption due to dissolved oxygen
Microorganisms adsorb or penetrate into the solid substrates	Microorganisms distributed uniformly in the mixed liquor
High production rate and high product yield	Low production rate and low product yield
Mixing is difficult and the growth is restricted by nutrient diffusion	Easy to mix; growth not restricted by nutrient diffusion
Difficult control of temperature	Easy control of temperature
Energy consumption and equipment investment are high	Low investment in equipment; low energy consumption
Low raw material cost	High raw material cost
Little waste organic water	Large amount of waste organic water
Heterogeneity	Homogeneity

2.1.6 Economic viability of SCP production

Estimating cost of SCP product is of extreme importance for the feed market industry. When considering SCP production it is relevant to accurately estimate the associated costs since in most cases the product will be competing with protein sources, mainly of plant origin, and the profit margins are predictably low. Several parameters can be used to estimate the economic viability of the process. In SCP production, with no pre-treatment or purification (e.g. nucleic acids removal) the substrate is the key element and accounts for nearly 62% of the total product cost followed by 19% of fixed costs, related to the production process (Stanbury, Whitaker, Hall, 2000; Ugalde & Castrillo, 2002).

To be market-competitive, at least with plant proteins, SCP produced should have at least the same price. As of December 2014, soybean meal (with 48% of protein), the major source of plant protein used as feed, was marketed in the USA at 340 euros per ton; fishmeal (with 60% protein), one of the prime animal protein sources used as feed, was sold at 1.940 euros per ton (Index Mundi, 2014).

Chapter 3

Materials and Methods

3.1 Microbiome, substrates and nutrients

3.1.1 Microbiome

A hydrogenotroph was used to inoculate the reactors.

The mixed liquors from the bioreactors were periodically harvested (typically 10% of the total volume) and stored in a cold room, for further inoculation. Prior to use, the inoculum was acclimated to 23 ºC (room temperature).

3.1.2 Substrates

Four types of industrial waste streams were tested to determine the best substrate for protein production: glycerol (65% purity from Proviron, Belgium), crude glycerol (technical grade from Delabie, Belgium), spent sulfite liquor and wood oil (Biomass Technology Group, Belgium).

Substrates were previously submitted to several analysis in order to do its characterization, namely, pH, total and soluble chemical oxygen demand (COD), total suspended solids (TSS), volatile suspended solids (VSS), ammonium nitrogen, orthophosphate and Kjeldahl nitrogen. The substrates were stored in a cold room and acclimated to room temperature (23 ºC) before analysis or feeding.

3.1.3 Nutrients

Apart from the inoculum and substrate, the reactor was fed with nitrogen and phosphate sources as well as trace elements. A trace elements solution (1 L) was prepared, according to Yu

et al. (2013) with 0,26 g CH_4N_2O, 0,6 g H_3BO_3, 0,4 g $CoCl_2 \cdot 6H_2O$, 0,2 g $ZnSO_4 \cdot 7H_2O$, 0,06 g $MnCl_2 \cdot 4H_2O$, 0,06 g $NaMoO_4 \cdot 2H_2O$, 0,04 g $NiCl_2 \cdot 6H_20$ and 0,02 g $CuSO_4 \cdot 5H_2O$; 1 mL of trace elements solution per liter of reactor was added. All solutions and mixed liquors were prepared with tap water.

3.2 Reactors operation

3.2.1 Continuous mode

A lab-scale continuous stirred-tank reactor (CSTR) was fed with a diluted solution containing all the nutrients and the substrate (with different concentration), as indicated in Figure 3.1. Aeration was made by means of a sparger of compressed air. The pH of the reactor was kept above 6,75 by automatically adding NaOH. The reactor was operated at room temperature (23 ºC). The reactor was inoculated with 10% of the total effective volume. The inoculum was previously acclimated to the respective substrate.

Sludge retention time (SRT) and hydraulic retention time (HRT) of the reactor are equal. This is guaranteed by feeding the influent continuously and removing the effluent via overflow. HRT was calculated according to Eq. 3.1.

$$HRT = \frac{V}{F} \qquad \textbf{Eq. 3.1}$$

Where:

- HRT represents the hydraulic retention time, in d;
- V is tł the reactor, in L;
- F is the influent flow rate, in L/d;

Organic loading rate (OLR) of the reactor was also calculated, in relation to HRT, as shown in Eq. 3.2.

$$OLR = \frac{C_{feed}}{HRT} \qquad \textbf{Eq. 3.2}$$

with:

- OLR representing the organic loading rate, in g COD/L·d;
- C_{feed} as the desired COD content, in g COD/L, of the influent (feed) stream;
- HRT representing the hydraulic retention time, in d.

By calculating the OLR it is possible to determine the sludge loading rate (SLR) as shown in Eq. 3.3.

Eq. 3.3

$$SLR = \frac{OLR}{VSS}$$

Where:

- SLR is the sludge retention time, in g COD/g VSS·d;
- OLR is the organic loading rate, as mentioned before;
- VSS represents the volatile suspended solids, in g VSS/L;

The amount of substrate to be added to the feed stream in order to achieve the desired COD in the influent was calculated according to Eq. 3.4.

$$V_{subs} = \frac{C_{feed}}{C_{subs}} \cdot V_{feed} \qquad \textbf{Eq. 3.4}$$

Where:

- V_{subs} represents the volume of substrate that should be added to the feed stream to obtain the desired COD content, in L;
- C_{feed} is the desired COD content, in g COD/L, of the influent (feed) stream;
- C_{subs} is the COD content, in g COD/L, of the substrate to be used;
- V_{feed} is the volume of feed, in L, to be prepared for a given test.
-

3.2.2 Batch mode

A reactor with the same characteristics as the one previously described was operated in batch mode. This typology was tested only with crude glycerol, with two different initial organic loads –*. All nutrients and the crude glycerol were provided in the beginning of the batch. The reactor was aerated with compressed air, through an air sparger, and the pH was kept above 6,8 by automatic addition of NaOH, as shown in Figure 3.2. The reactor was operated at 23 ºC (room temperature).

3.2.3 Follow-up of CSTR and batch reactors

Both operation modes were monitored regarding the same parameters: soluble chemical oxygen demand (sCOD), total suspended solids (TSS), volatile suspended solids (VSS), dissolved oxygen (DO) and pH.

Soluble COD was used to calculate the COD removal efficiency (in percentage) of each reactor according to Eq 3.5.

$$\text{COD removal efficiency} = \frac{C_{feed} - C_{ML}}{C_{feed}} \times 100\% \qquad \textbf{Eq. 3.5}$$

Where:

- C_{feed} is the soluble COD in the influent (feed), in g COD/L;

- C_{ML} is the soluble COD in the mixed liquor, in g COD/L.

3.3 Analytical techniques

3.3.1 Total suspended solids (TSS)

Total solids content in the samples was determined by the dry residue method as described by Clescerl *et al.*, 1999. Dry residue contains all suspended solids as well as soluble compounds. The dry residue was obtained after the evaporation of the water contained in a certain sample volume, after drying in a kiln at 103–105 ºC until constant weight. According to this method, a previously dried and empty crucible was weighted, by means of an analytical balance (Sartorius TE64, Germany), and then filled with a certain sample volume. The crucible was then placed in a kiln (Memmert, Germany) at 105 ºC overnight, until constant weight. After drying, the crucible was placed in a desiccator in order to cool down until ambient temperature and then weighted. Total solids (TSS), expressed in g/L, can then be calculated according to Eq. 3.6.

$$\text{Total suspended solids (TSS)} = \frac{m_2 - m_1}{V_S} \qquad \textbf{Eq. 3.6}$$

where: m_2 = weight of the crucible with the sample after drying at 105 ºC (g);

m_1 = weight of the pre-dried crucible (g);

V_S = volume of sample used in the determination (L);

3.3.2 Volatile suspended solids (VSS)

Volatile solids content was determined by the ash content method, as described by Clescerl *et al.*, 1999. The ash content corresponds to the residue left after incineration of the dry residue (previously obtained by the dry residue method) in the muffle oven at 600 ºC ± 50 ºC. Subsequently, the porcelain crucible with the dry matter was placed in the muffle oven LE 4/11/R6 (Nabertherm, Germany) at 600 ºC for 2 hours. The procedure was then the same as for the dry residue method: after 2 hours the crucible was placed in the desiccator to cool down until ambient temperature. After cooling down the crucible was weighted and the ash content (volatile suspended solids, VSS) was calculated according to Eq. 3.7.

$$\text{Volatile solids (VSS)} = \frac{m_2 - m_1}{V_S} \qquad \textbf{Eq. 3.7}$$

where: m_2 = weight of the crucible with the sample after incineration at 600 ºC (g);

m_1 = weight of the crucible with the sample previously dried at 105 ºC(g);

V_S = volume of sample used in the determination (L);

3.3.3 Dissolved Oxygen (DO)

Dissolved oxygen (DO) was measured using a DO meter (WTW Oxi 315i, Germany). DO in the reactors was kept above 2 mg O_2/L.

3.3.4 pH

pH was measured using the pH meter (Consort C535, Belgium).

3.3.5 Chemical Oxygen Demand (COD)

Chemical oxygen demand was determined by two different methods, depending on the samples. Chemical oxygen demand (COD) of mixed liquors was measured photometrically using a spectrophotometer (HACH Lange DR 3900, USA) and easy-to-use kits LCK014 (range: 1.000 – 10.000 mg O_2/L) and LCK 514 (range: 100 – 2.000 mg O_2/L) as specified by the manufacturer; solid samples and substrates were analyzed by means of the reference method. COD standard method is based on the oxidation (destruction) of organic matter by potassium dichromate, catalyzed by silver sulfate. Destruction was followed by a colorimetric titration with a ferroin indicator of the excess of non-reduced potassium dichromate with iron ammonium sulfate. The COD can then be calculated as shown in Eq. 3.8 and expressed in mg O_2/L or, in case of a solid sample, in mg O_2/g sample.

$$COD = \frac{(A - B)\cdot t\cdot 8000}{V_S} \qquad \textbf{Eq. 3.8}$$

where: A = volume of iron ammonium sulfate solution used for titrate the blank (mL);

B = volume of iron ammonium sulfate solution used for titrate the sample (mL);

t = normality of the iron sulfate ammonium solution (N);

8000 = milliequivalent weight of oxygen

V_S = amount of sample used in the determination (L or g);

The normality (t) of the iron sulfate ammonium solution is calculated according to Eq. 3.9.

$$t = \frac{10 \times 0{,}25}{n} \qquad \textbf{Eq. 3.9}$$

with n = volume of iron ammonium sulfate solution for the normality determination (mL).

