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Evaluation of the Cosmetic Potential of the Cyanobacterium *Spirulina platensis*.

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"The cosmetic products do less than we say, but more than we think".
Researcher from a French company.

Summary

List of Abbreviations

TPCS – Toiletry, Perfumery Cosmetics Sector
EPS - Exopolysaccharide
GRAS – Generally Recognized as Safe
FDA – Food and Drug Administration
LPS – Lipopolyssacharide
CPS – Capsular Polyssacharyde
ROS – Reactive Oxygen Species
DNA – Desoxiribonucleic Acid
ABTS - 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
FRAP - Ferric reducing antioxidant power
ORAC - Oxygen Radical Absorbance Capacity
IC 50 - Half maximal inhibitory concentration
UFSM-RS – Universidade Federal de Santa Maria – Rio Grande do Sul
MERCOSUL – Mercado Comum do Sul
EU – European Union
RDC - Resolução da Diretoria Colegiada da Anvisa
SEBRAE – Serviço Brasileiro de Apoio às Micro e Pequenas Empresas
SECEX - Secretaria Executiva do Ministério do Desenvolvimento Agrário
BHA - 2 and 3-terc-butil-4-hidroxianisol
BHT - Butil Hidroxi Tolueno
ANVISA - Agência Nacional de Vigilância Sanitária
ABIHPEC - Associação Brasileira da Indústria de Higiene Pessoal, Perfumaria e Cosméticos
LEB – Laboratório de Engenharia de Microalgas
HPLC - High Performance Liquide Chromatography
DPPH - 2,2-diphenyl-1-picrylhydrazyl
UHT – Ultra High Temperature
PDA – Potato Dextrose Agar
NA – Nutrient Agar

Chapter 1

General Introduction

Toiletry, Perfumery and Cosmetics Sector (TPCS) occupies a prominent place in modern society. The search for beauty and wellness, combined with increased life expectancy of the population, causes the intensive search to products that improve appearance, hygiene and health. Thus, the development of cosmetics is very stimulated. Another important feature is the continuously pressure from consumers and companies to this development of new and innovative products, raising competitiveness. Based on this, large sums are being annually invested in this area.

Innovation is the world in cosmetic industry. One of the biggest sources of innovation are the additives, which play a role ranging from improvement sensory properties to protection of the skin against free radicals.

With respect to these additives, most are of synthetic origin. Nowadays, their safety and the way they are obtained is being questioned, once negative effects of classical substances used through years are being founded. Thus, attempts to find natural substances capable of replacing these synthetic ingredients are being made, in association with principles of environmental sustainability. Therefore, developing biotechnological products has emerged as an important alternative for achieving efficient and safe additives for cosmetic use. This work fits in this context, aiming to study the potential application of *Spirulina platensis* biomass and exopolysaccharide (EPS) in cosmetic products.

The general objective of this work is:

- Evaluate the effects of the cyanobacteria *S. platensis* biomass and EPS addition in cosmetics searching a new cosmetic formulation.

The specific objectives were determined as:

- Promote microalgae growth and evaluate growth parameters, EPS production and biomass composition;
- Asses the phenolic compounds in *S. platensis* biomass and EPS and their antioxidant potential;
- Evaluate the rheological characteristics of the addition of biomass and EPS in a moisturizer for the face skin and propose a formulation;
- Assay cream stability through pH, color, odor, general aspect, spreadability, volatile acids and microbiology;
- Evaluate the new formulation for skin irritability in guinea pigs;
- Sensory analysis of the new formulation in panel members.

Chapter 2

Cultivation of *Spirulina platensis* and the obtaining of its Biomass and Exopolysaccharide (EPS)

2.1 Introduction

Biodiversity represents the biological diversity of a particular place and is yet not enough explored and elucidated on the planet. The value associated to it exploration is immeasurable, since even many compounds that could be used in medicines, foods, fertilizers, pesticides, cosmetics, solvents, plastics, energy, etc, can be discovered. However, it is difficult to measure how much this untapped biodiversity worth. Some studies have estimated its value at about U$ 4 trillion (Cunha, 2009). However, the most important thing is not to determine its real economic value, but to relate the findings to development for mankind through its sustainable use.

2.1.1 Microalgae and Cyanobacterium

Microalgae are quite diverse form of life, including nine phyla of eukaryotic organisms and two prokaryotes phyla. These organisms occupy virtually all types of biosphere environments, such as fresh and salt water, ice, soil, rocks and tree bark, occurring even in extreme environments such as deserts and polar regions, thanks to its efficient morpho-physiological adaptations (Van den Hoek et al., 1995). From a phylogenetic standpoint, microalgae range from very primitive (such as euglenoids) to modern lineages (such as some green algae) (Van den Hoek et al., 1995), setting more functional classification than taxonomical.

This evolutionary lines variety, shapes and physiological mechanisms, makes microalgae a group that produces a wide variety of chemical compounds with potential application in the food, cosmetic, pharmaceutical and even energy production (Olaizola, 2003). Coupled with the large production potential of microalgae as industrial producer of interesting compounds it is observed the ease isolation from natural environments and ease cultivation in culture collections (Lourenço, 2004).

2.1.1.1 Spirulina platensis Biomass Characteristics and Composition

Spirulina platensis is a bacterium belonging to the cyanobacterium. It is unicellular but grouped to form trichomes or filamentous forms (Figure 2.1). Their cell wall contains peptidoglycan in its composition and structure, thus classified as Gram negative. In the cytoplasm there are inclusions bodies (granules of polyphosphate,

glycogen and cinanophycine), the carboxysome polyhedral bodies (where RuBisCO, the main enzyme responsible for photosynthetic fixation of carbon dioxide, is situated) and 70S ribosomes.

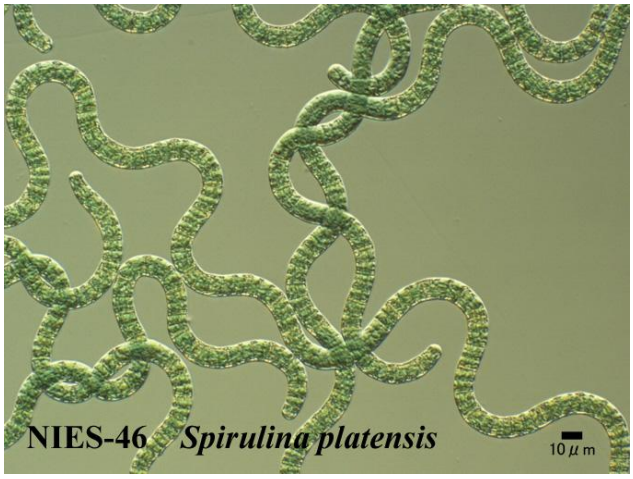


Fig. 2.1: *Spirulina platensis* micrography (http://www.shigen.nig.ac.jp/algae/top.jsp)

The use of *Spirulina* sp. for food is not new, since there is evidence of its use by that the Aztecs. Also other ancient societies in the region of Lake Chad, in Africa, also included in their usual diet *Spirulina* in the form of cakes.

Despite knowing this bacterium for centuries, it was in the last half of the twentieth century that it began to be used industrially. This is a crop suitable for dry areas where the salinity of the water is not fit for their traditional agricultural employment. Indeed, Spirulina industrial cultivation did not begin until 1962 in the area of Chad.

Nowadays its main application is in feeding, mainly in the form of pills or tablets, but it's also used as a source of pigments, such as phycocyanin or xanthophylls, and polyunsaturated fatty acids. Due to the high protein content it is also studied as a substitute of soy protein and as food for fish. Lots of other possible applications are under cevelopment.

Spirulina platensis is a microalga whose composition is suitable for use as food supplement and can be used to combat malnutrition (Fox, 1996). It's composition includes high levels of protein (64-74%), polyunsaturated fatty acids and vitamins (Cohen, 1997), and antioxidant compounds (Collar et al., 2007). This microalgae is classified as GRAS (Generally Recognized as Safe) by FDA (Food and Drug Administration), which ensures its use as food without risk to health.

In microalgae culture, the source of nutrients is economically the second influence in high production costs (Vonshak, 1997). Cost reducing studies were performed, showing that reducing the Zarrouk medium (the standard medium for

Spirulina cultivation) composition to 10 or 20% of its original medium, does not influence significantly biomass productivity (Pinto, 2004).

The carbon source in the standard medium for growth of Spirulina is sodium bicarbonate (Zarrouk, 1966), which supplies CO_2 for photosynthesis. Recently other carbon sources, such as CO_2 have been studied for the cultivation of microalgae (Morais & Costa, 2007). Although it uses photosynthesis metabolism to obtain carbon, Spirulina is able to assimilate organic substrates in mixotrophy cultures (Andrade & Costa, 2007; Marquez et al., 1993).

2.1.2 Polysaccharides Sources

Polysaccharides with industrial applications are usually extracted from plants (including algae), animals, or obtained through microbial fermentation, such as microalgae, bacteria and fungi. In higher plants they can be obtained from exudates, seeds, fruits and tubers.

Algae, specially red and brown ones, produce various types of polysaccharides, such as agaranas, carrageranas and alginates. Plant seeds structural or reservation polysaccharides include galactomannans, xyloglucans, mannans and glucans; the first two being used in industrial applications. Some plants produce exudates, which contains polysaccharides containing glucuronic and galacturonic acids, that function in the defense against stress caused by physical injuries and even microbial attacks. Fruits are important sources of pectin, a heteropolysaccharide constituent of their cell wall. Tubers and cereals are the most important sources of starch, the most important reserve polysaccharide of plants. (Cunha, 2009)

Polysaccharides such as hyaluronic acid and heparin can be obtained from animals. extracted from the vitreous humor of cattle or poultry crests and from the lungs of cattle and pig intestine, respectively.

2.1.3 Purification and Structural Analysis

Awareness of the importance of analyzing the structure of saccharide substances in recognition of their properties and biological roles has promoted the increasing development of methods to analyze the structure and stereochemistry of complex oligo and polysaccharides. Polysaccharide analysis is complicated due to its branching and monosaccharide bindings, unlike nucleic acids and proteins. The analysis of oligosaccharides should be done after the withdrawal of other related biomolecules such as proteins and/or lipids. It is then subjected to stages of degradation, with specific reagents, which show the position of the bounds and/or the

stereochemistry of the molecule. Other techniques such as mass spectrometry and NMR spectrometry also bring relevant analysis to assist in the elucidation of the structures of molecules. (Lehninger, 2003)

2.1.4 Applications

Extracellular polymeric substances produced by many microorganisms are applied widely in adhesives, food, and beverage industries (Sutherland 1996), pharmaceuticals (Benedetti et al. 1989), oil and metal recovery from ore and industry (Gutnick 1997), because of its special physico-chemical and physiological activation characteristics, such as stabilizing, suspending, gelling and water-retention capability (Morgante et al. 2007).

Polysaccharides have a wide range of applications in the areas food, pharmaceutical, cosmetic, especially as stabilizing agents, thickeners, gelling and retaining moisture. Biotechnological applications are the most extensive, ranging from immobilization of enzymes, biosensor, drug delivery vehicles, to substitute for blood plasma and preparation of resins used for the separation and purification of substances. Furthermore, the polysaccharides have been investigated for their potential as antiviral agents, stimulating the immune system, antitumor, antioxidant, antithrombotic and anticoagulants.

2.1.5 Microbial Polysaccharides

2.1.5.1 Definitions, Characteristics and Classification

Carbohydrates are the most abundant biomolecules on Earth. They are predominantly cyclic polihidroxialdehyde or polihidroxicetones, or substances that release these compounds by hydrolysis. Many carbohydrates are described by the general formula $(CH_2O)_n$, but some also contain nitrogen, phosphorus or sulfur. They are divided into three main classes according to their size: monosaccharides, oligosaccharides or polysaccharides.

Monosaccharides, also called simple sugars, consist of a single molecule of polihidroxialdehyde or ketone. The most abundant in nature is the D-glucose, also called dextrose.

Oligosaccharides are composed of short chains formed by monosaccharide units, which are joined by glycosidic bonds. Among the oligosaccharides, one example is the disaccharides, formed by two monosaccharides (Lehningher, 2003).

The polysaccharides, also called glycans, are composed of at least 20 monosaccharide units, reaching hundreds or thousands. These chains can be linear or

branched, and the linkages between the monomers can be of different types. . Polysaccharides differ based on the monosaccharide composition, the length of the chains and their degree of branching. Considering monosaccharide composition, polysaccharides can be classified in homo-polysaccharide (a single kind of monomer unit) or hetero-polysaccharide (two or more kinds of monomeric units).

Polysaccharides usually have defined molecular weights, a consequence of its the assembly mechanism. During the synthesis, enzymes that catalyze the polymerization of monomer units are regulated by specific programs to build the molecules (Lehningher, 2003).

Polysaccharides have important functions, such as reserve of energy (starch, glycogen) and as structural materials (cellulose, chitin, peptidoglycan). They can also play a role as intracellular indicators and mediators in specific cell-cell and cell-extracellular matrix interactions. They can bind to other biomolecules such as lipids and proteins, forming glycoconjugates, which are commonly bioactive biomolecules (Lehningher, 2003).

Microbial polysaccharides are present as cell wall components (lipopolysaccharide or LPS), linked covalently to the cell surface (capsular polysaccharides or CPS), or secreted into the extracellular medium (exo-polysaccharides or EPS) (Boels et al., 2001). While the polysaccharide associated with cell (CPS and LPS) have medical importance, many EPS have wide spectrum of applications in the food industry and cosmetics, being used as thickeners, stabilizers, emulsifiers, coagulants, films formers, suspending agents and dispersants, (Sutherland, 1998; Stredansky et al., 1999).