Mostly of the COD analysis performed were regarding the soluble COD. Analysis of soluble COD are performed in the supernatant of a previously centrifuged sample (usually 20 mL volume) at 10.000 rpm for 10 min.

3.3.6 Ammonium nitrogen

Ammonium nitrogen (NH_4^+-N) was measured photometrically using a spectrophotometer (HACH Lange DR 3900) and the commercial easy-to-use kits LCK302 (range: 47 – 130 mg NH_4^+-N/L), LCK303 (range: 2,0 – 47 mg NH_4^+-N/L) and LCK304 (range: 0,015 – 2,0 mg NH_4^+-N/L), according to the manufacturer indications. In order to remove suspended solids, the samples were previously centrifuged at 10.000 rpm for 10 minutes (Eppendorf 5810, Germany).

3.3.7 Nitrate and nitrite as nitrogen

Nitrate as nitrogen (NO_3^--N) was measured photometrically using a spectrophotometer using the easy-to-use kits LCK339 (range: 0,23 – 13,5 mg NO_3^--N/L) and LCK340 (5 – 35 mg NO_3^--N/L), according to the manufacturers indications.

Nitrite as nitrogen (NO_2^--N) was measured photometrically using a spectrophotometer using the easy-to-use kits LCK341 (range: 0,015 – 0,6 mg NO_2^--N /L) and LCK342 (range: 0,6 – 6 mg NO_2^--N/L) as indicated by the manufacturer. Samples were previously centrifuged at 10.000 rpm for 10 minutes in order to remove suspended solids.

3.3.8 Phosphate as orthophosphate

Phosphate as orthophosphate (PO_4^{3-}-P) was measured photometrically using a spectrophotometer using the easy-to-use kits LCK 348 (range: 0,5 – 5 mg PO_4^{3-}-P /L) and LCK350 (range: 2 – 20 mg PO_4^{3-}-P /L) as specified by the manufacturer.

3.3.9 Kjeldahl-Nitrogen and protein content

Kjeldahl method is used to determine organic and ammoniac nitrogen content of organic and inorganic substances. This method is based on the conversion of organic nitrogen in ammonium nitrogen under the form of ammonium sulfate, by means of destruction using concentrated sulfuric acid (98%) and a Kjeldahl catalyst (catalyst tablet containing 5 g K_2SO_4 and 0,5 mg $CuSO_4.5H_2O$) at high temperatures (400 ºC). After destruction, ammonium is released in alkaline solution as ammonia, distilled and bound as borate. The nitrogen content was determined with an acid-base titration. Nitrogen content, expressed as Kjeldahl nitrogen (Kj-N), was calculated according to Eq.3.10.

$$Kj\text{-}N = \frac{(A - B)\cdot t\cdot MM_N\cdot 1000}{V_S}\cdot f \qquad \textbf{Eq. 3.10}$$

With A = volume of acid (HCl) titrated for the sample (mL)

B = volume of acid (HCl) titrated for the blank (mL)

t = titre of the HCl solution (usually 0,02 N)

MW_N = molecular weight of Nitrogen (g/mol)

V_S = volume of the sample in mL.

f = dilution factor

Protein content of biomass was determined using Kjeldahl method. Although, this method does not measure the protein content directly, therefore a conversion factor (F_K) was applied in order to convert the measured nitrogen concentration to protein concentration, as shown in Eq. 3.11.

$$P=F_K \times N \qquad \textbf{Eq. 3.11}$$

with P = Protein content (mg/L)

F_K = Kjeldahl conversion factor

N = content of nitrogen (mg/L)

The magnitude of the Kjeldahl factor depends on the sample matrix (e.g. amino acid composition of the proteins) but a conversion factor of 6,25 is usually accepted, for simplification reasons.

3.3.10 Total fat content determination

Total fat content in biomass was determined using a lipids extraction method – Soxhlet method. According to this method lipids were extracted from the sample by multiple rinsing out with an organic solvent. The organic solvent used was fat-free petroleum ether (PE) with a boiling point range between 40 and 60 ºC. At the end of the extraction process the organic solvent was distilled out from the extracted lipid. The glass flask was then dried in the oven, at 105 ºC overnight, until constant weight. After cool down, the mass of the extract (total fat) was then measured and the percentage of extractable lipid (EL) in the initial sample was calculated according to Eq. 3.12. The fat content can also be expressed in miligrams of extractable lipids per gram of sample (mg EL/g sample).

$$EL = \frac{(m_2 - m_1)}{m_S} \times 100\% \qquad \textbf{Eq. 3.12}$$

where,

m_2 is the mass, in grams (g), of the extraction flask containing the extracted lipid after solvent evaporation (distillation and oven-drying) and cooled down to room temperature until constant weight;

m_1 is the mass, in grams (g), of the clean extraction flask (tare);

m_S is the initial mass, in grams (g), of the sample used for the extraction.

3.3.11 Carbohydrate concentration

The determination of the carbohydrate concentration was performed using a method implemented and validated in Avecom throughout this work (See 4. Standard Operation Protocol).

3.4 Harvesting, drying and grinding

Reactors were harvested and the mixed liquor was kept in a beaker to let it settle gravitationally for approximately - minutes. The supernatant was then discharged and the settled biomass was centrifuged during - minutes at - rpm using - mL bottles. In order to wash the pellet before drying, the pellet was centrifuged one time with water in the same conditions. Three different drying processes were tested: (1) tumble drying, (2) microwave drying, and (3) oven drying. The dried product was then ground by means of a pestle and a mortar.

3.5. SCP characterization

The characterization of the single cell protein – ProMic pure – was performed according to content of protein (Kjeldahl method), lipids (Soxhlet method), carbohydrates (Dubois method), ash and solubility in water.

3.6 Economic evaluation

Economic viability of the project was evaluated according to calculations regarding the process operation costs (e.g. costs of substrate, base/acid, oxygen, centrifuging and drying) and the price of the final product. The total process operation costs (Total OPEX) and the price of the final product were then compared regarding the substrates tested.

Chapter 4

Results and Discussion

4.1. Characterization of the waste streams

Prior to use, pure glycerol (PG), crude glycerol (CG), spent sulfite liquor (SSL) and two different types of wood oil (WO-1 and WO-2) were characterized in order to evaluate its applicability as carbon source for the reactor. In Table 5.1 are shown the main characteristics (pH, tCOD, sCOD, TSS, VSS, ammonium nitrogen, orthophosphate, total and soluble Kjeldahl nitrogen, color and scent) of the possible suitable substrates for production of proteinaceous biomass.

Table 4.1 - Characterization parameters (pH, total and soluble COD, TSS, VSS, ammonium nitrogen, phosphate, total and soluble Kjeldahl nitrogen, color and scent) of pure glycerol (PG), crude glycerol (CG), spent sulfite liquor (SSL) and wood oils (WO-1 and WO-2).

	Sample				
	PG	**CG**	**SSL**	**WO-1**	**WO-2**
pH					
tCOD (g O_2/L)					
sCOD (g O_2/L)					
NH_4^+-N (mg/L)					
PO_4^{3-}-P (mg/L)					
TSS (g/L)					
VSS (g/L)					
Kj-N_t (mg/L)					
Kj-N_s (mg/L)					
Color					
Scent					

As shown in Table 4.1, pure glycerol, crude glycerol and SSL both have high COD (higher than - g O_2/L) which indicated they were promising substrates for the intended goal; on the other hand, both wood oils (WO-1 and WO-2) had low COD which indicated that these two substrates would not be a good choice as carbon source for the reactor.

4.2 Waste stream selection

In order to be selected as suitable substrates, there were some requirements that the waste streams had to meet: (1) high COD content; (2) no presence of toxins or other potential harmful compounds; (3) low cost per kilogram of COD; and (4) high availability (high volumes, constant supply and no rupture of stocks). The COD content is shown in Table 4.1. Regarding this parameter, as previously explained, WO-1 would be the stream to be early discarded due to a low COD content. Although, since both wood oils had an unpleasant smell they were both discarded as possible substrates, since it is an unwanted characteristic of feeding products.

The presence of toxins is hard to determine therefore this parameter was not used to decide the suitability of the streams as substrate. Since all the waste streams were readily available, their availability could not be used as an excluding factor. Considering the price of the different waste streams (in euro per ton of COD), crude glycerol appeared to be the most promising waste stream given that it had the lowest price per ton of COD (200 €/ton) comparing to 350 €/ton and 360 €/ton for PG and SSL, respectively. Although PG was purchased at 250 € per ton the market price had to be discussed with the supplier. The initial market price of PG was 650 € per ton which would be unfeasible. In Table 4.2 is shown the price overview of the substrates tested: PG, CG and SSL.

Table 4.2 – Price overview (as €/ton and €/ton COD) of the substrates tested.

Industrial waste stream	Source	ton COD/ton stream	Price (€/ton)	Price (€/ton COD)
PG	Proviron	0,72	250	350
CG	Delabie	0,67	135	200
SSL	Greensource	0,55	200	360

Comparing the characteristics of the five acquired waste streams, the substrates selected to be tested were the pure glycerol, crude glycerol and SSL. A mixture of pure glycerol and SSL was also tested.

4.3 Bioreactors operation

4.3.1 Continuous mode

CSTRs were tested since it is one of the most widely typologies used for SCP production. Continuous operation was only tested with SSL, PG and an equal mixture of both. The reactors were followed-up during several weeks in order to achieve a steady-state biomass, thus protein, production. Previously to this work, the CSTRs were operated under the same process conditions, as evidenced in Table 4.3.

Table 4.3 – Previous process conditions of the CSTRs operated in Avecom

Parameter
Volume (L)
Flow (L/d)
Substrate concentration (g COD/L)
HRT (d)
OLR (g COD/L·d)
C/N/P

Although this organic concentration – - g COD/L – was not inhibitory of bacterial growth, the substrates were too recalcitrant (results not shown). Therefore the initial substrate concentration provided to the reactors was decreased from - g COD/L to - g COD/L. Since there was also a high amount of ammonium nitrogen left in the effluent (results not shown), the nitrogen source was reduced. The C/N/P was then changed from -/-/- to 100/5/1. The flow rate was increased from - L feed/d to - L feed/d. The reactors already showed a constant performance to the previously imposed - g COD/L·d. Thus this one was increased to - g COD/L·d.

4.3.1.1 Spent sulfite liquor

Two tests, with different feed concentrations were performed with spent sulfite liquor. Feed solutions were prepared with - g COD/L (- mL SSL/L) and - g COD/L (- mL SSL/L). Feed flow rate, HRT, OLR and C/N/P were kept constant in both tests. Ammonium chloride was used as nitrogen source and dipotassium phosphate was used as phosphate source. The - L CSTRs fed with SSL were tested according to the process parameters described in Table 4.4.

Table 4.1 - Process parameters of the SSL–reactor fed with 20 gCOD/L (A) and 10 g COD/L (B).

Parameter	A	B
Flow (L/d)		
Feed concentration (g COD/L)		
Nitrogen (g N/L)		
Phosphate (g P/L)		
HRT (d)		
OLR (g COD/L·d)		
C/N/P	100/5/1	100/5/1

The results of the follow-up, namely the COD and the COD removal efficiency, of the CSTR fed with - g COD/L are shown in the graph of Figure 4.1.

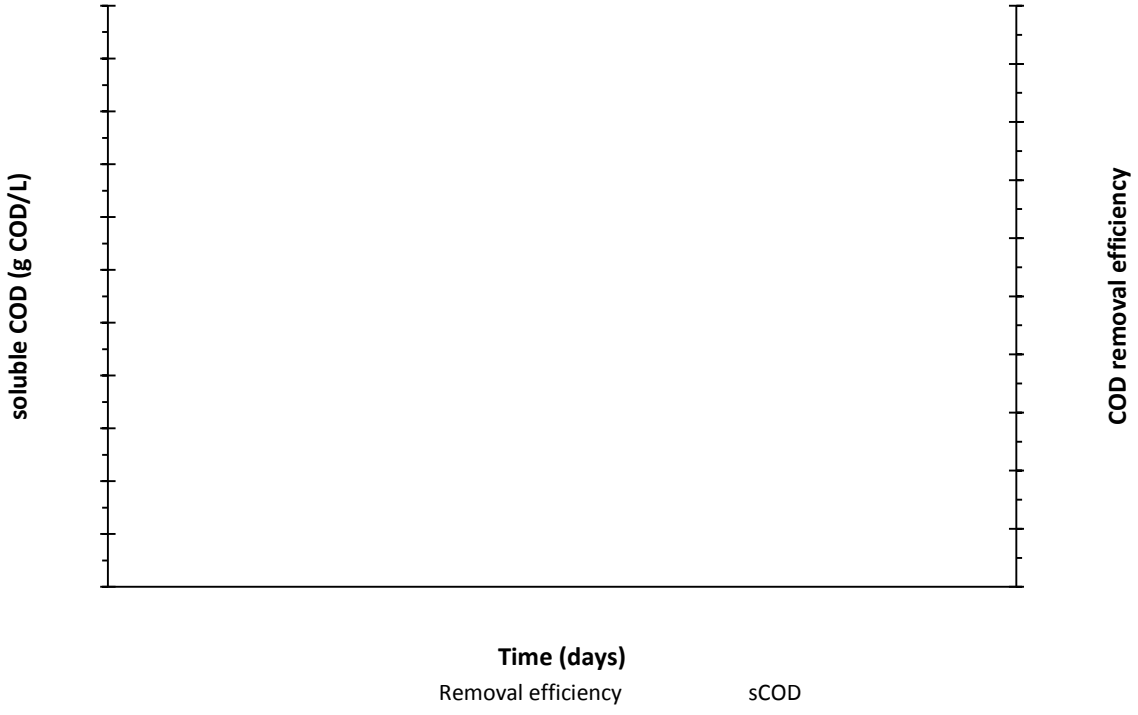

Figure 4.1 – Follow-up of the SSL-fed reactor (20 g COD/L): soluble COD (g COD/L) and COD removal efficiency (%).

As evidenced in Figure 4.1, the highest SSL consumption rate was obtained between day - and day - (removing - % of the COD of the influent). In the following days, the COD removal efficiency continued to increase, although at a slower rate. It reached a maximum peak at day -

with - % of COD removal from the influent. After day -, the consumption of substrate stabilized around - g COD/L. In Figure 4.2, is shown the cell dry weight (CDW) as well as the biomass yield.

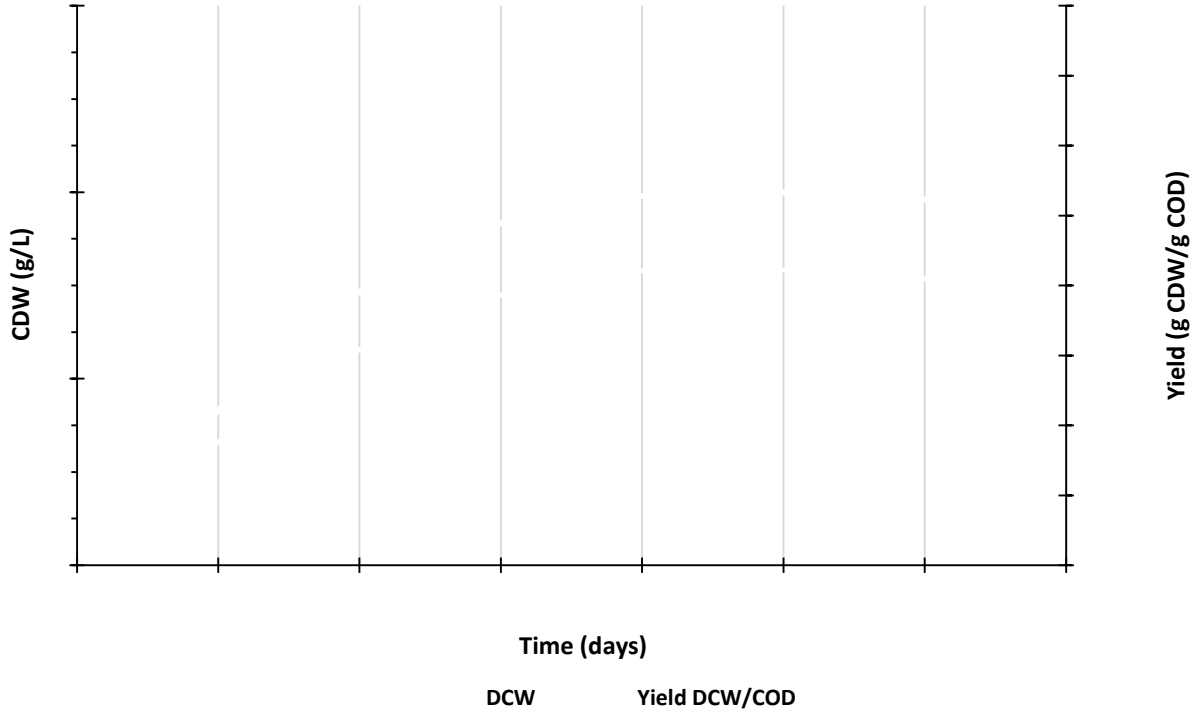

Figure 4.2 – Cell dry weight, in g CDW/L, and yield of biomass, in g CDW/g COD

Maximum cell dry weight – - g CDW/L – was achieved in the - day of operation. This corresponded to a yield of approximately - g CDW/g COD. The maximum yield obtained was within the range usually reported in literature (Guo & Olsson, 2014; Helle, Lin, Duff, 2008). After day -, biomass concentration showed a decreasing tendency.

A test with a stream with lower COD content (- g COD/L) was also performed. The results are shown in Figure 4.3.

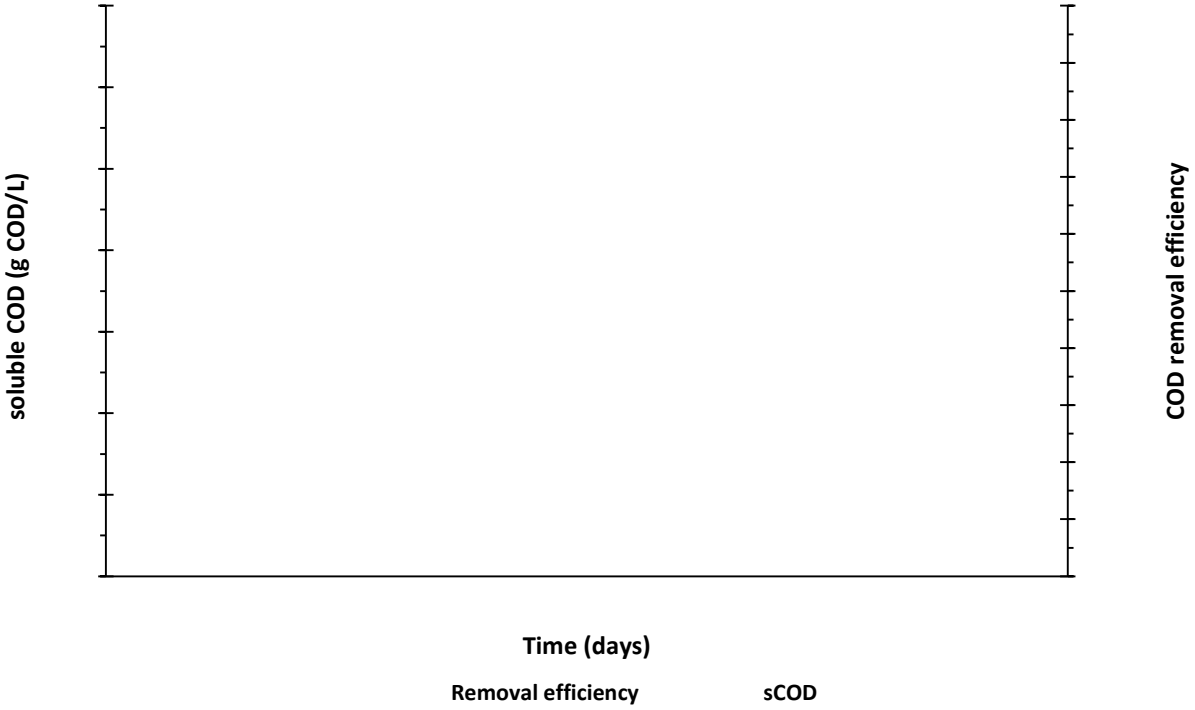

Figure 4.3 – Soluble COD, in g COD/L, and COD removal efficiency (%) of a reactor continuously fed with - g COD of SSL per L.

Comparing the graphs of Figures 4.1 and 4.3 one can tell that both reactors showed a similar performance. The reactor fed with - g COD/L reached the maximum of COD consumption at day - of operation, with a removal efficiency of -%. From day - onwards COD consumption stabilized: the COD left in the effluent was around - g/L; the COD removal efficiency was approximately - %. Cell dry weight and respective yield CDW/COD was not considered in this test.