The EPS are long chain polysaccharides secreted mainly by bacteria, although they may also be produced during growth of microalgae, yeasts and fungi. They are not linked to microbial cell surface (Laws et al., 2001) and, among other properties, promote protection against limiting and/or toxic environments (Looijesteijn et al., 2001) and contribute to colonization and cellular recognition (Roberts, 1996). Furthermore, it should be noted that the polysaccharides extracted from plants and algae still dominate the gums industry due to low production cost. The production cost is the main limiting factors for the use of microbial EPS (Stredansky et al., 1999, De Vuyst et al., 2001).

The microbial biosynthesis of EPS is very complex. The biosynthetic pathway can be divided into four separated sequences of reactions: the reactions involved in sugar transport to the cytoplasm, the synthesis of sugar-1-phosphate activation and binding of sugars, and the processes involved in the secretion of EPS (Kleerebezem et al., 2000; De Vuyst et al., 2001, Laws et al., 2001). The EPS production seems to

occur during the logarithmic phase, and for some bacteria, continues during the stationary phase of growth (Laws et al., 2001).

The fermentation conditions strongly influence the production of EPS. Studies indicate that the best conditions for the production are usually different from the optimum condition for cell growth due to the fact that production is related to responses of microorganisms to stress conditions (Gamar et al., 1997). The effect of pH on EPS production is based in the activity of enzymes glicosil-hydrolases responsible for degradation of EPS after long fermentations. The optimum pH for EPS production will be the one at which production and degradation are balanced (Pham et al., 2000, Laws et al., 2001).

Commercial exploitation of exopolysaccharides as materials to improve the texture and sensory characteristics of foods and cosmetics requires the synthesis of EPS with adequate physical properties and in sufficient quantities to meet demand. A major problem observed is the low level of production, varying from a few milligrams to about 1 g/L (De Vuyst et al., 2001, Laws et al. 2001).

An increase in EPS production can be achieved through genetic manipulation or through metabolic engineering. The determination of the mechanisms responsible for controlling and regulating the biosynthesis of EPS, the level of genes and proteins, is necessary for the production of EPS can be optimized (De Vuyst and Degeest, 1999; Boels, et al. 2001; Laws et al., 2001).

2.1.5.2 Algal Polysaccharides

Microalgal polysaccharides are important metabolites, representing 40-90% of organic compounds produced by them and showing a huge spectrum of compositions and different molecular masses (Myklestad, 1995). In general polysaccharides from microalgae can be divided in three major groups: reserve, structural and extracellular.

The main intracellular polysaccharides are the reserve ones, usually composed by homogeneous glucans such as starch and its variations in cyanobacteria and rodophites and crisolaminarina in heterokonts (diatomaceaes, crisoficeas, xantoficeas and others) (Percival, 1979).

The structural polysaccharides, present mainly in cell walls, can greatly vary in composition depending on the taxonomic group, including xylans, mannans, ramnans (Carlberg and Percival, 1977) and various glycoproteins (Morita et al., 1999), being used as an important criterion in the definition of phyla, classes and orders of microalgae (Reviers, 2006).

Extracellular polysaccharides, which are quite representative in the quantitative point of view (Paulsen and Vieira, 1994), also have a very variable composition and

can form compounds as homogeneous fucans (Giroldo and Vieira, 2002), mannans (Vieira et al. 2006), arabinogalactan (Kiemle et al., 2007), and heteropolysaccharide with five or more components (Giroldo and Vieira, 2005). This group of sugars is less known, both in terms of composition, structure and biological functions (Giroldo et al., 2003).

The lack of information about extracellular polysaccharides in most species of microalgae is a consequence of the no relation between the kind of polysaccharide production and the phylogenetic classification, mainly in reserve and structural polysaccharides. In addition, there are few studies on characterization of these polysaccharides in the taxonomic groups.

Besides the interesting functions performed by these extracellular polysaccharides, many of these compounds have known biological activity (Olaizola, 2003). The wide range of fractions with different molecular weights and different compositions, produced by several not studied species of microalgae, shows the possibility of discovering innumerous bioactive compounds. Among the potential polysaccharides with biological activity commonly observed in microalgae there are fucans, arabinans, arabinogalactan and ramnogalacturanos (Paulsen, 2001).

Fucans are polysaccharides rich in fucose and has a broad spectrum of biological activities including anticoagulant effect, anti-inflammatory, immunostimulatory, anti-viral, anti-tumor and anti-metastasis (Cumashi et al., 2007). The main sources are the brown algae (Class Phaeophyceae), particularly of the Orders Laminariales and Fucales (Cumashi et al., 2007), which produces high sulfated fucans known as fucoidan. Several species of microalgae can also produce fucans with bioactivity, but tests in this area are still quite rare. *Cryptomonas obovata*, *Tetrapyrenoidosa cryptomonas* (Cryptophyceae), *Duostra thalassiosira* (Bacillariophyceae) and *Staurastrum orbiculare* (Zygnematophyceae) are examples of freshwater microalgae producers of fucose-rich polysaccharide (Giroldo and Vieira 2002; Giroldo et al., 2003).

Arabinans, arabinogalactan and ramnogalacturanos are often associated with a class of compounds known as pectins, characterized by the presence of arabinose, galactose and galacturonic acid. These compounds are very common in higher plants and plants used in traditional medicine. Several species of algae, especially those phylogenetically near higher plants, present cell wall pectins (Domozych et al., 2007). Moreover, other green microalgae exhibit cell wall and extracellular polysaccharides with characteristics similar to pectin, such as the glycoproteins (pherophorins) that form the cell envelope typical of Bars and Volvocales Chlamydomonadales (Morita et al., 1999). The immunostimulating activity of Chlorella sp. (Chlorococcales) is directly

related to the presence of arabinose and galactose rich polysaccharide (Kralovec et al., 2007).

Considering the aspects described, it is evident that the production of bioactive polysaccharide by microalgae should be urgently explored. Bioprospecting shows out as an indispensable tool for the discovery of new substances of human interest.

2.1.5.2.1 *Spirulina platensis* Exopolysaccharides

Spirulina sp. secretes sulfated exopolysaccharides through capsule formation and gradually releasing in the culture medium, where it is first dissolved by making it more thick and ends by forming yellow-brown microscopic lumps of varying size (Pinotti, 2004).

EPS production frequently occurs at high and low pH and under strong light (Cornet, 1992). Strong production of EPS is an efficiency loss and led to harvesting difficulties, such as an inability to wring pressing the biomass. Otherwise, the presence of a certain amount of EPS in medium appears to facilitate the harvest, occasionally causing flocculation.

The promising applications of the *Spirulina* EPS are related to your biological activity, and so many properties are being investigated, like antitumor, antioxidant, antibiotic, antiparasitic, etc. (Pinotti, 2004)

2.1.6 Antioxidant Potential

Several studies have pointed out the crucial role of free radicals and other oxidants as responsible for aging and the cause of many degenerative diseases such as cancer, cardiovascular diseases, immune system reduced efficiency, cataracts, brain dysfunction, etc. These free radicals, called reactive oxygen species (ROS), can damage DNA, oxidize lipids and proteins. They attack the polyunsaturated fatty acid chains of phospholipids and cholesterol, removing one hydrogen atom of the bis-allylic methylene group, initiating the process of lipid peroxidation in cell membranes. The carbon radicals formed through this reaction can react with oxygen, leading thus to peroxyl radicals, which in turn can attack new chain polyunsaturated fatty acids, propagating the reaction, and the result is the oxidation of various molecules fatty acids. These hydroperoxides formed by lipid peroxidation have short life and when they react with metals, form aldehydes (eg malondialdehyde, acrolein and crotonaldehyde) and epoxides, which are reactive and cause damage to the “DNA again”. (Atoui, 2005, Sousa et al, 2007)

The body free radical production is controlled by various compounds and antioxidants, which are endogenous (eg the enzyme superoxide dismutase) or from feeding (eg tocopherols (vitamin E), ascorbic acid (vitamin C), polyphenols, selenium and carotenoids). Antioxidants are able to stabilize or disable free radicals. The lack of antioxidants causes a cumulative damage (Atoui, 2005).

According to Souza et al (2007), antioxidants can be considered substances that, in low concentrations, significantly retard or inhibit the oxidation of the substrate, whereas the reaction product between subtances capable of producing radicals and an antioxidant radical is stable and do not propagate in chain. The antioxidant capacity of a substance is the potential to capture and stabilize a given quantity of free radicals. There are several methodologies available to evaluate this capacity. According to Thaipong et al (2006), the following chemicals or processes are used to generate free radicals: 2,2 - azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS); 2,2-diphenyl-1-picrylhydrazyl (DPPH), Ferric Reducing Antioxidant Power (FRAP) and oxygen radical absorption capacity (ORAC).

The chemiluminescence method is based on the principle of absorption of photons emitted by the luminol when it reacts with the free radicals produced by the combination of hydrogen peroxide and a peroxidase enzyme. The addition of an antioxidant substance, leading to the neutralization of free radicals, leads to a decrease in the emission of photons (Cheng et al., 2003).

The ability of a substance to neutralize free radicals is expressed in terms of IC_{50}, which expresses the amount of sample required to inhibit 50% of free radicals to which it is exposed. Thus, the lower is the substance's IC_{50}, the greater is its antioxidant potential (low sample concentration is able to inhibit a large amount of free radicals). Strong antioxidant substances such as rutin and gallic acid, have IC_{50} values about 27.80 mg/mL and 24.27 mg/mL, respectively (Souza et al, 2007).

In the food industry, lipid oxidation is undesirable and is inhibit through the use of free radical scavengers. The most common substances used for this purpose are butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT), tert-butyl hydroxy quinone (TBHQ) tri-hydroxy-butyl fenona (THBP) and epigallocatechin gallate propyl (GP) (Souza et al, 2007). Some studies have demonstrated toxic effects of synthetic antioxidants (Botterweck et al, 2000, Cruces-Blanco et al, 1999). Therefore, intensive researches of natural sources of antioxidants are being done, aiming to reduce or even avoid the use of synthetic compounds.

2.2 Materials and Methods

2.2.1 Biomass

S. platensis strain LEB-52 were obtained from Federal University of Santa Maria (UFSM, Brazil) and cultivated in 6L Erlenmeyer (4L working volume) at 30°C in Zarrouk medium. Illumination was provided by eight cool white 32W fluorescent lamps (3500 lux) in 12:12 h (light/dark) photoperiod. Experiments duration were 15 days.

The agitation was realized with sterile air injection at 0.2-0.3vvm. The initial concentration of *S. platensis* was 0.23 $g.L^{-1}$. Biomass growth was monitored daily by dry weight. Evaluations of maximum biomass concentration (X_{max}, $g.L^{-1}$) and maxiumum productivity (P_{max}, $g.L^{-1}.day^{-1}$) were carried. The maximun productivity was obtained according to the equation $P = (X_t - X_0)/(t - t_0)$, where X_t is the biomass concentration ($g.L^{-1}$) at time t (day), and X_0 was the biomass concentration ($g.L^{-1}$) at time t_0 (day). The maximum specific growth velocity ($\mu_{máx}$, day^{-1}) was obtained by linear exponencial regression applied to the logarithmic growth phase (Bailey & Ollis, 1986).

$$P = \frac{\log N - \log N_i}{t - t_i} \cdot 2{,}303$$

Samples were withdrawn daily and centrifuged in a Sorvall Legend Mach 1.6 R centrifuge (Sorvall, Germany) at 16800g for 15 min. Cells were washed once and dried at 60°C until constant weight, while the cell-free medium was used for further analysis of total sugars by the phenol–sulfuric method (Dubois et al., 1956), predicting the EPS production.

The dried biomass was analyzed for chlorophylls, carbohydrates, proteins, lipids and ash. Pigments were extracted with 90% acetone and the quantification followed the equations suggested by Strickland and Parsons (1968). Lipids were determined by extraction with methanol:chloroform 1:1 (Bligh and Dyer, 1959) followed by a liquid–liquid extraction with hexane (Sydney et al 2010). Ash was quantified by the AOAC 941.12 method, while phenol–sulfuric method (Dubois et al., 1956) was used for total carbohydrate and the Lowry method (Lowry, 1951) for protein determination. The phenolic compounds were quantified by the Folin-Ciocalteu method (Costa, 2010), and the antioxidant potential was determined using two different methodologies: the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay system and chemiluminescence assay (Cheng, 2003) using a 10% (w/v) biomass aqueous extract.

2.2.2 Exopolysaccharides

Samples collected at various intervals from shake flask were vacuum filtered by filter-paper (Whatman 1). The resulting culture filtrate was concentrated to ¼ of the original volume by rotary evaporator below 50°C. The filtrated was dialyzed in a membrane with 12-14 KDa cutoff. For EPS precipitation, four volumes of absolute ethanol was used, stirring vigorously and left overnight at 4°C. After centrifugation at 16800xg for 10 min, the supernatant was discarded. The pure EPS was lyophilized and the weighted.

The extracellular polysaccharide concentration was determined according to the classical method of Dubois et al. (1956) using glucose solution as a standard reference.

The EPS composition analysis was performed in a Shimadzu HPLC (*High Performance Liquid Chromatrography)* equipped with a refractive index detector. The column used was Aminex HPX87H. Analytical conditions were: H_2SO_4 5mM, 60°C, 0,6mL/min.

The antioxidant potential of samples was determined using the DPPH and the chemiluminescence method. The analysis of phenolic compounds was performed through the Folin-Cicauteau method.

2.3 Results and Discussion

2.3.1 Biomass

2.3.1.1. Growth and Composition Analysis

The *S. platensis* growth pattern can be seen in figure 2.2. It's an expected behavior, showing a lag phase (until day 5), a log phase (between days 5 and 12) and the beginning of the stationary phase.