A great amount of base was consumed in order to control the pH of the reactors. Approximately - mL of NaOH were pumped to the reactor per day (considering the one fed with - g COD/L). This highly influences the process costs since a higher amount of base is linked to higher process costs. SSL and the high amount of base consumed combined with its high cost (350 € per ton of COD), SSL was economically unfeasible and should not be considered for scale-up (for detailed economic viability please refer to 4.6 Economic evaluation). The process could still be considered feasible if the biomass yield was high since the high amount of cell produced would compensate the high costs. Though, this was not verified since the biomass yield was too low.

4.3.1.2 Pure glycerol

Pure glycerol was tested an influent stream containing - g COD/L. The process parameters applied were the same as described before (see Table 4.4).

If diluted, glycerol is known to be very sensitive to contamination at room conditions. Refrigeration of the feed container and tubing could be a possibility. Although this would increase process costs which was undesirable. Hence, the design of the reactor was changed. Instead of feeding the CSTR with a single influent stream (diluted stream of glycerol, nutrients and micronutrients) the CSTR was fed with two separated streams: a stream with pure, concentrated glycerol and a stream of nutrients and minerals solution (see Figure 4.4). This eliminated the contamination problem since concentrated glycerol is not susceptible to contamination.

Figure 4.4 – Schematic diagram with the changed CSTR design with two separate influent streams: one with pure glycerol and a solution containing the nutrients.

The results of the soluble COD and COD removal efficiency are shown in the graph of Figure 4.5. The CSTR fed with pure glycerol (- g COD/L) demonstrated an excellent performance regarding COD consumption. After - days of operation, the COD left in the effluent was approximately - g COD/L; the maximum COD removal efficiency was - %. Even though the COD removal efficiency decreased in the following days, it remained above - % which was still an excellent removal efficiency.

Comparing the graphs of Figure 4.1 (CSTR fed with - g COD of SSL per liter) and Figure 4.5 (CSTR fed with - g COD of pure glycerol per liter) it was evident that the reactor fed with pure glycerol showed a far better performance than the SSL-fed. The maximum COD removal in the SSL reactor was - % (when fed with - g COD/L) against - % in the glycerol reactor (fed with a higher COD content: - g COD/L).

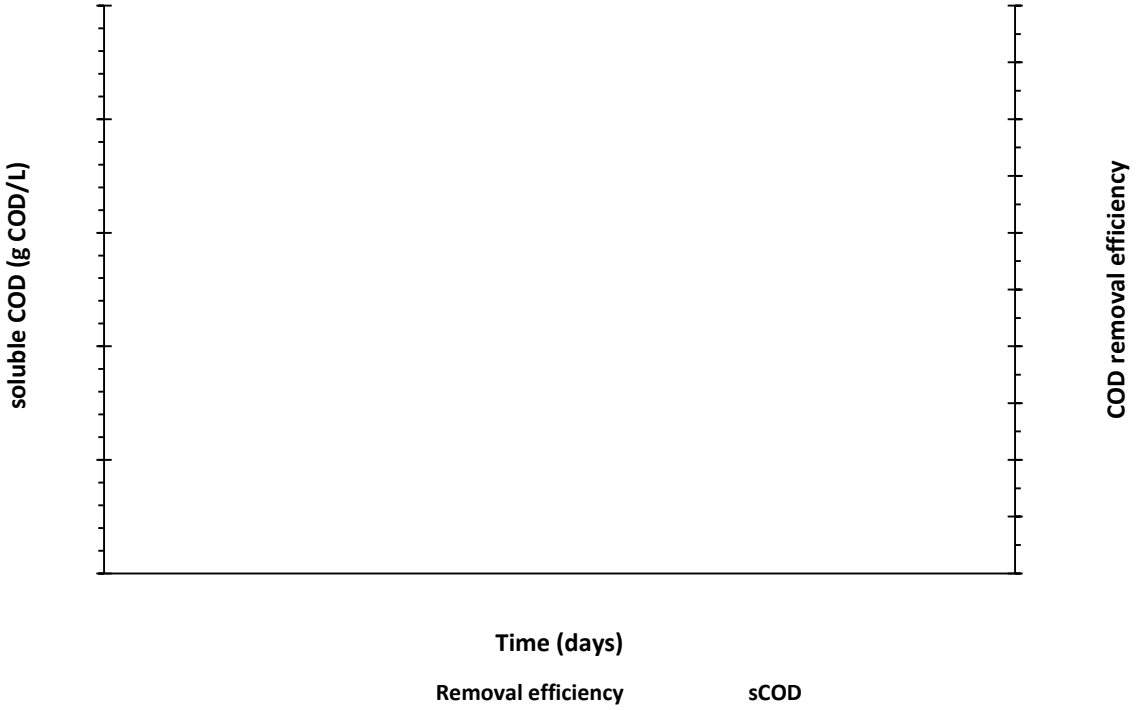

Figure 4.5 – Removal efficiency (in percentage) and soluble COD (in g COD/L) of a - L CSTR continuously fed with pure (concentrated) glycerol.

The cell dry weight (CDW) and respective yield of mass of biomass per mass of COD provided to the reactor is represented in the graph of Figure 4.6.

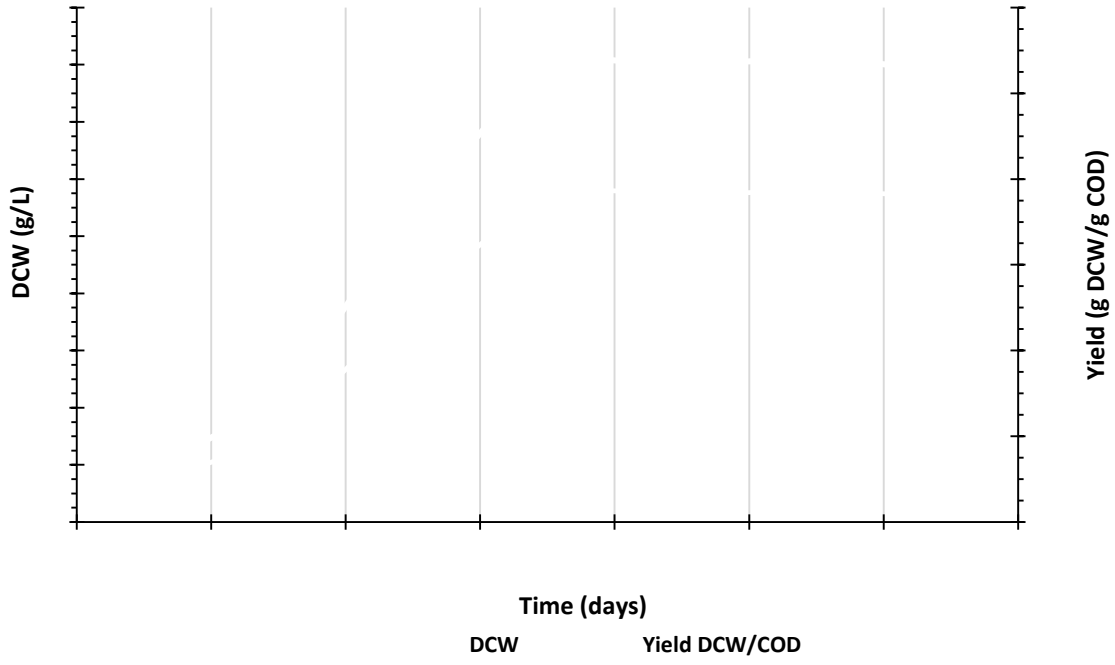

Figure 4.6 – Cell dry weight, in g CDW/L, and yield of biomass, in g CDW/g COD, of the CSTR fed with - g COD/L of pure glycerol.

The maximum biomass concentration was obtained at day - of operation – approximately - g CDW/L. This was equivalent to a yield of biomass of - g CDW/g COD. Besides, even though the biomass concentration and yield decreased after day -, they remained constant afterwards.

Although, the glycerol reactor also consumed a great amount of base – - mL per day – to increase the pH. Once again this was undesired since it has influence in the process costs. Even the considerably high biomass concentration (- g CDW/L) was not enough to compensate the process costs (see 4.6 Economic evaluation, for more detailed information on the process costs).

4.3.1.3 Crude glycerol

Crude glycerol was tested in the same process conditions as mentioned before for both SSL and pure glycerol. The COD content of the feed stream was -g COD/L. Similarly to pure glycerol, to avoid contamination, the CSTR was tested according with the diagram of Figure 4.4. Soluble COD of the effluent and the respective COD removal efficiency of the CSTR is represented in the graph of Figure 4.7.

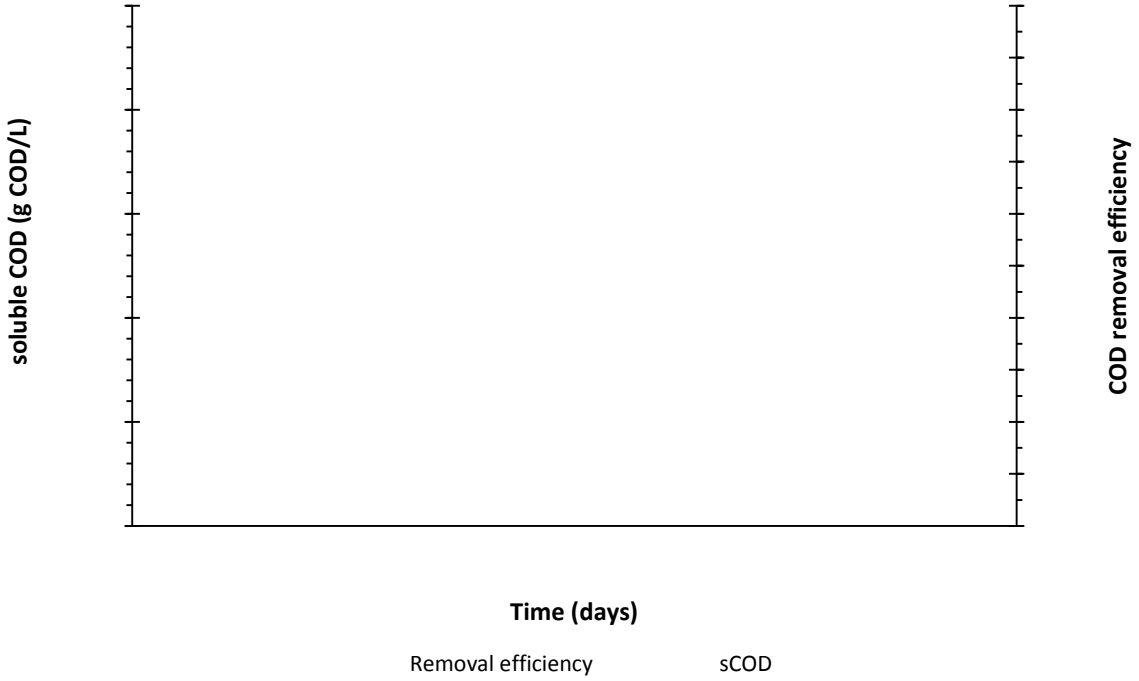

Figure 4.7 - Removal efficiency (in percentage) and soluble COD (in g COD/L) of a - L CSTR continuously fed with crude (concentrated) glycerol.