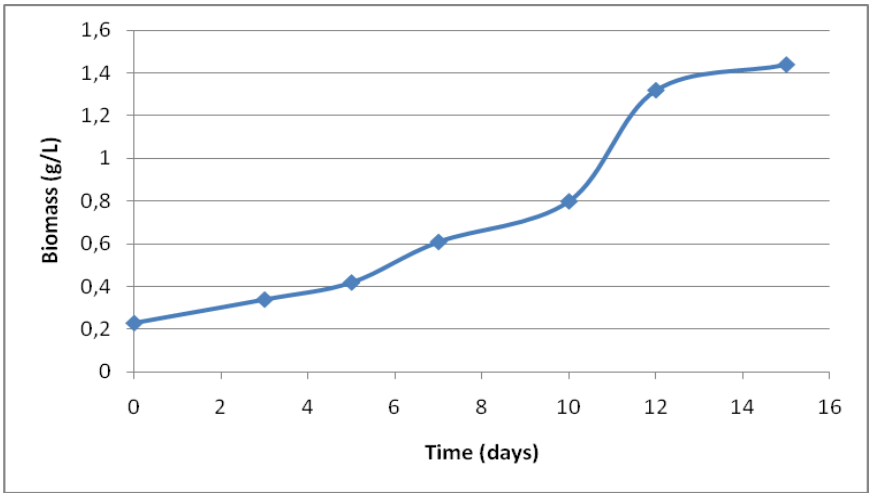


Fig. 2.2: *S. platensis* grow pattern, during 15 days.

The X_{max} achieved was 1,44g.L^{-1}, and the maximum productivity P_{max} was 0,42g.L^{-1}.d^{-1}, and μ_{max} 0,43 day^{-1}.

The *S. platensis* general biomass composition in terms of protein, sugars, pigments, lipids and ash is showed in table 2.1. The composition is in accordance to other works (Andrade & Costa, 2008; Sydney et al, 2010).

	Protein (%)	**Sugars (%)**	**Pigments (%)**	**Lipids (%)**	**Ash (%)**
Spirulina platensis LEB-52	52.33 ± 1.5	10.6 ± 0.92	16.32 ± 2.1	10.3 ± 1.7	6.81 ± 1.6

Table 2.1: *S. platensis* biomass centesimal composition.

2.3.1.2 Phenolic Compounds

Phenolic compounds test was performed in the and in the polysaccharides, using a biomass water dissolution at 10% (v/w). *S. platensis* contains many pigments, including chlorophyll-a, xanthophyll, betacarotene, echinenone, myxoxanthophyll, zeaxanthin, canthaxanthin, diatoxanthin, 3'-hydroxyechinenone, beta-cryptoxanthin and oscillaxanthin, plus the phycobiliproteins c-phycocyanin and allophycocyanin (Vonshak, 1997). All the enumerated pigments have unless a phenolic characteristic region and they can play a role as antioxidants. The total phenolic compounds was equal to 150.39 mg in 100 g of biomass.

The amount of Chlorophylls a and b and the Carotenoids are shown in Table 2.2.

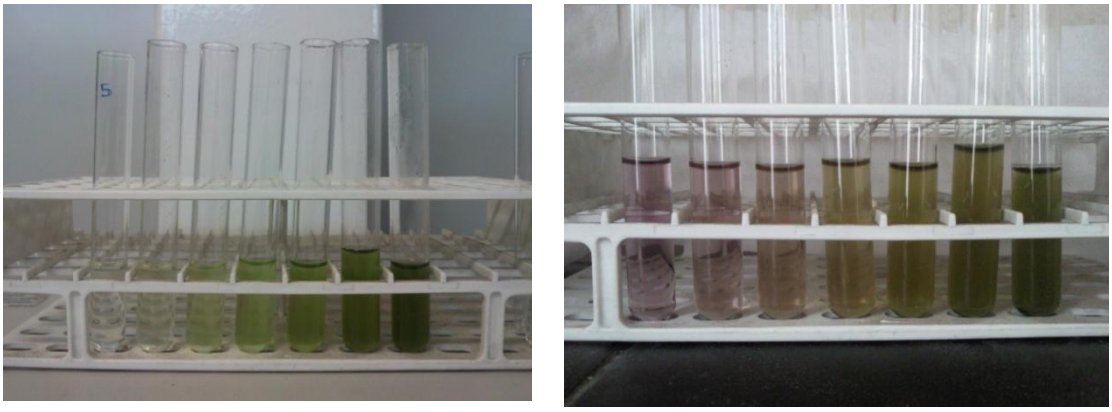
Pigments	**(mg/m³)**
Chlorophyll a	297,8
Chlorophyll b	133,78
Carotenoids	68,44

Table 2.2: **Chlorophyll and Carotenoids quantification *S. platensis* biomass.**

2.3.1.3 Antioxidant Potential

Initially, qualitative tests were performed with aqueous biomass and polysaccharide extracts. A 10% water dissolution of these samples were tested against 0.2 µL of reagent DPPH. The biomass sample caused the discoloration in a test tube (it was originally purple), and thus was deemed to contain antioxidant potential. However, perhaps due to the existing color in this extract of biomass, the final color in the tube was not yellow (corresponding to the coloring of the total free radical inhibition) (Figure

2.3). Despite the indication of potential antioxidant, in this sample it was not possible to obtain the antioxidant potential curve due to the method used, DPPH.



(a) (b)

Fig. 2.3: Aqueous biomass extract dilutions to perform the DPPH test (a) and the DPPH test results (b). Note the purple color disappearance in tubes and the green color influence (b).

Thus, we used another method to quantify the antioxidant potential of the biomass of *S. platensis*: the method of luminol-dependent chemiluminescence. This method is fast, reliable and effective in determining the ability of some antioxidants is to neutralize free radicals (Cheng et al, 2003).

Bar graphs shown in figure 2.4 indicate the signal measured by the equipment (Greater area under the curve AUC-detected), proportional to the free radical content. Thus, higher antioxidant power of the sample will result in lower AUC. The solvent used in dilutions was phosphate buffer, pH 7.4. A control was made with distillated water.

It was conclude (Figure 2.4) that the biomass sample had a capacity of almost 100% inhibition of free radicals by at a concentration of 2.0 mg/mL. Thus, the sample's IC_{50} can be calculated, which is equivalent to 0.962 mg/ml.

As a parameter, are normally used in food industry the BHT (di-tert-butyl-methyl phenol) with an IC_{50} of 12.5 µg/mL, and BHA (tert-butyl-4-hydroxyanisole), with an IC_{50} of 2.97 µg/mL.

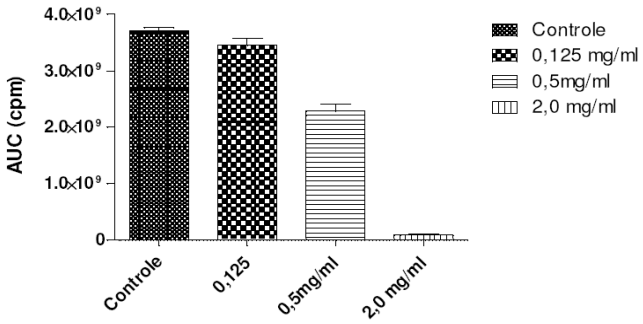


Fig. 2.4: The luminol-dependent chemiluminescence assay results of *S. platensis* aqueous biomass extract in different concentrations.

2.3.2 Exopolysaccharides

2.3.2.1 Production

The *S. platensis* EPS production was accompanied during 14 days, reaching 0.46g.L^{-1} (Figure 2.5), what means 0,183gEPS/gbiomass (18.3%). Neto et al (2004) reached 20% to the cianobacterium *Mastigocladus laminosum*. The theoretical balance for *Spirulina platensis* (Cogne, 2003) shows that this value can reach 51%. It indicates that EPS production can be much more enhanced.

In figure 2.5, it is possible to see the enhanced EPS production in the middle-end of the growing period. EPS production is associated to nitrogen deficiency or phosphate absence (De Phillipis; Vincenzini, 1998). This explains why, besides its proportionality to biomass concentration, EPS production was greatly increased after the beginning of the stationary phase of growth.

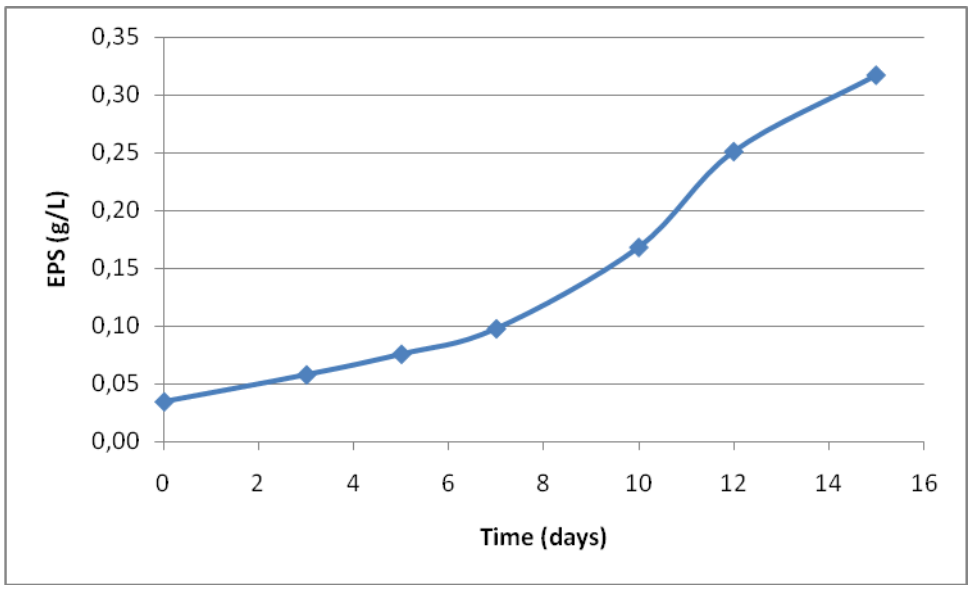


Fig. 2.5: *S. platensis* EPS production curve in the 15-days experiment.

2.3.2.2 Appearance

The no-dialyzed lyophilized EPS has a very similar appearance to kaolim. Both are powdered, clear and low density (Fig. 2.6).

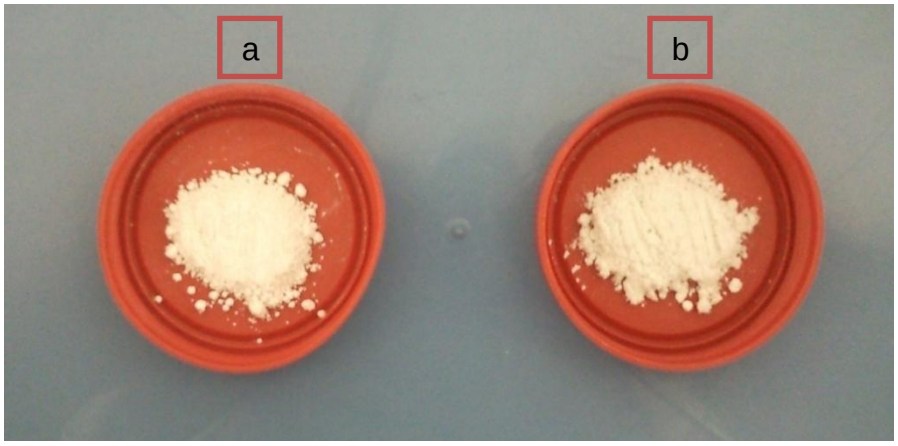


Fig. 2.6: The general powdered appearance of Kaolin sample (a) and liophilizated *Spirulina platensis* EPS (b).

2.3.2.3 Monomers Composition (HPLC)

Until 1988, 70 cyanobacteria strains were studied for EPS production (de Philippis e Vicenzini, 1998). Glucose is the monosaccharide most found in cyanobacterial EPS is glucose (90%), followed by galactose, mannose and rhamnose (80-85%).Cyanobacterial EPS showed the following monosaccharides in their composition:

(i) Hexoses: glucose, galactose and mannose;

(ii) Pentoses: ribose, xylose and arabinose;

(iii) Deoxhyhexoses: fucose and rhamnose;

(iv) Acid monosaccharides: glucuronic and galacturonic acids.

The monosaccharide composition of the EPS produced is showed in table 2.3. Hexoses was founded to be the main constituents of the EPS (glucose, mannose and galactose), but high amount of pentoses was also observed (rhamnose and fucose).

Compounds	%
Protein content	49.2
Carbohydrate content	42.1
Monosaccharide	**mol %**
Rhamnose	11.5
Fucose	13.4
Ribulose	1.7
Arabinose	1.7
Xylose	6.5
Mannose	**25.1**
Glucose	**24.3**
Galactose	16.0

Table 2.3: EPS protein and carbohydrate content and its monosaccharide composition.

2.3.2.4 Phenolic Compounds and Antioxidant Activity

The phenolic quantity in the EPS was equal to 7.44 mg (in 100 g of EPS sample).

To the DPPH assay a dissolution of 10% (w/v) was prepared. This solution was diluted (fig. 2.7 – a) and the assay was carried. It was observed a slight decrease in the purple coloration (fig. 2.7 – b) and also the appearance of turbidity. So this test was only used as a qualitative analysis: the free radical scavenging exists for this sample, but can't be measured by this method.

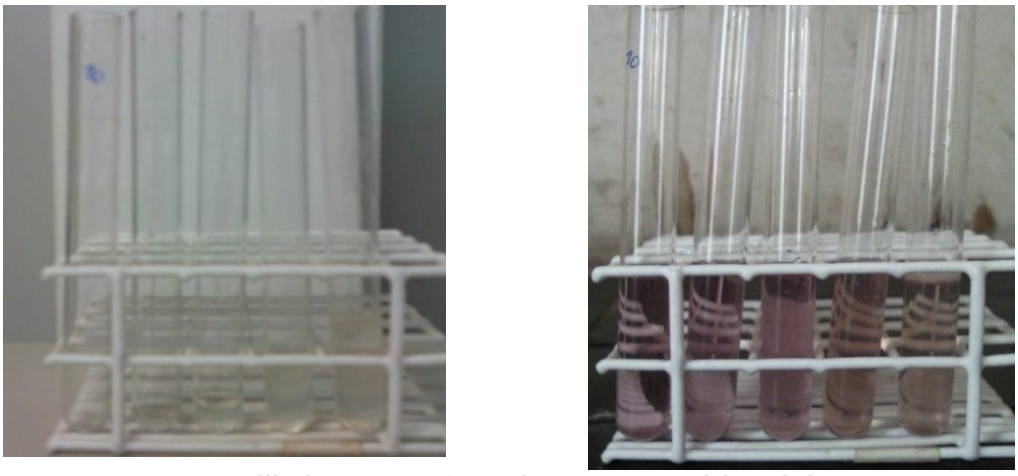


Fig. 2.7: Aqueous EPS dilutions to perform the DPPH test (a) and the DPPH test results (b). Note the purple color disappearance in tubes and turbidity appearing (b).