When running on crude glycerol the reactor showed a better performance than the reactor fed with SSL. Although, the reactor fed with pure glycerol was still better regarding COD removal and COD left in the effluent. However, this was already expected. As described on literature by several authors, reactors running on crude glycerol usually have a worse performance than those running on pure glycerol. In fact, in some cases crude glycerol could have an inhibitory effect in the microbiome (Chatzifragkou, A. & Papanikolaou, S., 2012).

The highest COD removal efficiency – - % - was recorded at day - which corresponded to - g COD/L left in the effluent. After day -, the COD content in the effluent showed a stabilizing tendency around - g COD/L. Likewise, the COD removal efficiency stabilized around - %. The biomass content and biomass yield are represented in the graph of Figure 4.8.

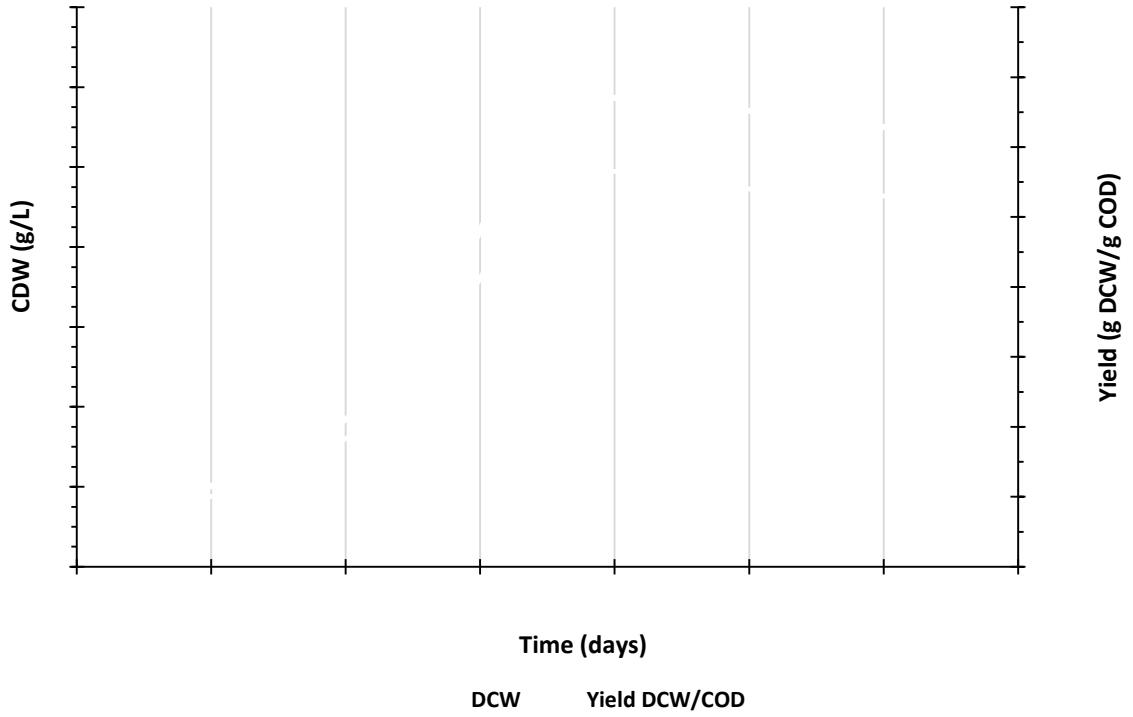

Figure 4.8 – Cell dry weight, in g CDW/L, and yield of biomass, in g CDW/g COD, in the CSTR fed with - g COD/L of crude glycerol

Even though the reactors fed with pure and crude glycerol had different biomass content and yield, the graphs showed similar performances. This indicates that there is no glycerol-inhibition. Similarly to the pure glycerol reactor, the crude glycerol also evidenced the highest biomass concentration at day - (- g CDW/L) with a slight decrease in the following days. The biomass content tended to decrease in the following days. Although, it started to show a stabilizing tendency around - g CDW/L. Maximum biomass yield was obtained at day - – - g CDW/g COD. Even though the yield decreased from - g CDW/g COD to - g CDW/g COD, it was visible stabilized around this final value.

Similarly, to the other two reactors, the high consumption of base represented a drawback. The reactor consumed around - mL of base per day.

4.3.1.4 Remarks on the CSTR operation

Since there was no resources in Avecom to centrifuge and then dry the harvested biomass continuously, there was great amount of wastes produced that had to be discarded. This was a major technical and economic drawback. The wastes could have been stored in a cold room for later centrifuging although this would only be feasible at lab-scale and not at bigger scale. Additionally, the disposal and then treatment of this wastes would represent an increased process cost. As there was no possibility to acquire a continuous system for centrifuging, in the time-lapse of this work, an alternative had to be addressed. Batch mode operation was then tested as a possible solution.

4.3.2 Batch mode

4.3.2.1 Crude glycerol

The batch operation mode consisted in providing the necessary COD and nutrients (nitrogen, phosphate and other micronutrients) in the beginning of the test. Batch tests were started in the same conditions as aforementioned for the CSTR operation. C/N/P was kept constant (100/5/1).. Two different initial COD contents were tested: - g COD/L and - g COD/L. Results of soluble COD, measured within - weeks, are shown in Figure 4.9.

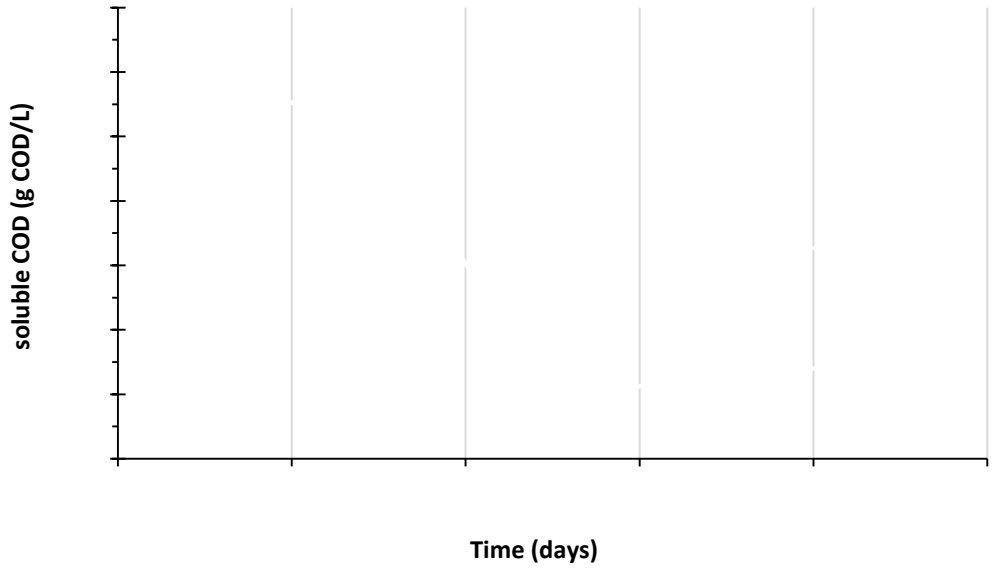

Figure 4.9 – Results of soluble COD of the crude glycerol batch reactor fed with two different initial glycerol concentration: - g COD/L and - g COD/L.

The reactor showed a similar performance when running with both concentrations of crude glycerol. The lowest COD content in the mixed liquor was measured at day - of operation: - g COD/L (when fed with - g COD/L) and - g COD/L (when fed with - g COD/L). The removal efficiency in both batches is shown in Figure 5.10. Since from day - onwards there was no COD

removal, the graph only shows the removal efficiency between the start of the batch and day -. COD removal efficiency was calculated considering the initial COD content of both reactors.

As shown in Figure 4.10 the batch started with - g COD/L showed a higher COD removal. However it was not a significant difference. Considering the batch started with - g COD/L, the

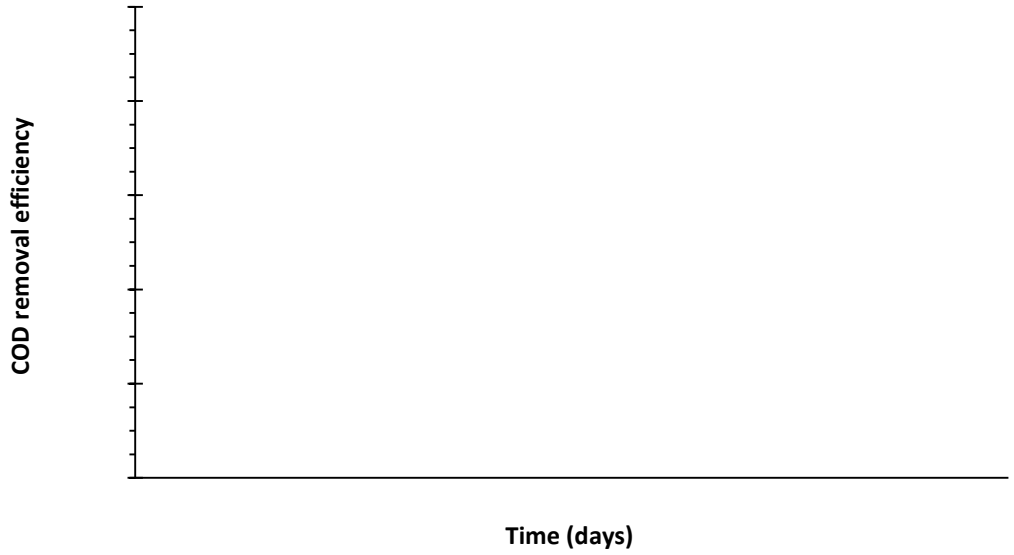

Figure 4.10 – COD removal efficiency in the reactors tested with two different initial COD contents: - g COD/L and - g COD/L

removal efficiency was approximately - %; as for the batch started with - g COD/L, the removal efficiency was - %.