To quantify the EPS antioxidant potential the luminol-dependent chemiluminescence method was performed. EPS IC_{50} was equal to 2.878 mg/ml. This value is three times higher than the biomass extract IC_{50}, what means that the biomass extract is three times more efficient against free radical than the EPS.

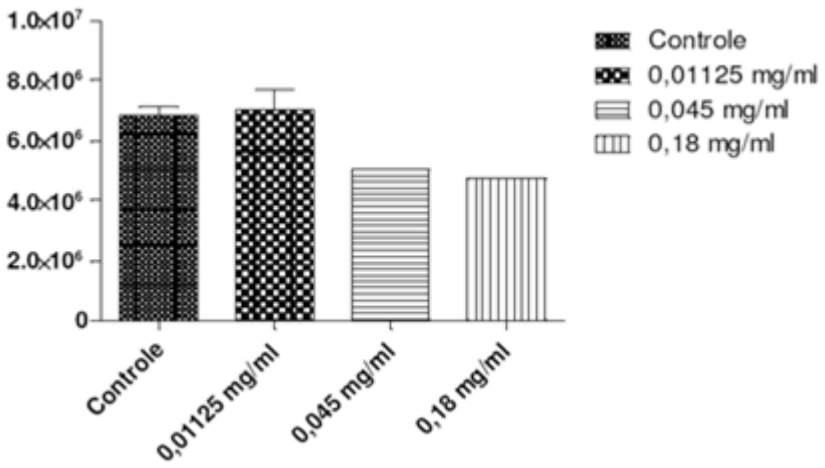


Fig. 2.8: The luminol-dependent chemiluminescence assay results of *S. platensis* EPS in different concentrations.

2.4 Conclusions

- It was possible to cultivate *Spirulina platensis* and obtain biomass and EPS at usual levels;
- The biomass and EPS analysis were performed and their composition are similar to those described in the literature;
- It was found that the *S. platensis* EPS has a protein-glucan nature;
- The antioxidant capacity test demonstrated the potential of *S. platensis* biomass and EPS for cosmetics application;
- It was observed potential possibility to replace or unless diminish the use of synthetic antioxidant substances, like BHA and BHT, by *S. platensis* biomass extracts;
- S. *platensis* EPS showed visual similarity to Kaolim, which is a mineral used in cosmetic industry to improve rheological and sensorial properties.

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Chapter 3

Cosmetic Products – An Overview

3.1 Cosmetics

3.1.1 History and Definitions

The use of cosmetics was present even in the more primitive societies, in body painting both for ornamental and religious purposes. Over time, cosmetic products were developed and improved. Around 180 A.C., the Greek physician Claudius Galen developed a cold cream, the first chemical-pharmaceutical product. Although during the Middle Ages the use of cosmetics was suppressed due to the ban the cult of hygiene and exaltation of beauty, their use was restimulated during the Crusades. In the late seventeenth century, it was considered a manifestation of witchcraft until the Contemporary Age (mid-nineteenth century). Cosmetics industries have emerged only in the early twentieth century. (SECEX, 2004; SEBRAE, 2007; Romanowski & Schueller, 2001. v.1).

Brazilian definition of cosmetic products is, according to Resolution RDC n°. 79 of August 28, 2000 (Brasil, 2000):

> "Cosmetics are products made with natural and synthetic substances or their mixtures, for external use in various parts of the human body (skin, hair system, nails, lips, genitalia, teeth and mucous membranes of the oral cavity), with the goal of cleaning them, perfuming them, alter their appearance, correcting body odors, protect them or keep them in good condition."

The EU definition is essentially the same adopted by Brazil and MERCOSUL, through Resolution n° 31, 1995.

3.1.1 Industry Overview

Over the past 14 years, the Brazilian industry of personal hygiene, perfumery and cosmetics deflated an annual average growth of 10.5%, much higher than the average growth of other industries (2.3%). This increase is attributed to several factors, such as increasing participation of women in the labor market, advanced technologies development and productivity increased, supporting good prices and constant new products release. Another relevant factor is the increase in life expectancy. In the last decade, Brazilian exportation of personal care products, fragrances and cosmetics had an accumulated increase of 249.7%, against a cumulative exportation of 121.0%. The surplus in 2009 reached R$ 131 million (ABIHPEC, 2010).

Table 3.1 describes Brazilian specific cosmetic products exportation in 2009:

		US$ Million	% Vs. 2008	% Vs. 2000
2009 – FOB	Hair Products	162.627	0,7	1.281,1
	Oral Hygiene	125.731	-16,6	217,1
	Soaps	113.513	-27,3	185,7
	Deodorants	47.668	4,3	766,2
	Disposable(*)	46.723	8,4	37,9
	Other	91.312	0,7	143,6
	TOTAL	587.575	-9,3	249,7

(*) Includes diapers, sanitary napkins, toilet paper and napkins, towels and wipes.

Table 3.1: TPCS products 2009 brazilian exportation (Source: ABIHPEC, 2010)

In relation to the world market, Brazil occupies a prominent place. According to Euromonitor (2009) Brazil is in third place (Table 3.2), representing 8% of global cosmetic exportations. The Brazilian performance also includes, in terms of world exportation: first place in deodorants, second in children's products, male enhancement products, oral hygiene, sunscreen, perfume and bath, and third in products for hair and stains, sixth in skin and eighth in depilatories.

Personal Hygiene, Perfumery and Cosmetics		2009 US$ Billion (consumer price)	%	
			Growing	Participation
Countires		350,3	-2,1	
1	United States	58,9	-1,3	16,8
2	Japan	39,9	6,6	11,4
3	Brazil	28,4	3,2	8,1
4	China	20,8	10,4	5,9
5	Germany	17,4	-4,2	5,0
6	France	16,3	-6,8	4,7
7	United Kingdom	15,0	-13,2	4,3
8	Italy	12,5	-5,4	3,6
9	Spain	10,7	-17,7	3,1
10	Russia	10,4	-9,2	3,0
Top Ten		230,37	-1,6	65,8

Table 3.2: Top ten world markets of HPPC. (Source: Euromonitor, 2009)

3.1.3 Cosmetics Classification

Cosmetic products can be divided into four categories according to their use (SEBRAE, 2007): hygiene products, cosmetics, perfumes, and products for babies. In relation to risk, they can be divided into two groups, according to Table 3.3 (SEBRAE, 2007; RDC 211/05, Annex II):

Level Risk	Type of Risk	Information Required	Examples
1	Mínimum	Elementary or basic properties, prove initially did not necessary; not required detailed information (mode and use restrictions).	Soaps, shampoos, shaving creams, after-shave lotions, toothbrushes, dental floss, powder, beauty creams, beauty lotions, oils, make-up, lipstick, lip pencils and eyeliners, eye products and perfumes .
2	Potential	Specific directions; proof of safety and efficacy, need for information and care, method and use restrictions.	Anti-dandruff shampoos, toothpaste anti-caries and anti-plaque, intimate feminine deodorants, underarm deodorants, chemical exfoliators for the skin, for protecting the lips with sunscreen, certain products for the eye area, UV filters, tanning lotions, tinctures hair, bleaching, permanent waving products, products for hair growth, hair removers, cuticle removers, stain removers, nail hardeners, insect repellents and all products for babies.

Table 3.3: Cosmetic product classification according to its potential risk to the human health.

The criteria for this classification were defined according to the probability of occurrence of unintended effects from the misuse of the product, its formulation, use intention, areas of the body application and precautions to be observed during its use. It is noteworthy that cosmetic products classified as Level 2 are submitted to technical requirements much more stringent. (Noguti et al, 2007).

3.1.4 Raw materials and cosmetic formulations

There are over 12,000 ingredients that can be used in cosmetics (ANVISA, 2008), such as thickeners, emulsifiers, dyes, scents, etc. Many of these ingredients are known, besides their chemical name, by its trade names. This variety of nomenclatures may cause confusion during buying and selling materials and can cause unwanted

changes in the final products. To minimize this problem, INCI - International Nomenclature of Cosmetic Ingredient – was created. It is an international coding system of nomenclature of cosmetic ingredients, widely recognized and used, created to standardize the names of ingredients and the products labeling (ANVISA, 2008). This nomenclature system has many advantages for the consumer (clearer identification and reduction of errors of interpretation), for health surveillance (greater agility in the identification of ingredients of cosmetic products in a more clear, correct and accurate way) and also for the scientific community (advice for consumers, and dynamic updating of scientific knowledge).

Different materials have different functions in cosmetics and may, for example, modulate the distribution of the active principles among the target cells of the skin, act as a matrix, increase the penetration speed and guide the location of certain biomolecules, among various other goals. These vectors are usually solutions, suspensions or emulsions. Solutions result from dissolution of solutes (gases, liquids or solids) in a liquid, called solvent, generating homogeneous mixtures. Suspensions are dispersions of fine solid particles (dispersed) in a liquid (dispersant) in which they are insoluble (Fresnel, 1999).

Emulsions are mixtures relatively stables of oil and water, produced by mixing water soluble and fatty soluble substances with an emulsifier agent. The substances affinity is manifested not only by solubility but also by "phase" formation. When two or more immiscible substances coexist in a solution, each one is regarded as a "phase". In two-phase systems, one substance is distributed in the other. In these circumstances, the first phase is known as "internal", "dispersed" or "discontinuous", and the latter phase as "external" or "continuous."

There are four types of emulsions: water-oil emulsions (W-O), oil-water emulsions (O-W), multiple emulsions and microemulsions. The choose between them depends on the active ingredient and which cells are target, or on the specific type of skin of the consumer.

The water-oil emulsions are an oily continuous phase with aqueous substances dispersed. This type of emulsions is the basis for greasy creams and are lipid soluble, insoluble in water and do not conduct electricity. In the oil-water emulsion, the continuous phase is aqueous and the dispersed phase is oily. Emulsions are less greasy than its components, are water-soluble, insoluble in lipids, and conduct electricity.

There are two types of multiple emulsions: hydrophilic-lipophilic-hydrophilic, where an oil phase separates two aqueous phases, and the lipophilic-hydrophilic-lipophilic, where an aqueous phase separates the oily phases. It is prepared with an

initial emulsion, which is subsequently used to prepare the second emulsion. (Fresnel, 1999)

Microemulsions consist of oil, water and surfactants. Its particular structure is due to the fact that molecules hydrophilic and lipophilic surfactants and are intertwined in each other, forming a homogeneous system. They are very stable and better absorbed by the skin, greatly improving its appearance. They are commonly used in bath oils. (Fresnel, 1999; Moore & Wilkinson, 1990)

3.1.4.1 Kaolin

According to Silva, 2001, the term kaolin or "china clay" derives from the Chinese word Kauling (which means high hill) and refers to a hill of Jauchau Fu, located in northern China, where the material was obtained long ago. It is one of the most important and one of the six most abundant minerals in the top of the Earth crust (10 meters depth). The same author reports that the first industrial use of this mineral was in the manufacture of ceramic and porcelain, many centuries ago, and that only in the 1920s it began to be used in the paper and rubber industry. Kaolin is now also used to produce plastics, pesticides, paints, adhesives, cement, animal feed, food and pharmaceutical products, including cosmetics, fertilizers and others, currently having a very large variety of industrial applications.

Kaolin has many industrial applications and new uses are constantly being researched and developed. Kaolin is a mineral with special features very interesting for many industrial applications, including cosmetic ones. Special features include chemically inertion over a wide pH range, white color, great hiding power when used as a pigment or extender, soft and slightly abrasive, low conductivity of heat and electricity and low cost (Silva, 2001). In terms of sensorial characteristics and rheological properties it gives finesse to the final product, being mostly indicated to people with sensitive skin, and can even be ingested.

World reserves of kaolin are abundant. Brazil is the sixth largest producer of kaolin, with approximately 2.8 million tons in 2008, or about 6% of world production, approximately. The United States is the largest global producer. The Brazilian reserves of kaolin reach 7.3 million tons. The detailed Brazil and World productions can be seen in figure 3.1, as the prices.

(a)

(b)

Figure 3.1: World x Brazil Kaolim Production in the last decade (a); kaolim's prices evolution since 2001 (b).

In 2008 Brazil exported 2.8 million tons of kaolin around (U.S. $ 305 million), representing an increase of 20% over the previous year. Countries that import Brazilian Kaolin are Belgium (21%), United States (20%), Japan (14%), Netherlands (13%), Finland (11%) and others (21%).