Comparing batch operation (initial COD: - g/L) with the continuous operation (influent stream: - g COD/L) it was possible to see that both showed approximately equal COD removal efficiencies. In continuous mode the removal efficiency was - % while in batch mode was - %. However, it took - days of operation in continuous mode to successfully remove the - % of the COD fed while in batch mode it was only necessary - days. The remaining soluble COD in both operation modes was approximately the same: - g COD/L in continuous mode and - g COD/L in batch.

The biggest difference found between both operation modes was the biomass concentration obtained. In continuous mode it was possible to obtain considerably higher biomass concentrations – - g CDW/L – while in batch mode it was lower – - g CDW/L. However, the main problem found in batch mode was the decrease of the biomass content (- % less biomass) after the - day of operation (see Figure 4.11). This decrease in the cell dry weight could be a result from accumulation of byproducts or other compounds that can be toxic for the microbiome. These products could result in cellular lysis leading to an increase of the soluble COD and decrease of CDW.

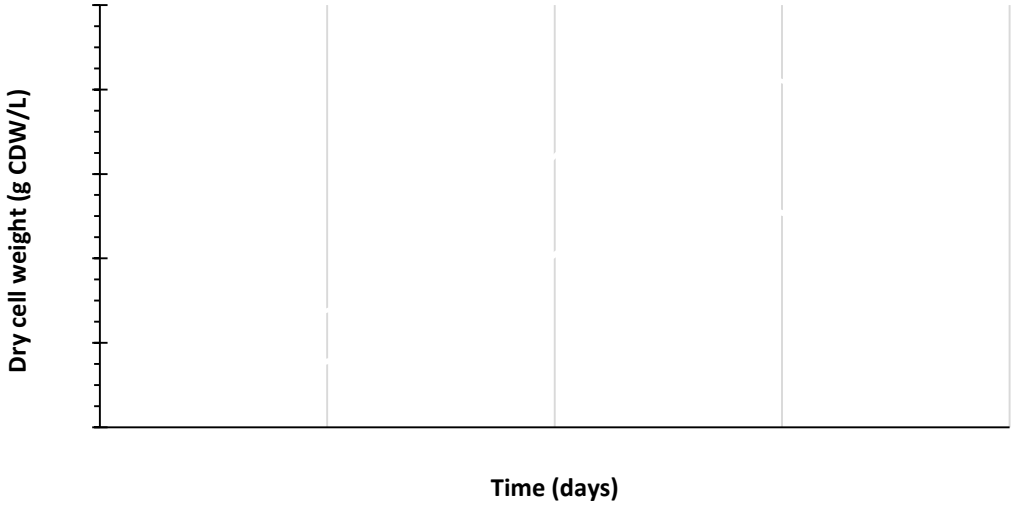

Figure 4.11 – Cell dry weight, in g CDW/L, during a - days batch-test with two different initial organic loads: - g COD/L and - g COD/L.

Since the biomass concentration after the - day of operation starts to decrease, the reactor should no longer operate. Thus, the reactors should be operated during - days, then harvested and restarted again.

5.3.2.2 pH control

Similarly to what happened in the continuous mode, there was a great consumption of NaOH to control the pH. Once again this was undesirable since it increases process costs. The reactor consumed - mL NaOH per day, making a total of - mL of base in the batch test (- days). Even considering that the reactor would only operate during - days that would result in - mL of base per batch or - L of base per week. Thus, a cheaper solution had to be addressed.

The alternative tested was the addition of calcium carbonate ($CaCO_3$) in the mixed liquor in the beginning of the batch. Calcium carbonate was added in different concentrations: - g/L, - g/L, - g/L, - g/L and - g/L. Results of pH (the lowest measured) and ash content are represented in Table 4.5.

Table 4.5 – Variation of pH and ash content with the different $CaCO_3$ concentrations.

CaCO3 content (g/L)	pH	Ash content (%)[1]
	6,17	
	6,29	
	5,95	
	6,33	

[1] Considering the highest TSS result and correspondent VSS

As shown in Table 5.5., the pH was stable – under 5,95 – for each concentration of calcium carbonate testes. However, as expected, the higher the concentration of calcium carbonate the higher the ash content. Since a high ash content was undesirable, the amount of calcium carbonate added per batch should not exceed - g/L. The process costs effectively decreased after replacing base as pH-buffer (as shown in 4.6 Economic evaluation)

4.3.2.3 Follow-up of nitrogen content

Firstly, monitoring of the nitrogen content in the mixed liquor is important to ensure that there was not high amounts of nitrogen sources left in the mixed liquor.

Additionally, there is a future interest to start to produce SCP enriched with polyhydroxybutyrate (PHB), the remaining nitrogen content of the reactor had to be controlled. Given that PHB usually accumulates in microorganisms under nitrogen limitation, the nitrogen left in the mixed liquor in the end of a regular batch (- days) needed to be kept close to zero. To ensure that no possible nitrogen source was present in the reactor, ammonium nitrogen, nitrate and nitrite were measured. The results of these analyses are shown in Table 4.6.

Table 4.2 – Follow-up of the different nitrogen sources (ammonium, nitrate and nitrite) during a single batch (- days).

	Ammonium nitrogen	**Nitrate, as nitrogen**	**Nitrite, as nitrogen**
	mg NH_4-N /L	**mg NO_3-N /L**	**mg NO_2-N /L**
start			
-			
-			

As shown in Table 4.6, ammonium nitrogen – the main nitrogen source – was completely consumed within the - days batch period. Although, nitrate and nitrite were not provided per se, they were probably present in the crude glycerol. However, the final concentration of the three nitrogen sources was considered residual.

4.4 Biomass harvesting, drying and grinding

4.4.1 Tumble-drying

The centrifuged mixed liquor was placed in a - mL plastic bottle with - metal spheres. One hole was drilled in both ends of the plastic bottle to allow air circulation inside the bottle. The drying test was performed in a regular tumble dryer during - hours, using the extra-dry program. The interior view of the bottle used in the drying test is shown in Figure 4.12. The goal of the metallic spheres was homogenization of the sample during the drying process.

Figure 4.4 – Inside view of the plastic bottle (with - metal spheres) after tumble-drying with the extra-dry program for - hours.

Even after - hours, in the extra-dry program, the sample was still wet and more than half of the initial volume, placed in the bottle, spilled from the holes drilled in its ends. Therefore this drying process could not be considered suitable for drying the biomass harvested from the reactors.

4.4.2 Microwave drying

Microwave drying test was performed during - minutes using a regular microwave oven. As shown in Figure 4.13, the outer portion of the sample was already dried while the central portion was still wet. In order to dry the central portion, the drying time should have been extended; although, by extending the drying time, the outer portion would be burned. Hence, microwave drying was also considered unfeasible for biomass drying.

Figure 4.5 – Centrifuged mixed liquor after drying in the microwave oven for - minutes.

4.4.3 Oven-drying

These drying tests were performed in a regular oven by using different temperatures: -, - and - ºC. The harvested biomass was spread in the oven trays as shown in Figure 4.14.

Figure 6 – Mixed liquor harvested from the reactor: (A) after centrifuging at - rpm for - minutes and before drying at - ºC for - hours; (B) after drying at - ºC for - hours.

After - hours at - ºC the sample was completely dried and ready to be ground. In opposition, the sample dried at - ºC required an extra hour to be completely dried. Additionally, at - ºC the required drying time was between - and - hours. To decide about the ideal drying temperature it was essential to determine the protein content in the powder after drying at different temperatures.

4.4.4 Drying temperature

Three different drying temperatures were tested. The protein content of the dried powder was determined using the Kjeldahl method and the results are shown in Table 4.7. The samples were analyzed in triplicate. The samples were also analyzed before drying to see whether the drying process affects the protein content.

Table 4.7 – Kjeldahl nitrogen and protein content according to three different drying temperatures: -, - and - ºC.

Drying temperature (ºC)	Kj-N (mg Kj-N/g sample)	Protein (mg/g sample)	Protein content (%)
-	- ± 0,80	- ± 5,00	- ± 5,00
-	- ± 0,79	- ± 5,00	- ± 5,00
-	- ± 0,93	- ± 5,81	- ± 5,81

As shown in Table 5.7, there is no evident influence of the drying temperature in the protein content of the final product. Hence, for a faster process, the drying temperature established was - ºC. Although this is not a suitable drying process for bigger scales. Thus, when scaling-up, alternatives should be addressed.

The sample analyzed before drying exhibited an average protein content of -%. Thus it was possible to observe that the loss of protein in the drying process was not significant (less than 10%).

4.4.5 Grinding

After drying, the biomass was ground with a pestle and a mortar originating a fine powder with particles lower than 0,5 mm, as shown in Figure 4.15. After drying, the ready-to-feed ProMic-pure powder exhibited a pleasant and cereal-like scent.

Figure 4.9 – ProMic-pure powder obtained after grinding the dried biomass

4.5 Nutritional value and safety of SCP

The nutritional value of the ground ProMic-pure was determined regarding protein content, lipid (fat) content, carbohydrates content and ash. Solubility in water was also determined. A microbiological quality control test was also performed in the final product to ensure its safety.

4.5.1 Nutritional value

The results of the composition of the ProMic-pure powder are shown in Table 4.8.

Table 4.8 – Composition of ProMic-pure. Comparison with other single cell proteins (*Kefir*, bacterial SCP, yeast SCP) and fishmeal.

	ProMic-pure		*Kefir* SCP[1]	Bacterial SCP[2]	Yeast SCP (*S. cerevisae*)[2]	Fishmeal[2]
Compound	**Content (g/g CDW)**	**% w/w**	**% w/w**	**% w/w**	**% w/w**	**% w/w**
Protein	-	- ± 1,80	53,9	73,1	46,8	66,7
Lipids	-	- ± 0,09	4,0	5,7	5,7	9,1
Carbohydrates	-	- ± 0,87	6,5	NA	NA	NA
Ash	-	- ± 1,02	7,3	11,7	6,2	14,9
Others	-	7,7	28,3	35	41,3	9,3

[1] Paraskevopoulo *et al*., 2003

[2] Tacon, 1987

The final product obtained – ProMic-pure – had an average protein content of - % which can be compared to the other protein sources displayed (see Table 4.8). Lipid content was lower than in other sources: - % lower when compared to traditional fishmeal. Additionally, ProMic-pure had a high carbohydrate content when compared with *Kefir* SCP, e.g. Ash content was also within the range. The other compounds, not measured, were probably nucleic acids. Although, there was no analytical method in Avecom to determine these compounds. However, in case it corresponds to nucleic acids, it falls within the range normally described in literature (Nasseri *et al.*, 2011).