The mining activity of kaolin strongly modifies the environmental conditions of where it is extracted. Although it starts to perceive an incipient environmental concern, the kaolin industry still cause disorders such as excessive production of particulates generated during transportation (raw gross) and production tailings (waste). These wastes contain, in addition to other contaminants, concentration of metals such as iron (Fe), aluminum (Al), zinc (Zn) and cadmus (Cd), above the legally allowed. In Brazil, the first legal provision in order to minimize the negative impacts caused by mining was based on the Mining Code of 1967, that provides, among other things, that the miner

should *" (...) prevent air pollution or water that may result from mining works; protect and conserve the sources and use the water according to the precepts technicians (…)."*

Typically, kaolin mines rejects are usually placed in adjacent areas with no criteria, contaminating the soil and water bodies in the region. Several old mines are abandoned without any action being taken to recover. The inspections are rare and inefficient. However, mining should be done based on the principle of rational use of natural resources, as described by the Brazilian Federal Constitution of 1988:

> *"Art 225, § 2 Those who explore mineral resources shall be required to restore the degraded environment, according to the technical solution required by the competent organ as provided by law."*

Thus, despite being a cheap and abundant element, solutions for the replacement of kaolin are important. The development and of materials with similar characteristics, but not causing environmental impacts, is indispensable. Society demands for environmentally friend and sustainable products.

3.1.5 Innovations in Cosmetics

Innovation, as may be appropriate for some instrument of intellectual property, can represent for a particular company or institution a right of exclusive economic exploitation, thereby inhibiting a possible opportunistic behavior of its competitors (Schumpeter, 1985). However, in addition to innovation, the cosmetic industry should seek to develop other strategies, including, for example, more efficient production and more effective, better sales structure (distribution channels, advertising, brand promotion), assists post sales and technical support, etc.

Thus, one of the major aspects involved in innovations in cosmetics is related to new raw materials and additives development, which gives different properties and characteristics capable of bringing better and faster results and that are consistent with contemporary concepts, such as improvement of quality of life and environmental sustainable.

The growing importance of such new products in recent years has led to the adoption of the term "cosmeceuticals", or cosmetics with therapeutic goals and that use active chemicals, a phenomenon parallel to that in the food industry ("nutraceuticals"). Industry professionals are almost unanimous in pointing out that this segment of the cosmetics industry has strong growth potential, which could generate business opportunities in the sector (Portal Educação, 2010).Thus, the search for applications of biomolecules, not only from extractive processes but also from other sources such as

biotechnological processes, is extremely important. They can provide better cosmetic properties and characteristics, and still leading a more environmentally and economically sustainable product with greater added value and market acceptance.

3.1.5.1 Microalgae and Algae Products in Cosmetic Application

Microalgae and their products use in cosmetic products as creams, gels, shampoos, etc is very disseminated. Heterogenic polysaccharides, called mucilage, are extracted from brown (Feoficeas) and red (Rodoficeas) algae. When in water contact these mucilage form colloidal not adhesive. Among these compounds, alginate, agar and carrageenan are the most used. Other species, like *Fucus vesiculosus* (a red algae with emollients properties), have the hability to accumulate iodine and are used in anticellulitic treatments.

The powdered *Macrohystis pyrifera* presents sebaceous secretion regulation properties. Transpiration reduction properties are assigned to the specie *Rhodymenia palmaria. Delesseria sanguínea* e *Undalaria punnatifada* act on skin toning, as moisturizing and as anti-wrinkles action. *Dunaliella salina* is a pro-vitamin A (beta-carotene) source and its extracts stimulate cell regeneration at the level of the epidermis or dermal connective tissue; moreover It also seems to stimulate the melanin production and has antioxidant power. *Plocanium coccineum* is a source of red pigments, which generate less risks than synthetics ones but shows problems of instability (Barata, 2000).

3.1.6 Human Skin

Skin is defined as an active barrier between the environment and the human organism. It is responsible to controls fluids loss, avoids dangerous substances penetration and protects against mechanical shocks, also regulating the thermic changes. The permeability barriers are cells, firmly packed, that forms the epidermis. (Moore & Wilkinson, 1990)

The epidermis is constituted by many layers, which are result of keratinocytes changes. They are borned in the basal layer and dead on the last layer (skin surface). There are another tree cells kinds in the skin: melanocytes (pigmented cells), Langerhans cells (dendritic, with no color) and Mekel cells (related to the tact) (Moore & Wilkinson, 1990).

The skin layers are: stratum basal (or germinativum), the stratum spinosum, the stratum granulosum, the stratum lucidus, and the stratum corneum. For this study, the most important is the Stratum Corneum. The queratinocits are anuclear cells that live in the Stratum Corneum and have great quantities of keratin. The corneum cells are

released continuously from the skin surface, and the moisturizer skin degree is directly related with the water found in this layer (Moore & Wilkinson, 1990).

3.2 Conclusions

- The mondial and brazilian cosmetic market have place to new and innovative products;
- The microalgae are beginning to be intensively researched as source for new and innovative cosmetic products and stand out as one interesting alternative for the cosmetic industries.

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Chapter 4

Creams Formulation and Experimental Design

4.1 Introduction

In cosmetic context, the “cream” term means a solid or semi-solid emulsion. If an emulsion has a so low viscosity as the gravity makes it to flow, this substance is called lotion, and don’t cream. The skin creams can be classified according to its function and composition: cleaners, to night, to massage, moisturizers, evanescent, hands and body protectors, and for general use creams. (Moore & Wilkinson, 1990)

According to Moore & Wilkinson, 1990, the proposed cream, for table 4.2, is an evanescent and base cream. This kind of cream is easily spread and has the sensation that disappears rapidly after applied. To reach a fast absorption effect, the fatty phase, besides having a low composition percentage, have emollient esters, as stearic acid, an emollient substance that generates smooth and well-being. It tenderizes the skin, leaving it non greasy, and makeup can be applied easily. The oil phase remains invisible during use, provides a film no-grease, and leaves a very attractive final product.

All cosmetic emulsions are made by two imiscibles liquids: one dispersed as little drops in the other, and separated by a tensoactive agent in each size of the liquid-liquid frontier, as explained in Chapter 3. The phases are mixed at turbulent conditions: one of them divides into little drops, because the shear force, and is distributed completely in another phase. During the agitation, the drops will divide into ever smaller drops, until the point at which the applied force is not able to reduce their size, and they are in almost same average diameter, with little variation.

The creams were prepared by using the ingredients showed in Table 4.1. The aqueous and oily substances were mixed, according to its affinity, and the two phases were warmed separately to 75.0°C, in bath. Under constant agitation, the oily phase was shed into the aqueous phase, and manual agitation was done until the mixture reaches room temperature.

After cream preparation, the additives (both EPS and aqueous biomass extract) were added, and cream’s appearance, color and homogeneity, observed. This addition caused emulsion instability, with viscosity loss and phase separation. So, it was chosen a second formulation, presented in table 4.2, which didn’t caused destabilization but improved general creams characteristics: it became softer, less shining and sticky (Figure 4.1).

Compound	%
Ethoxylate Stearyl alcohol	1
Stearyl alcohol	1
EDTA	0,05
Isopropyl Palmitate	2
Triethanolamine	6
Propylparaben	0,02
Methylparaben	0,18
Propylene glycol	3
Polymer Carboxivinilic	15
Distilled water	q.s.p.

Table 4.1: Composition of formulation # 1

Compound	%
Ethoxylate Stearyl alcohol	3
Stearyl alcohol	6
Silicone Fluid (dimethicone copolyol)	1
Cetyl palmitate	3
Isopropyl myristate	4
Butylhydroxytoluene (BHT)*	0,05
Propylparaben	0,02
Methylparaben	0,18
Propylene glycol	3
Ethoxylated lanolin 50%	2
Distilled water	q.s.p.

Table 4.2: Composition of formulation # 2
*BHT wasn't added, not to interfere in the cream antioxidant quantification.

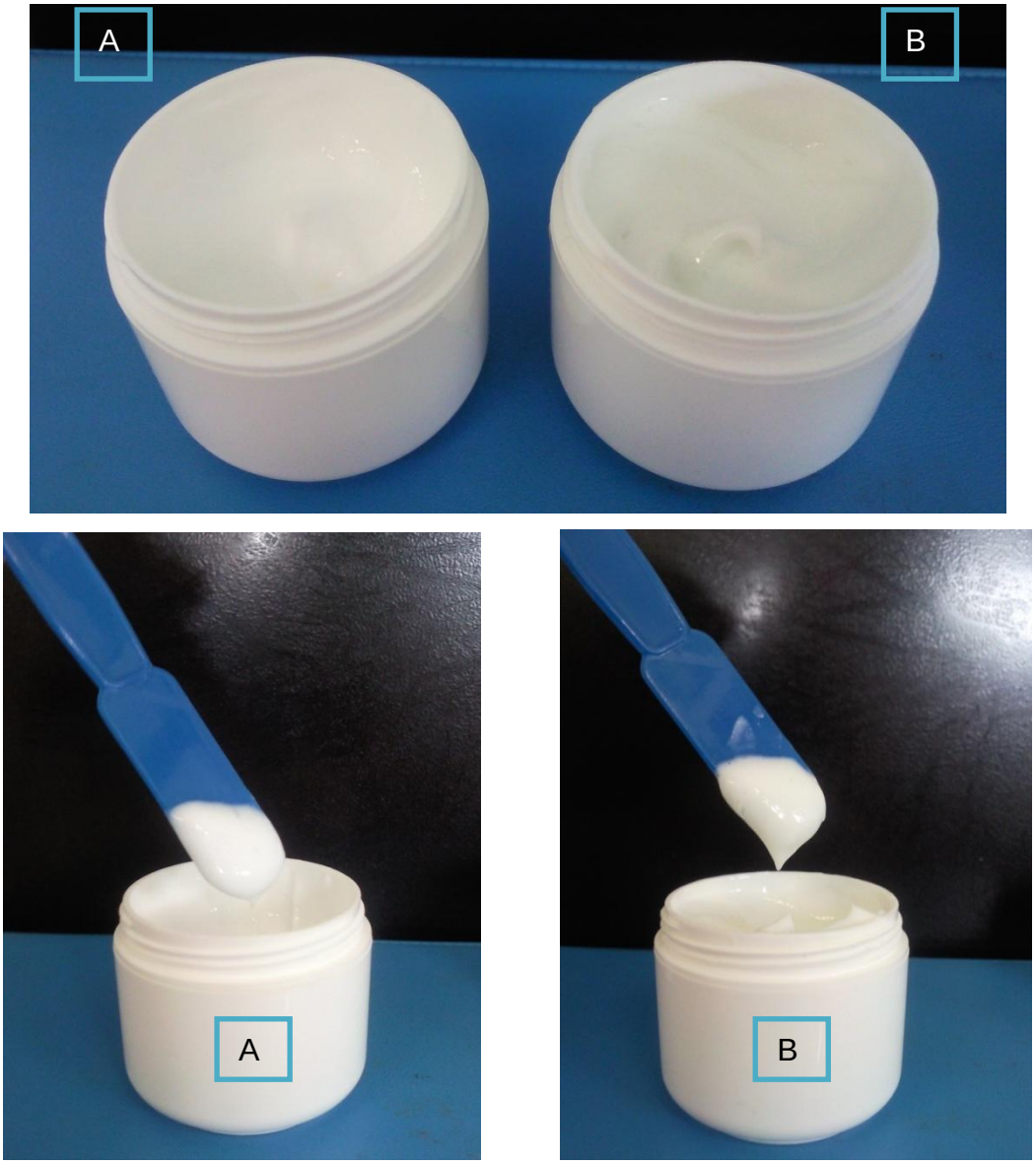

Figure 4.1: The general appearance of Base Cream (A) and Spirulina Cream (B). A slight green color can be observed in (B) due to the aqueous *S. platensis* biomass extract.

4.1.1 Biomass Extract in Cream Formulation

A biomass extract was prepared by adding water in a dry biomass. After vigorously agitation (3 or 4 times in 30 minutes), the mixture was centrifuged (Sorvall Legend Mach 1.6 R, 5000rpm, 5 minutes). Two milliliters of the supernatant was used for each 50 grams of cream. Choosing this quantity was based in the tests of antioxidant power, which were carried and are described in Chapter 2.

4.1.2 EPS in Cream Formulation

EPS was added to the cream formulation in concentrations of 0.5, 2.5 and 5.0% (w/w). Rheological analysis were performed, trying to predict any destabilization problem and are described in Chapter 6. From here, the so-called “Base Cream”

designates the Cream prepared according to Table 4.2, without any addictive, while the "Spirulina Cream" has the same basic composition as the Base Cream but added with *Spirulina platensis* aqueous extract and EPS at determined concentrations.

4.1.3 Experimental Design

Formulating a new product involves not solely the correct formulation chosen, acceptable to the formula owner and apparently having good properties and characteristics. As it can be a commercial product, stability tests may be performed, to guarantee that in this shelf life, any problem occurs and the consumer's acceptance continue being the same.

With this aim, once the EPS concentration was defined, in Chapter 5, preliminar and accelerated stability tests were performed to foresee the cream stability against severe transport and storage conditions, giving important information about the cream shelf life and evolution through the time (Chapter 6). With these results, could be needed reformulations, or changes in the formula, aiming to maintain the physical, chemical and biological creams properties.

Also, in vivo tests may be performed, to foresee the cream irritability and to give reliable information about the allergenic Spirulina cream potential. This test was carried in guinea pigs (Chapter 7), before the cream's contact with human. So, if there are any irritant response in the guinea pigs skin (that is more sensible then the human skin) reformulations can yet be done.

After all, the consumer's acceptance is a crucial factor. So, sensorial tests were performed (Chapter 8), to evaluate consumer's impressions and opinions about the new developed cream. The expected enhanced properties were evaluated by the volunteers, comparing the Base and Spirulina Cream, directly seeing and using the products. The effects of biomass extract and EPS in moisturizing power was also evaluated and described in this Chapter.

4.2 Conclusions

- It was possible to find a formulation that, with the EPS addition, there were no emulsion problems in viscosity, aspect, pH or color.
- The EPS addition can cause emulsion destabilization, and before test a new formulation, preliminary tests has to be performed.

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Chapter 5
Rheological Analysis

5.1 Rheology

According Schramm (2006), rheology is the science that describes body deformation, whether solid, liquid or gas, under the influence of stresses. To Bretas and D'Avila (2005) ideal solid deforms elastically and the energy required for its deformation is completely recovered when the stress is removed. Ideal fluids such as liquids and gases deform irreversibly, draining, and the energy required for deformation is dissipated within the fluid as heat and cannot be restored merely by the tensions removal.