The solubility of the ProMic-pure in water was determined by dispersing - g of powder in 1 L of distilled water. The protein content in the supernatant was determined using the Kjeldahl method. The content was calculated by means of a ration between the protein in the supernatant and the protein content in the dispersed sample. Hence, the ProMic-pure had a - % solubility in water.

4.5.2 Safety and quality

A microbiological control test was performed in order to confirm the absence of pathogens in the final product. To perform the test - g/L of dried ground powder were dispersed in sterile distilled water. The mixture was then pasteurized at - ºC for - h and homogenized in a shaker overnight.

A serial dilution set was prepared and plated out on the selective agar media for known pathogens as described in Table 4.9.

Table 4.9 – Putative pathogenic microorganisms and corresponding specific agar plates

Microorganism detected	Agar plate
Salmonella typhimurium ATCC®14028	Brilliance *Salmonella* Agar
Listeria monocytogenes ATCC® 13932, *Listeria monocytogenes* ATCC® 19111 and *Listeria innocua* ATCC®33090	Chromogenic *Listeria* Agar (ISO)
Enterococcus faecalis ATCC 29212 and *Enterococcus /faecium* ATCC 19434	VRE Selective
Staphylococcus aureus MRSA	Brilliance MRSA 2 Agar
Escherichia coli ATCC	TBX Medium

The plate counting results for putative pathogenic microorganisms (in colony forming unit per mL, CFU/mL) is shown in Table 4.10.

Table 4.10 – Results of the plate counting, in CFU/mL, for supposed pathogenic microorganisms.

Agar plate	Concentration (CFU/mL)
Brilliance *Salmonella* Agar	-
Chromogenic *Listeria* Agar (ISO)	-
VRE Selective	-
Brilliance MRSA 2 Agar	-
TBX Medium	-

4.6 Economic evaluation

After testing the performance of the reactors, both in continuous and batch mode, there was the need to evaluate their economic feasibility. Economic feasibility of the process was determined for the three substrates tested and for both operation modes. The evaluation was based on the price of substrates and several process costs, such as, price of biomass produced, cost of pH buffers and cost of aeration, centrifuging and drying. The goal of the economic evaluation was

to determine which substrate and operation mode resulted in the most market-competitive product. This was determined based on the price of the final product (in €/ ton protein). Other costs such as cost of workers and rent were not addressed.

The prices of the substrates tested as well as the price of the biomass produced, considering the biomass yield obtained, are indicated in Table 4.11.

Table 4.11 – Price of the substrates (€/ton and €/ton COD), biomass yield according to the operation mode (in ton CDW/ton COD) and price of biomass (€/ton CDW).

Operation mode	Substrate	Price (€/ton)	Price (€/ton COD)	Biomass yield (ton CDW/ton COD)	€/ton CDW
Continuous	Pure glycerol	250	350	0,40	-
	Crude glycerol	135	200	0,27	-
	Spent sulfite liquor	200	360	0,09	-
Batch	Crude glycerol[1]	135	200	0,28	-
	Crude glycerol[2]	135	200	0,23	-

[1] Buffered with $CaCO_3$

[2] Buffered with - N NaOH

As aforementioned, from an economic point of view, the most promising substrate was the crude glycerol. The low price per ton of COD and a good biomass yield, especially in batch mode, confirmed it as a top choice for SCP production. In fact, crude glycerol is the substrate that produced a biomass with lower price: - €/ton CDW in batch mode when buffered with calcium carbonate. When buffered with base, the price of biomass was - €/ton CDW. In continuous mode the price of biomass was the same as the latter. Additionally, pure glycerol could also be chosen as a feasible substrate. The high biomass yield (- ton CDW/ton COD) compensated the high cost per ton of COD of the pure glycerol (350 €/ton). Therefore, the biomass produced had similar price to the one resulting from the crude glycerol: - €/ton CDW.

Spent sulfite liquor was the substrate which produced the priciest biomass. The high cost per ton of COD (360 €/ton COD) combined with the low biomass yield (- ton CDW/ton COD) resulted in an exorbitant price of - €/ton CDW. Considering a protein content of -% (- g protein/g CDW), the protein produced via SSL would be marketed at - €/ton, even without accounting other process costs. This would not be viable since fishmeal, one of the primary protein sources for animal feed, was marketed at 1.940 €/ton. The following calculations confirmed the unfeasibility of SSL.

In Table 4.12 are shown the process costs of the CSTRs tested with the three substrates. The calculations were made based on prices and the biomass yield described in Table 4.11.

Process costs were calculated based on the electricity prices for households (- €/kWh); if calculated with industrial electricity prices (approximately 0,06 €/kWh), the process costs would be lower.

Table 4.12 – Process costs of the CSTRs fed with SSL, pure glycerol (PG) and crude glycerol (CG).

		Continuous		
Parameter		**SSL**	**PG**	**CG**
Buffer	Type	Base	Base	Base
	Price	60 €/m^3	60 €/m^3	60 €/m^3
	Volume	- mL/d	- mL/d	- mL/d
	Volume per CDW	- m^3/ton CDW	- m^3/ton CDW	- m^3/ton CDW
	Price per CDW (€/ton CDW)	-	-	-
Process	Oxygen (€/ton CDW)	-	-	-
	Drying (€/ton CDW)	-	-	-
	Centrifuging (€/ton CDW)	-	-	-
	Total process (€/ton CDW)	**-**	**-**	**-**
OPEX[1] cost	**Substrate + Buffer + Process (€/ton CDW)**	**-**	**-**	**-**
Final product (ProMic-pure) (€/ton protein)		**-**	**-**	**-**

[1]Operational expenditure – non-capital expenses for running a product/business.

The price of the final product was similar for both: - €/ton protein (pure glycerol) and - €/ton protein (crude glycerol). However, both substrates resulted in a product with a non-competitive price for the aquafeed market since it is higher than fishmeal, e.g. As predicted, SSL resulted in an extremely expensive product: - €/ton protein.

In Table 4.13 are presented the same process costs for a batch operation with crude glycerol. The parameter changed between both tests was the pH buffer. One was buffered with base (- N NaOH) while the other was buffered with calcium carbonate.

Table 4.13 – Process costs of the batch operation considering the two pH buffers: - N NaOH and $CaCO_3$.

		Batch	
Parameter		**CG**	**CG**
Buffer	Type	Base	$CaCO_3$
	Price	60 €/m^3	250 €/ton
	Volume		
	Volume per CDW	-	-
	Price per CDW (€/ton CDW)		
Process	Oxygen (€/ton CDW)	-	-
	Drying (€/ton CDW)	-	-
	Centrifuging (€/ton CDW)	-	-
	Total process (€/ton CDW)	-	-
OPEX cost	**Substrate + Buffer + Process (€/ton CDW)**	-	-
Final product (ProMic-pure) (€/ton protein)		-	-

As shown in Table 4.13 there was a significant difference in both reactors. The one buffered with calcium carbonate (g $CaCO_3$/L) produced ProMic-pure that could be sold at - €/ton. This product can be highly competitive with the current commercial fishmeal (market price of fishmeal: 1.940 €/ton). On the other hand the reactor buffered with base resulted in a product with a rather pricey protein (- €/ton protein). The biggest was found in the calculations of the buffer consumption. The price per ton of CDW of the batch reactor buffered with base was an order of magnitude higher than the one buffered with calcium carbonate.

However the process was considered feasible, profitability was not ensured. Considering a pilot-reactor of - m^3 (active volume) operating in batch, it would only be possible to produce - kg per batch. In terms of sales, this would only represent - €/month (- €/year). As a result, to be considered profitable, a much bigger scale would need to be implemented. If considering a reactor with - m^3 (active volume), it would be possible to produce - ton/month resulting in - €/month (- €/year). Several reactors running at the same time should also be considered.

Chapter 5

Conclusions and future work

5.1 Conclusions

The goal of this work was to study the technical performance and economic feasibility of bioreactors running on different substrates. The desired product was protein enriched biomass – known as single cell protein (SCP) – to be marketed as feed, particularly for aquaculture.

Pure glycerol (PG), crude glycerol (CG), spent sulfite liquor (SSL) and two different types of wood oils were acquired. Prior to use, the waste streams were characterized according to their COD content, ammonium nitrogen, phosphate, total and volatile suspended solids, Kjeldahl nitrogen and color and scent. After characterization, both wood oils were discarded as potential substrates due to their low COD content (lower than - g O_2/L) and the unpleasant smell. Following characterization, SSL, PG and CG were tested as substrates.

Two operation modes were tested: continuous and batch. SSL, PG and CG were tested in continuous mode; the only substrate tested in batch was CG.

In continuous mode, SSL proved to be unfeasible. The COD was too recalcitrant: of the - g COD/L fed to the reactor, there were still around - g COD/L in the effluent (-% COD removal efficiency. The maximum cell dry weight (CDW) reached was - g CDW/L with a low biomass yield of - g CDW/g COD. The low cell density and biomass yield combined with the high price per ton of COD (- €/ton COD) resulted in high process costs. Hence, the obtained SCP was too expensive: - €/ton protein. Removal efficiency in this reactor was always higher than - %. When fed with - g COD/L the maximum COD content measured in the effluent was - g COD/L. Plus, it reached a cell density of - g CDW/L and a high biomass yield of - g CDW/g COD. It was possible to produce SCP priced at - €/ton protein. Crude glycerol showed a worse performance compared to CG but better in relation to SSL. The COD removal efficiency was around - %: - g COD/L in the effluent out of - g COD/L in the influent. Biomass concentration and biomass yield was also good: - g CDW/L and - g CDW/g COD, respectively. With the low price per ton of COD (200 €/ton COD) it

was possible to produce SCP priced at - €/ton protein. SCP produced via glycerol, both pure and crude, evidenced a similar final price.

The major technical drawback of the continuous mode was the lack of equipment to harvest, centrifuge and dry the mixed liquor continuously in Avecom NV. Therefore, batch operation was addressed as a solution. Since SSL proved to be unfeasible and PG had a higher price, they were not tested in the batch operation mode. Only crude glycerol was tested.

In batch mode, CG proved to be feasible for the production of SCP. After - days of operation, maximum biomass concentration – - g CDW/L – and maximum biomass yield – - g CDW/g COD – were reached. The COD removal efficiency within the - days was - % corresponding to - g COD/L left in the mixed liquor. After day -, the biomass concentration started to decrease which could indicate some sort of inhibition. In this operation mode it was possible to obtain a SCP priced at - €/ton protein. It was considered market-competitive with other protein sources such as fishmeal (priced at 1.940 €/ton protein). Although, to be considered profitable, the process should be implemented at a big scale.