Many of the textural properties that humans perceive when they consume food, cosmetics, and various other products, are basically the rheological ones, including creaminess, juiciness, tenderness, softness and hardness (Haminiuk, 2007). According to Fields and colleagues (1989), the rheological data are essential to:

• Calculations in process engineering, involving a large variety of equipment such as pumps, pipes, extruders, mixers, heat exchangers, among others;

• Determination of ingredients functionality in product development;

• Control intermediate or final product quality;

• Shelf life testing;

• Evaluation of the products texture (foods, cosmetics, etc.) and correlation with sensory tests.

5.1.1 Fluids Rheology

Isaac Newton expressed the basic viscometry law, describing the flow behavior of an ideal liquid, (equation 5.1), where t is the shear stress, h is the dynamic or apparent viscosity and γ is the rate of shear or deformation.

$$t = h \cdot \gamma \qquad [5.1]$$

The fluid deformation generates modifications through the continuous force action at a constant rate. This condition can be designed and monitored by using two parallel plates, with fluid placed in the space between them. The bottom plate is fixed and the top moves at a constant speed, and deformation in the fluid is evaluated.

The shear stress (t) relates to a force (F) applied parallel or tangential to the face of a material (A). The application of the force F produces a flow of the material,

generating also a resistance force, the so called shear stress. By keeping F constant, the flow rate can be maintained. The share stress (t) is also a consequence of the internal resistance of the liquid, ie the viscosity (Navarro, 1997). The shear stress generates different responses in solids or liquids. The firstdeformed, while the other flows.

The shear stress acting on the fluid makes it flow in a certain pattern. The maximum speed is observed far away from the stationary surface, while the minimum speed is observed in the liquid in contact with the stationary surface. This velocity gradient is defined as shear rate (γ); by increasing the shear rate, the material viscosity can increase or decrease (Navarro, 1997).

Viscosity is a rheological property defined as the resistance to flow, it becomes apparent when a fluid layer moves relatively to another. Thus, with increasing the fluid viscosity, it also increases the frictional forces, requiring more energy to fluid displacement.Consequently, highly viscous fluids require more force to flow than less viscous materials (Bretas; D'Avila, 2005).

Viscosity is expressed through the mathematical formula presented in equation [5.2], where h is the dynamic or apparent viscosity, t is the shear stress and γ is the rate of shear or deformation.

$$h = t / \gamma \qquad [5.2]$$

Among the parameters that affect the viscosity of polymers are the type of molecular structure, temperature and concentration of polymer solution analyzed. Temperature can cause a decrease in viscosity, while concentrated polymer solutions tend to have higher values of h (Pinto et al, 2002; Silva 2006), for example. Polymeric structures can have their arrengement undone by increasing shear rates, due to the orientation of molecules in the direction of flow (Figure 5.2). Spherical particles can be deformed into a more elongated, and aggregates may be broken (Bretas; D'Avila, 2005).

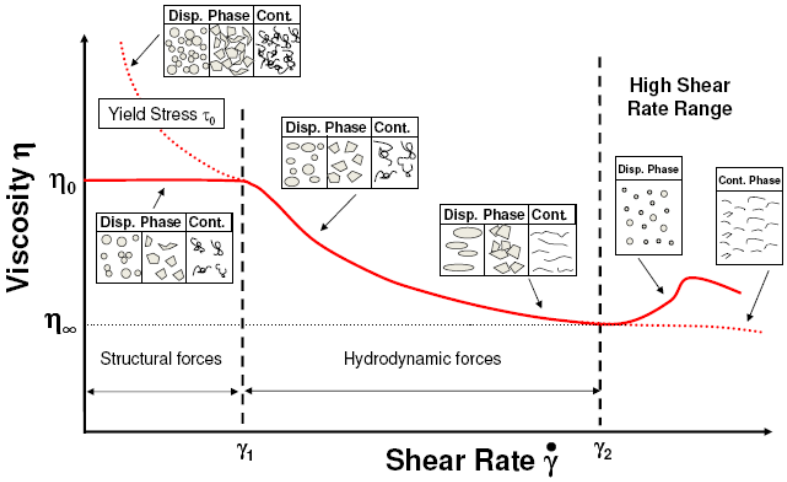


Fig. 5.1: Viscosity Behavior of Multiphase Dispersed Systems (emulsions).

Fluids can be defined as Newtonians and non-Newtonians, in accordance to their rheological characteristics (figure 5.2).

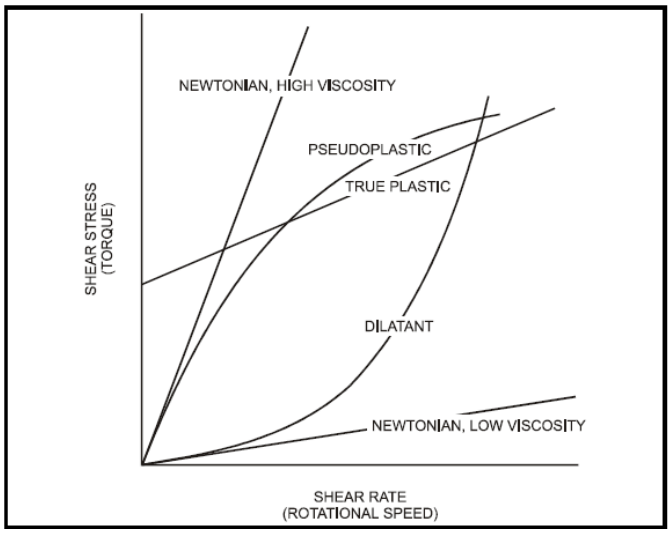


Fig. 5.2: Rheological behaviors of fluids at different shear rates and shear stress, according to their characteristics.
Source: http://www.globalspec.com/reference/10735/179909/chapter-3-physical-properties-of-fluids-vapor-pressure-and-boiling-point-of-liquids

5.1.1.1 Newtonian liquids

In his studies about the properties of materials, Newton assumed that the line generated by plotting (equation [5.1]) for an ideal liquid would be a straight line, starting at the origin and with inclination α. Thus any point of this line defines pairs of values (t x γ), and from this we can obtain h (from the equation [5.2]).

By means of an ideal liquid, it can be inferred that the viscosity (h) is not affected by changes in shear rate, as it is kept constant at all points. All fluids for which

this statement is true are called Newtonian, such as pure liquids, very dilute solutions and true colloidal systems (water, mineral oil, etc).

5.1.1.2 Non-Newtonian Fluids

Liquids that do not exhibit the behavior of "ideal" flow as described in the previous section, are called non-Newtonians. They are, in number, a larger group than the Newtonian fluids.

Non-Newtonian fluids are defined as those where the ratio t / γ is not constant. This means that when the shear rate varies, the shear stress does not vary in the same proportion (or necessarily in the same direction) (Mano, Mendes, 1999), causing viscosity changes during variation of shear rate.

The non-Newtonian fluids can be classified as viscoelastic or inelastic. The viscoelastic fluids exhibit both properties of fluid (viscous) and solid (elastic) (Barnes, Hutton, Walters, 1989).Inelastic non-Newtonian fluids are classified based on dependence of duration of force F application: the apparent viscosity of the independent ones, under conditions of constant temperature and composition, depends only on the magnitude of F, while the apparent viscosity of the time-dependent depends on the magnitude and on the duration.

Among the inelastic fluids independent of F duration, there are pseudoplastics, dilatants and plastics. For pseudoplastics, such as paints, emulsions and dispersions in general, the viscosity decreases with increasing shear rate. For dilatants, such as caramels and corn starches in water, viscosity increases with increasing shear rate. Plastic fluids, such as creams, ketchup, mayonnaise, toothpaste, etc, behave like solids under static conditions, with internal forces that prevent it from flowing until a certain shear stress, after which they begin to flow (Shaw, 1975). The F duration-dependent inelastic fluids are those in which there are changes in viscosity over time and shear rate and temperature variation, and can be divided into thixotropic and rheopectics. In thixotropic fluids viscosity decreases with time for a constant shear rate (Costa, 2006), while for the rheopectics the opposite occurs, i.e. the viscosity increases with time for a constant shear rate.

The microbial polysaccharides normally show pseudoplastic and viscoelastic characteristics (Covielo et al., 2007; Toneli et al 2005, Sutherland 2001). Some advantages of the microbial EPS use in industrial products are: low concentration needed to form viscous solutions, high solubility, viscosity and stability present in a wide range of pH, temperature and in the presence of salts.

5.1.1.3 Viscoelastic Behavior

The viscoelastic behavior of an emulsion can be defined through studies of rheological dynamic analysis and/or oscillatory tests for the evaluation of its proportional solid and fluid behavior: the elastic shear modulus (G') concerns the character of solid behavior, while the viscous shear modulus (G") refers to the character liquid behavior. The energy used in the deformation of an elastic solid is recovered when it returns to its original state, while for an ideal liquid there is no such recovery and the energy is lost. Thus, the modules G 'and G" are also called modules of storage and loss, respectively (Schramm, 2006 and Brummer, 2006). For a perfect elastic material all the energy is stored, the stress and deformation are in phase and G" is equal to zero. On the other hand, for no elastic liquids all the energy is dissipated as heat, G' is equal to zero, and the tension and strain are out of phase (Iagher, 2000, Brummer, 2006).

The elastic shear modules (G') and viscous (G") are given by the following equations:

$$G' = (t_0/g_0).\cos d \qquad [5.3]$$

$$G'' = (t_0/g_0).\cos d \qquad [5.4]$$

$$G''/G' = \text{tg } d \qquad [5.5]$$

where:

G'= dynamic elastic shear modulus

G = dynamic viscous shear modulus

t_0 = stress amplitude

g_0 = amplitude of deformation

d = phase angle

The response of a sample to oscillatory strain deformation can be characterized by complex dynamic shear modulus G *, which is given by the following equation:

$$G^* = (G'2 + G''2)\ 1/2 \qquad [5.6]$$

where:

G* = complex dynamic shear modulus

G'= dynamic elastic shear modulus

G = shear modulus dynamic viscous

The complex dynamic modulus G* can define a complex viscosity (h*), which describes the total resistance for a dynamic measurement and is expressed by the equation:

$$h^* = G^*/ f \qquad [5.7]$$

where:

h* = complex dynamic viscosity

G* = complex dynamic shear modulus

f = frequency

Dynamic analysis of shear modules G' and G'' and complex dynamic viscosity, allows viscosity and elasticity characterization (Morris, 1996). Variations in G', G'' and h* also give important information to characterize the time scale of molecular interactions in emulsion systems.

The thixotropy is a viscous and time-depending variable, and shows the material's ability to deform under a constant shear rate. A thixotropic product tends to have longer shelf life because, during storage, has constant viscosity, which hinders the formulation constituents separation.

The most appropriate way to measure the thixotropy is achieved by the flow curve with shear rate controlled. The shear rate increases with time until it reaches a maximum shear value. Subsequently, without any disturbance, the process is reversed, decreasing the shear rate, leading to the formation of curves up and down. The area enclosed by the curve up and down is referred to as hysteresis (Schramm, 2006).

A thixotropic product becomes more fluid during application, facilitating spreading, and restores the original viscosity by the end of application. This prevents product draining (Martin, 1993, Gaspar, Maia Campos, 2003; Leonardi et al., 2000). Very high thixotropy values make product flow over the skin after application. In contrast, very low values of thixotropy generate low spreadability and do not allow homogeneous application on the skin (Gaspar & Maia Campos, 2003).

Substances addition to a cosmetic formulation, especially in the case of complex ones (such as microbial polysaccharides) may cause instability. Rheometry allows the verification of the cosmetic response, to ensure the reproducibility of properties. By checking parameters such as viscosity and thixotropy, the product physicochemical nature can be understood, the raw materials quality can be controlled and final product failures or instabilities can be detected. Instability problems can cause

failures in the product as creaming, flocculation and coalescence of phases, reactions that are easily detected and reproved by consumers (Dukhin et al., 2003).

5.2 Materials and Methods

Four creams sets were prepared, using the formulation in table 3.2 (Chapter 3). To 3 of these creams Spirulina EPS was added in crescent concentrations of 0.5%, 2.5% and 5.0%. The cream without EPS addition was used as control. The creams were maintained in opaque flasks at 4ºC. Four hours before the analyses, creams were left at room temperature until come into equilibrium.

The analysis was performed in a Brookfield Mod. RV III rheometer, using a cone-plate system, Software Rheocalc 3.0. and a CP 52 spindle. Triplicate analysis were carried using 0.4 g of sample at 25°C. The variables considered in this study depended on the formulation obtained.

5.3 Results and Discussion

Rheology is useful for predicting emulsions physical instability in long-term (Tadros, 2004). From the observation of the Rheograms ascending and descending patterns, presented and discussed below in this section, it was found that both do not obey Newton's law and are classified as non-Newtonian fluids.

5.3.1 Stress Ramp Test

In the stress ramp test, the shear stress is increased continuously until it reaches a maximum value ($100s^{-1}$), resulting in a torque which is measured (Fig. 5.3). Based in these intervals the viscosity was calculated. After reaching the maximum, the shear stress applied was continuously decreased and the torque decreasing ramp is also measured (Fig. 5.3). In Figure 5.4 all patterns are plotted in the same area allowing better comparison between them.

There are several models available for interpreting measure curves, and different results are found based in the way the data is plotted. If the shear stress is plotted against the torque, it’s possible to see an area in which the force or shear stress applied to the sample do not cause any detectable deformation, showing that the system remains at rest.