In both operation modes pH buffering with base (- N NaOH) represented an economic drawback. Using calcium carbonate as pH buffer (- g $CaCO_3$/L) in the batch reactor proved to be efficient and economically feasible.

The final product – ProMic-pure – had a rather high protein content of - ± 1,80 %w/w. Lipid and carbohydrate content was - ± 0,09 and - ± 0,87 %w/w, respectively. Ash content was around - ± 1.02 %w/w. ProMic-pure had a nutritional value comparable to other protein sources (i.e. different SCP and fishmeal) as described by Paraskevopoulo *et al.* (2003) and Tacon (1987). However, digestibility and amino acids composition should be further analyzed. A microbiological quality control test ensured that there was no presumptive pathogens in the final product.

Following this work, the reactors were scalled-up to pilot-scale (working volume: - m^3) and are currently under tests.

6.2 Future work

Although the process is already being tested at pilot-scale there are still some further tests that can be done.

The main interest should be the production of SCP enriched with polyhydroxybutyrate (PHB). According to Defoirdt *et al.* (2009) and Liu *et al.* (2010), PHB has the potential to be used as a supplement for animal feed as an anti-infective strategy in order to discard antibiotics in animal production. Therefore, PHB could be used to add extra value to the SCP produced increasing its price thus increasing the profitability of the process.

Besides that, shifting from batch to a continuous process should be done. This would increase the amount of SCP produced. A strategy to effectively control the pH should be addressed. Buffering with calcium carbonate could be a solution. Strategies to increase the biomass content of the reactors should also be taken into account (i.e. recirculation system).

References

Alexandratos, N. & Bruinsma, J. (2012). *World Agriculture Towards 2030/2050*. FAO ESA Working Paper no. 12-03

Anupama & Ravindra, P. (2000). Value-added food: single cell protein. *Biotechnology advances*, 18, 459-479

Brown, L.R. (2006). *Plan B 2.0: Rescuing a Planet Under Stress and a Civilization in Trouble*. Washington DC, USA: Earth Policy Institute

Chatzifragkou, A. & Papanikolaou, S. (2012). Effect of impurities in biodiesel-derived waste glycerol on the performance and feasibility of biotechnological processes. *Applied Microbiology and Biotechnology*, 95, 13-27

Chen, H. (2013). *Modern Solid State Fermentation: Theory and Practice*. Springer

Chen, H.Z. & Xu, J. (2004). *Modern Solid State Fermentation: Theory and Practice*. Beijing, China: Chemical Industry Press

Cooney, C.L. & Tannenbaum, S.R. (2012). Variation in the composition of bacteria and yeast and its significance to single-cell protein production. In N.W. Pirie (Ed.) *Food Protein Sources*. Great Britain: Cambridge University Press

Defoirdt, T., Boon, N., Sorgeloos, P., Verstraete, W., Bossier, P. (2009), Short-chain fatty acids and poly-beta-hydroxyalkanoates: (New) Biocontrol agents for a sustainable animal production. *Biotechnology Advances*, 27(6), 680-685

Diana, J.S., Egna, H.S., Chopin, T., Peterson, M.S., Cao, L., Pomeroy, R., Verdegem, M., Slack, W.T., Bondad-Reantaso, M.G., Cabello, F. (2013). Responsible Aquaculture in 2050: Valuing Local Conditions and Human Innovations Will Be Key to Success. *BioScience*, 63(4), 255-262

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F. (1956).Colorimetric Method for Determination of Sugars and Related Substances. *Analytical Chemistry*, 28(3), 350-356

Feed International (2012). World Feed Panorama: Once again, industry increases its volume.WATT Global Media

Food and Agriculture Organization (2004). *FAO Animal Production and Health Proceedings: Protein Sources for the Animal Feed Industry*. In FAO Expert Consultation and Workshop, Bangkok, 2002. Rome, Italy: FAO

Food and Agriculture Organization (2006). *Livestock's Long Shadow: Environmental issues and options*. Rome, Italy: FAO

Food and Agriculture Organization (2012). *FAO Yearbook: Fishery and Aquaculture Statistics 2012*. Rome, Italy: FAO

Food and Agriculture Organization (2014). *The State of World Fisheries and Aquaculture: Opportunities and Challenges*. Rome, Italy: FAO

Food and Agriculture Organization (2014). *The state of World Fisheries and Aquaculture – Opportunities and challenges*. Rome, Italy: FAO

Glazer, A.N. & Nikaido, H. (2007). *Microbial Biotechnology – Fundamentals of Applied Microbiology*. (2nd Ed.). USA: Cambridge University Press

Glazer, A.N. & Nikaido, H.N. (2007). *Microbial Biotechnology – Fundamentals of Applied Microbiology* (2nd Ed.). Cambridge University Press

Guo, Z. & Olsson, L. (2014). Characterization and fermentation of side streams from sulfite pulping. *Process Biochemistry*, 49, 1231-1237

Hamdan, I.Y. & Senez, J.C. (2009). The economic viability of Single Cell Protein (SCP) production in the twenty-first century. In E.J. DaSilva, C. Ratledge, A. Sasson (Eds.), *Biotechnology: Economic and Social Aspects*. Cambridge University Press

Hasan, M.R. & New, M.B. (2013). *FAO Fisheries and Aquaculture Technical Paper: On-farm feeding and feed management in aquaculture*. Rome, Italy: FAO

Helle, S.S., Lin, T., Duff, S.J.B. (2008). Optimization of spent sulfite liquor fermentation. *Enzyme and Microbial Technology*, 42, 259-264

Index Mundi (2014). *Commodity Prices: Soybean vs Fishmeal*. Retrieved January 18, 2015 from http://www.indexmundi.com/commodities/?commodity=soybean-meal&currency=eur&commodity=fish-meal

International Feed Industry Federation (2013). *International Feed Industry Federation Annual Report 2012/2013*. Retrieved January 7, 2015 from http://www.ifif.org/uploadImage/2013/10/1/c838a3d3dbb286acb4685f331c1b70241380656385.pdf

International Grains Council (2014). *Five-year global supply and demand projections*. Retrieved January 7, 2015 from http://www.igc.int/en/downloads/grainsupdate/igc_5yrprojections2014.pdf

Jamal, P., Alam, M.Z., Salleh, N.U. (2008). Media Optimization for Bioproteins Production from Cheaper Carbon Source. *Journal of Engineering Science and Technology*, 3(2), 124-130

Jay, J.M., Loessner, M.J., Golden, D.A. (2005). *Modern Food Microbiology* (7th Ed.).New York, USA: Springer Science+Business Media, Inc

Lee, J.Z., Logan, A., Terry, S., Spear, J.R. (2015). Microbial response to single-cell protein production and brewery wastewater treatment. *Microbial Biotechnology*, 8(1), 65-76

Liu, Y, Schryver, P., Delsen, B., Maignien, L., Boon, N., Sorgeloos, P., Verstraete, W., Bossier, P., Defoirdt, T. (2010). PHB-degrading bacteria isolated froim the gastrointestinal tract of aquatic animals as protective actors against luminescent vibriosis. *FEMS Microbiology Ecology*, 74(1), 196-204

Mitchell, D.A., Berovič, M., Krieger, N. (2006). Solid-State Fermentation Bioreactors: Fundamentals of Design and Operation. Germany: Springer

Najafpour, G. (2015). *Biochemical Engineering and Biotechnology* (2nd Ed.). Elsevier

Nasseri, A.T., Rasaul, S., Morowvat, M.H., Ghasemi, Y. (2011). Single cell protein: production and processes. *American Journal of Food Technology*, 6(2), 103-116

Naylor, R.L., Hardy, R.W., Bureau, D.P., Chiu, A., Elliott, M., Farrell, A.P., Forster, I., Gatlin, D.M., Goldburg, R.J., Hua, K., Nichols, P.D. (2009). Feeding aquaculture in an era of finite resources. *Proceedings of the National Academy of Sciences of the United States of America*, 106 (36), 15103-15110

Paraskevopoulo, A., Athanasiadis, I., Kanellaki, M., Bekatorou, A., Blekas, G., Kiosseoglou, V. (2003). Functional properties of single cell protein produced by kefir microflora. *Food Research International*, 36(5), 431-438

Quorn (2014). *Sustainable Development Report 2014*. North Yorkshire, UK: Quorn Food Ltd

Rose, A.H. & Harrison, J.S. (Eds.) (1993). *The Yeasts: Yeast Technology* (2nd Ed.). Academic Press

Rust, M.B., Barrows, F.T., Hardy, R.W., Lazur, A., Naughten, K. and Silverstein, J. (2011). *The Future of Aquafeeds*. USA: National Oceanic and Atmospheric Administration (NOAA) and United States Department of Agriculture (USDA)

Stanbury, P.F., Whitaker, A., Hall, S.J. (2000). *Principles of Fermentation Technology* (2nd Ed.).

Tacon, A., Metian, M., Hasan, M. (2009). Feed ingredients and fertilizers for farmed aquatic animals: sources and composition. *FAO Fisheries and Aquaculture Technical Paper, 540*

Tacon, A.G.J. & Metian, M. (2008). Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. *Aquaculture*, 285 (1-4), 146-158

Tacon, A.G.J. (1987). *The nutrition and feeding of farmed fish and shrimp – A training manual. 2. Nutrien sources and composition*. Brasilia, Brazil: FAO

Tacon, A.G.J., Hasan, M.R., Metian, M. (2011). *FAO Fisheries and Aquaculture Technical Paper: Demand and supply of feed ingredients for farmed fish and crustaceans*. Rome, Italy: FAO

Trinci, A.P.J. (1992). Myco-protein: a twenty-year overnight success story. *Mycological Research*, 96, 1-13

Ugalde, U.O. & Castrillo, J.I. (2002). Single cell proteins from fungi and yeasts. Applied Mycology and Biotechnology, 2, 123-149

United Nations (2013). World Population Prospects: The 2012 Revision (Volume I: Comprehensive Tables). New York, USA: United Nations

Volova, T.G. & Barashkov, V.A. (2010). Characteristics of Proteins Synthesized by Hydrogen-Oxidizing Microorganisms. *Applied Biochemistry and Microbiology*, 46 (6), 574-579

Ware, S.A. (1977). *Single Cell Protein and Other Food Recovery Technologies from Wastes*. USA: Environmental Protecting Agency

World Bank (2013). *World Bank Report number 83177-GLB: Fish to 2030 – Prospects for Fisheries and Aquaculture.* USA: World Bank

Yu, J., Dow, A., Pingali, S. (2013). The energy efficiency of carbon dioxide fixation by a hydrogen-oxidizing Bacterium. *International Journal of Hydrogen Energy*, 38, 8683-8690