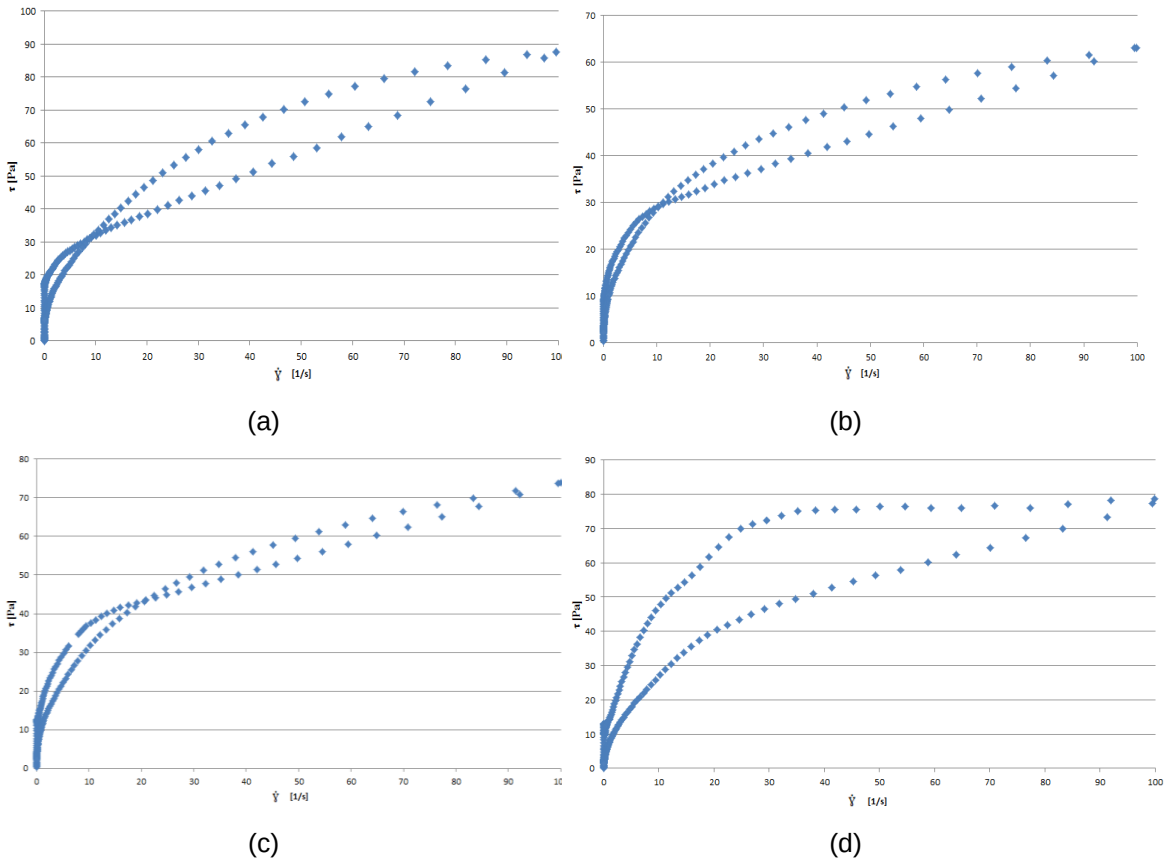


Fig. 5.3: Flow Curves. Control (a); 0.5% Spirulina EPS (b); 2.5% Spirulina EPS (c); 5.0% Spirulina EPS (d).

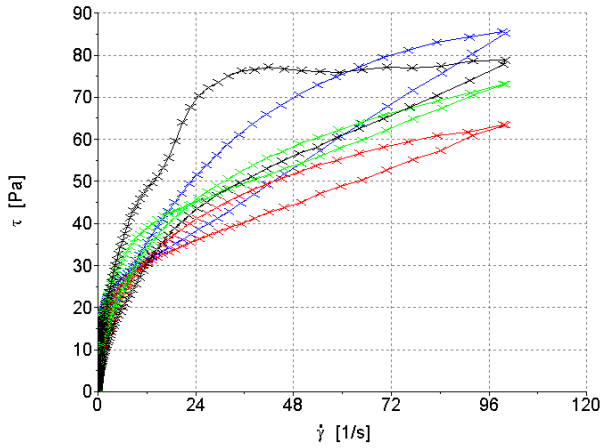


Fig. 5.4: Flow curves of all formulations plotted together, helping the comparison among them.

In this work, the thixotropy was quantified through the measurement of the hysteresis area (between the ascending and descending curves). All the emulsions showed pseudoplastic behavior and the presence of hysteresis areas, showing its thixotropy. This area pattern is a typical characteristic of thixotropic formulations. The hysteresis area calculated for each sample is showed in table 5.1.

During the shear rate time the bounding forces between molecules/particles decrease and, when it ceases, reagroupment and restoration of the initial

characteristics of the fluid can be observed. This reagroupment and the viscosity restauration were observed (Figure 5.3).

The hysteresis area presented by the 0.5% EPS Cream presented a slightly decrease when compared with the base cream (fig. 5.3 a). The cream with EPS 2.5% presented a very small hysteresis area, while the cream with EPS 5.0% had a very big hysteresis area. The last two behaviors are not desired: very small or very big hysteresis areas indicate problems (enormous difficulty to flow or draining) interfering directly in the process stability. So, for the tree formulations, the one with the best hysteresis area pattern is the EPS 0.5%. At this concentration, only a slight decrease in the hysteresis area was observed, not affecting the cream spreadability.

Sample	Hysteresis Area (Pa/s)
Base Cream	723.4±61.0
EPS 0.5%	514.6±36.1
EPS 2.5%	188.4±43.5
EPS 5.0%	1721.0±73.4

Table 5.1: The hysteresis area average and deviation for each formulation.

5.3.2. Minimum Apparent Viscosity

Another result that gives information about the stability of the formulation is the viscosity, which is always related with a strain rate (Martin, 1993). The sample minimum apparent viscosity (Pa.s) is calculated during the Stress Ramp test, at the highest shear used (100 s^{-1}). At this point the viscosity is at the lowest value, being called minimum apparent viscosity. In all the formulations the minimum apparent viscosity was diminished (table 5.2), comparing to the base cream, and the smaller value was found in the 0.5% EPS cream. This indicates that this cream may present better spreadability and flow properties than the others.

Sample	Minimum Apparent Viscosity (Pa.s)
Base Cream	0.874±0.015
EPS 0.5%	0.631±0.003
EPS 2.5%	0.734±0.005
EPS 5.0%	0.787±0.008

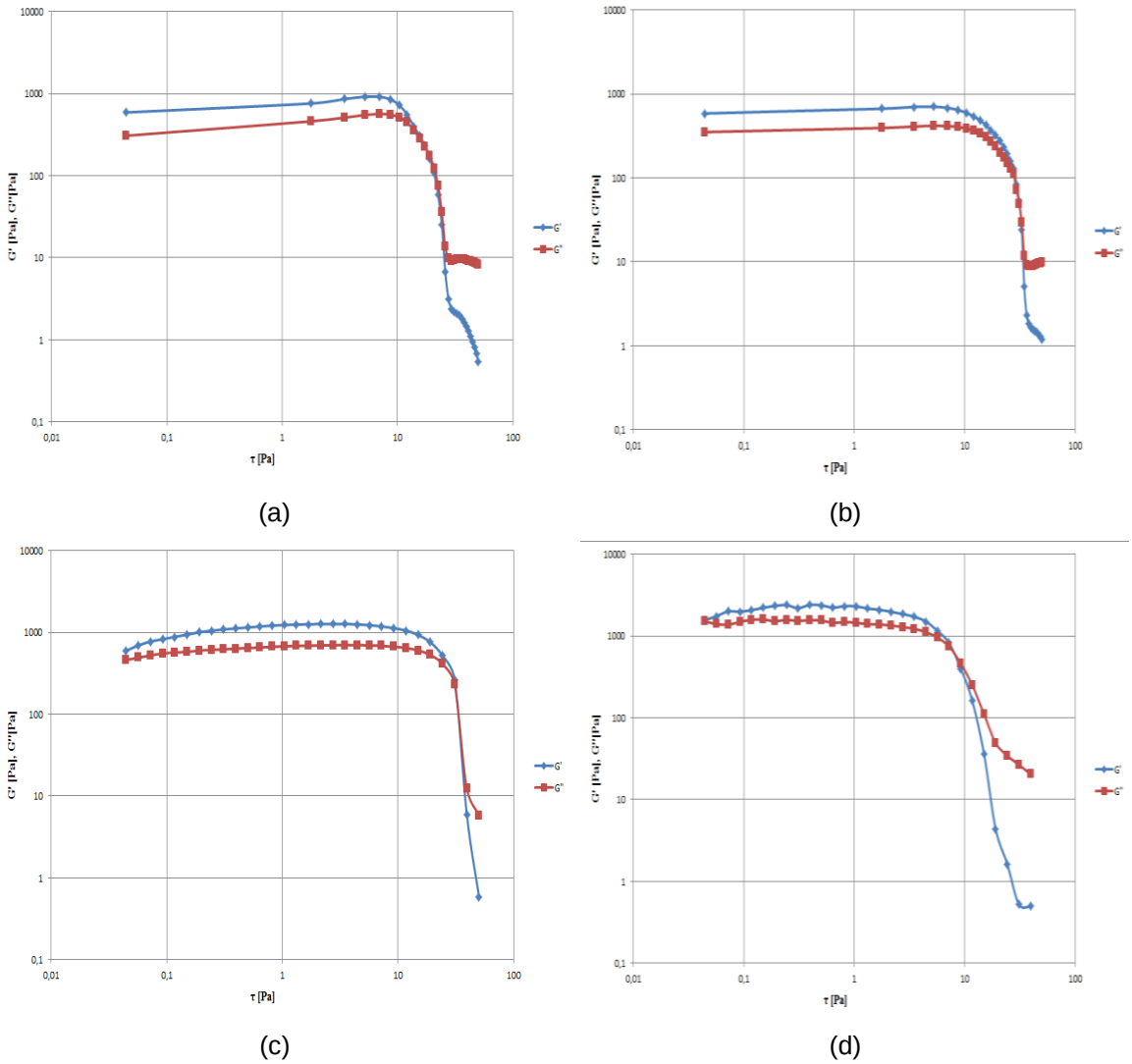
Table 5.2: The minimum apparent viscosity average and deviation for each formulation.

5.3.3 Tension Scan

The tension scan may be performed before the frequency scan and the creep and recovery tests, because it is used to determine the ideal zone tension to the

sample's rheological analyses. This ideal zone tension is that where the tension applied is not high enough to cause sample destructurization, but sufficient to allow sample to flow. This test, also called amplitude variation, or strain test, is performed at constant temperature and frequency. The response obtained are two moduli G' e G", which, when plotted against the tension applied, must appear as parallel curves. To achieve this, random frequencies were chosen, increasing tensions were applied and the obtained curves analyzed. The tension amplitude was varied from 0.01 to 100Pa. Figure 5.5 shows the best pattern for the tension scan achieved.

From figure 5.5 it was observed that a linear pattern was achieved in the region between 0.01 and 10 Pa tension. When more tension was applied it was observed a destabilization in viscosity (not showed), G' and G" in all samples. Thus, for the following rheological analysis, a random share tension from the linear phase response was chosen: 5Pa.



(a) (b)

(c) (d)

Fig. 5.5: Tension Scan. Base Cream (a); EPS 0.5% (b); EPS 2.5% (c); EPS 5.0% (d).

5.3.4 Frequency Scan

After fixing the tension in the tension scan, the frequency effect was studied. Measurements usually begin at the highest frequency followed by logarithmic reductions.

The frequency scan testing is a useful tool in evaluating elastic (G ') and viscous (G'') modulus and the apparent viscosity. Therefore, it is possible to say whether the formulation has elastic, viscous or viscoelastic behavior. In the case of viscoelastic behavior, we are able to evaluate which behavior prevails: elastic or viscous.

The frequency scan showed (Fig. 5.6) that in the established range (1-10 Hz) there was virtually no change in the behavior of elastic and viscous moduli both in Base Cream and 0.5% EPS samples. On the other hand, for the samples containing 2.5% and 5.0% EPS, the viscous modulus G'' suffered a slight increase. This behavior may be due to increased particulate matter in the emulsion, which turns the sample more viscous. Furthermore, a strong viscoelastic behavior was observed in the range established, since G' (elastic modulus) are higher than G'' (viscous modulus). This shows that the sample is in semi-solid state because the modulus G" would always be larger than the modulus G' if it was a fluid.

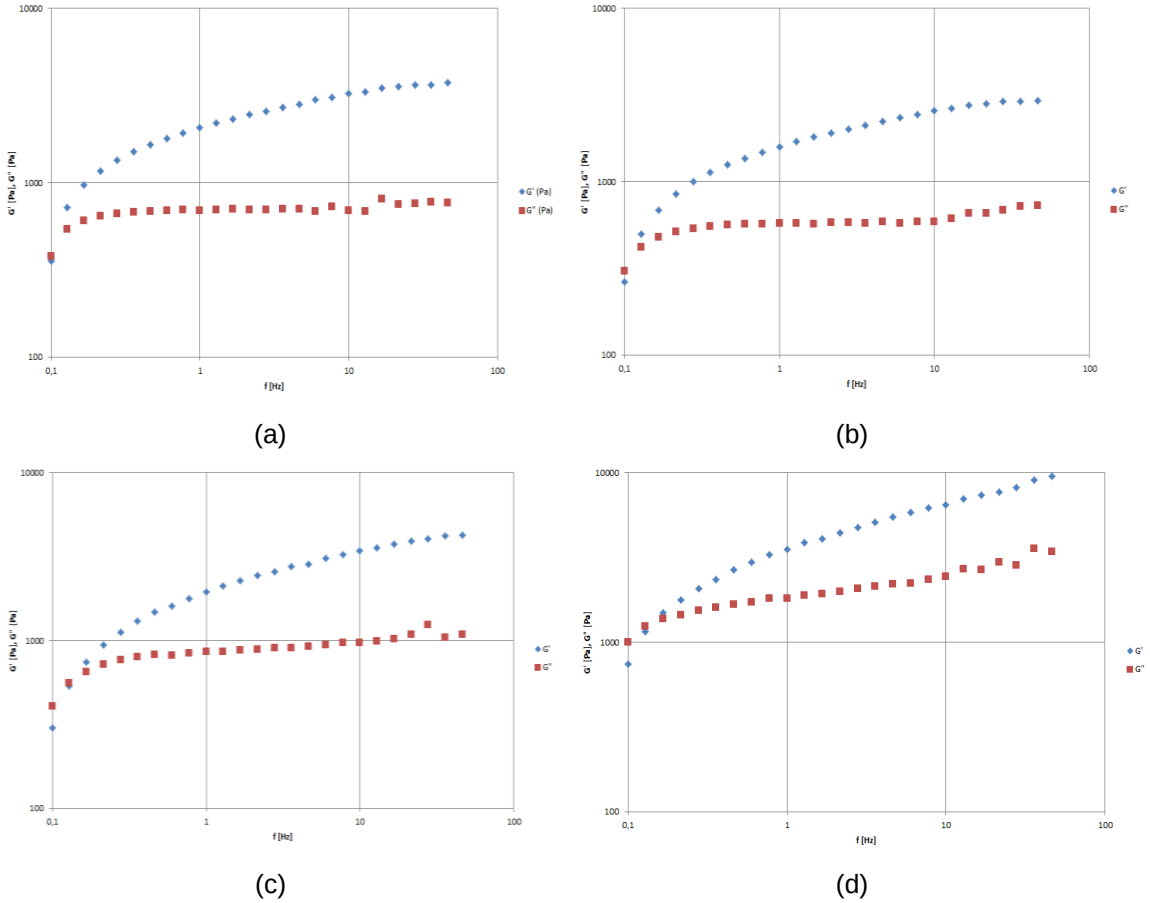


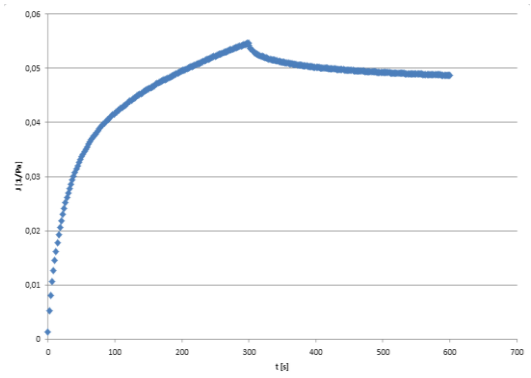
Fig. 5.6: Frequency Scan. Base Cream (a); EPS 0.5% (b); EPS 2.5% (c); EPS 5.0% (d).

5.3.5 Creep and Recovery

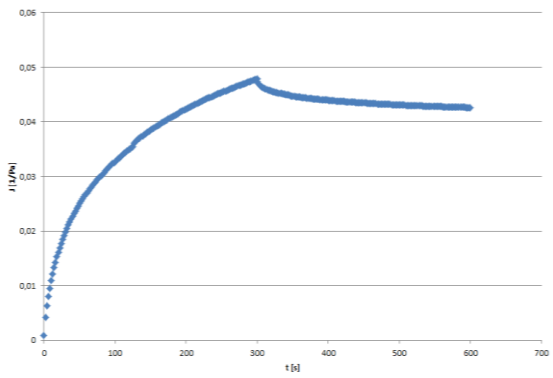
The creep test is a simple and quick test for obtaining relevant information on the viscoelastic properties of a sample (opposed to oscillation measurements). In this experiment a constant force (shear stress) is applied to the sample at time t_0 and removed at time t_1. Maximum time recovery (t_2) is recorded.

The creep and recovery test consists in applying an increasing tension in the sample for a certain period of time, after which the tension ceases gradually until it reaches zero. It is useful to evaluate the formulation viscoelastic characteristic and the sample structure resilience when the shear ceases. There are three different types of creep and creep recovery curves: the ideal elastic behavior, the ideal viscous behavior, and the real viscoelastic behavior.

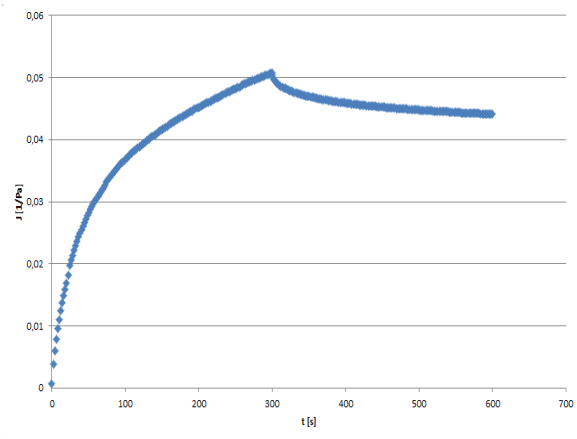
A real body is both viscous and elastic. This means that when a force is applied (t_0), after some time (t_1) the deformation to become constant, approaching a constant slope. When the force is removed (automatically), part of the energy stored in the body is released. This results in a recovery of the elastic part γ_e and a permanent deformation of the viscous part γ_v. A viscoelastic solid will therefore recover after a time lag but not completely. The patterns obtained for the Creep and Recovery test are shown in Figure 5.7.



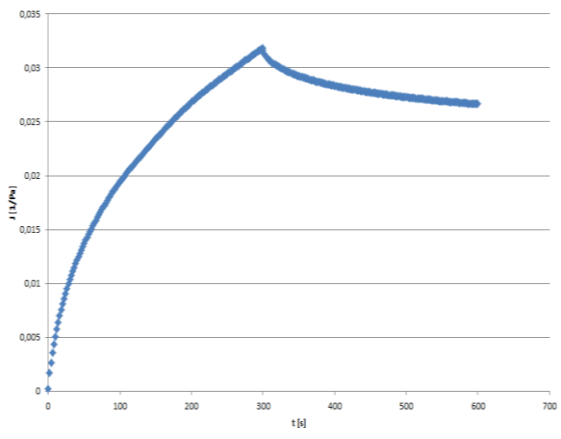
(a)



(b)



(c)



(d)

Fig. 5.7: Creep and recovery tests. Base Cream (a); EPS 0.5% (b); EPS 2.5% (c); EPS 5.0% (d).

From the data obtained, the sample's recovery percentage can be calculated (Table 5.3). It's possible to see that for the Base Cream and the EPS 0.5% Cream almost the same recuperation was achieved. For the two other samples, the recoveries were smaller, indicating that they must be more fluid than the other ones, causing problems during its application such as draining and less smooth appearance.

Sample	Recovery (%)
Base Cream	89.1%
EPS 0.5%	89.0%
EPS 2.5%	86.9%
EPS 5.0%	83.6%

Table 5.3: The percentage recovery achieved by the creep-recovery test.

5.3.6 Rheological and Sensorial Data Correlation

The relationship between structure and sensory emulsion is difficult to extract. A correlation between rheology and sensory testing could therefore help achieve the desired sensory product properties. After being established a relationship, it would be possible to correlate the ingredients used and the processes involved to have the final sensory attributes of personal care products. Then, the chemical formulator can choose the best ingredients and the best process to achieve the desired goal of sensory attributes. This knowledge, therefore, should facilitate the development of innovative cosmetic formulations.

4.5 Conclusions

- All the creams tested (with or without Spirulina EPS addition) showed a non-Newtonian, pseudoplastic and viscoelastic behavior. All of them also showed thixotropic behavior. The more adequate hysteresis area was achieved with 0.5% *Spirulina platensis* EPS addition to the Base Cream;
- The 0.5% of *Spirulina platensis* EPS cream presented the best Minimum Apparent Viscosity, indicating its better spreadability;
- The frequency and tension scans were useful to observe the G' and G" moduli behavior of each sample. The base cream and the 0.5% EPS cream showed the more constant pattern, what indicates best emulsion stability (distorted in the other creams probably by the great amount of solid particles in it).
- The higher recovery was achieved for the Base and 0.5% EPS creams. The results achieved in these tests showed that the cream with 0.5% EPS presented general better results in relation to the Base Cream. The 0.5% EPS cream was chosen for the next tests. By analyzing the rheograms, some sensorial and physico-chemical stability improvements of the addition of 0,5% EPS could be predicted: it can make the cream smoother and improve spreadability.
- Further evaluation of the improvements in cream properties proposed by the addition of Spirulina EPS will be handled in the next chapter.

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Chapter 6

Accelerated Stability Analysis

6.1 Introduction

Product stability can be defined as the maintenance of its key features and functions through time. Many variables can be related to the product properties, like its manufacturing process, material bottle packaging, transport and environmental conditions, and all can influence in its stability. (Isaac, 2008) In product development, especially when a substance is added, like antioxidants, rheological and sensorial improvers, plant extracts, etc., many studies may be performed aiming to obtain a stable, secure and effective product.

Changes that occur in cosmetics products can be caused by external factors (extrinsic) or related to the formulation's nature (intrinsic). Extrinsic factors could be time, temperature, light, oxygen, microorganisms and vibration, and the reactions caused by these factors are summarized in table 6.1. On the other hand, intrinsic factors are related to ingredients interactions or to the conditioning material, such as redox reactions, hydrolysis, pH changes, etc. (Isaac, 2008, Brasil, 2004).

Factor	Problem
Time	Product aging.
Temperature (high or low)	Accelerating the chemical and physical-chemical reactions, changes in viscosity, aspect, color, odor, precipitation, crystallization.
Light and oxygen	Initiate the redox reactions.
Humidity	Changes in rheological properties, making the product more softened or sticky, or modifying its weight or volume. It can potentiate the microbiological contamination.
Packing Material	Interactions between the product and the packaging material.
Microorganisms	Contamination, causing product deterioration, health consumers problems, toxic substances appearance, changes in color, odor and aspect.
Vibration	Emulsion separation phases, compaction, changes in viscosity, and changes in temperature.

Table 6.1: Extrinsic factors and how they can change the product.

The cosmetic stability analysis provides information that indicates the relative product stability in different conditions to which it may be subjected, since its production until the end of its validity. However, stability assessments are always relative, because

product properties varies with time and depend on various factors. However, some modifications, provided within pre-established parameters, can be accepted.

The purpose of cosmetic stability studies is to be a guide to formula development and appropriate packaging material choose. It also provides subsidies for the formulations improvement; validity estimation, assists in monitoring organoleptic activity, physico-chemical and microbiological properties, producing information on the reliability and product safety.

6.1.1 Preliminar Stability

The preliminar stability test is used to select the best product formulation, and is one of the first analysis to be made. It consists in submitting a sample to extreme temperature conditions and make assays about many parameters, according to the kind of cosmetic product and the analysis aim. The test is made through 15 days, being the first analysis 24h hours after the product manipulation or preparation (maturation time), and the following analysis each 24 hours. The parameters that can be analyzed are: aspect, color, odor, after the freeze-thaw and centrifugation. Measurements must be done in triplicate and the arithmetic average shown.

6.1.1.1 Freeze-thaw

According to Friedrich et al (2007), freeze-thaw cycles evaluate the stability by anticipating temperature shock that the product could suffer, in its storage or transport, which can cause problems like phase separation, crystals formation, compression, loss of sensorial and rheological properties and active principles damage. In this test, the samples are stored in alternate temperatures at regular time intervals, and the cycle numbers are variable. The suggested limits are:

- Cycles of 24 hours at room temperature and 24 hours at -5 ± 20°C.
- Cycles of 24 hours at 40 ± 20°C and 24 ± 20 hours at 4°C.
- Cycles of 24 hours to 45 ± 20°C and 24 hours at -5 ± 20°C.
- Cycles of 24 hours at 50 ± 20°C and 24 hours at -5 ± 20°C.

After the temperature cycles, the physical and organoleptic characteristics are evaluated. Great changes indicate that a re-formulation may be done.

6.1.1.2 Centrifugation

The centrifugation test aims to determine the cream behavior at the end of the storage conditions. The gravity force acts in the sample making its particles to move inside. It generates sample stress, simulating a growth in gravity force, increasing the

particle mobility and producing instabilities. This can cause precipitation, phase separation, caking formation, coalescence, etc. The sample is centrifuged in pre-determined time, temperature and velocity, followed by visual evaluation. (Friedrich et al, 2007)

6.1.2 Accelerated Stability

This test is carried to foresee product's stability, such as the life time and the compatibility between formulation and its packing, using non-extreme conditions (Brasil, 2004). This predictive study can be also used to evaluate modifications in product's formulation or production process. The samples are packed in glass flasks or in the final product pack. It's important not to complete the total package volume (left around a third free) to possible gaseous changes (Brasil, 2004).

The accelerated stability test is carried through 90 days, although it could be extended to six months or to a year, depending in the product's characteristics (Brasil, 2004). The samples may be submitted to heating (in incubators at controlled temperature), cooling (in refrigerators), light radiation and to environmental conditions. It's also possible to simulate product transportation, submitting the samples to a real movement (placing them in a transportation truck, for example) or to artificial movement, like in shakers.

There are many parameters that could be followed in accelerated stability analysis. They are defined by the evaluator and depend on the product characteristics and the ingredients used in the formula. The sample must be analyzed in relation to the pattern in order to check for instability signs. This is an important evaluation because changes are undesirable, since a cosmetic product normally is used for many days, usually for weeks or months.

Generally, are evaluated the following parameters (Brasil, 2004):

- Organoleptic: appearance, color, odor and flavor, if applicable;
- Physicochemical: pH, viscosity, density, and in some cases, the monitoring of ingredients of the formulation;
- Microbiological: microbial count and the challenge test preservative system (Challenge Test).

6.1.3.1 Aspect

The product general aspect may be evaluated for the non-occurrence of phase separation, precipitation or turbidity, what should be an indicative of the stability of the sample. The appearance can be described as grainy, dry powder, wet powder,

crystalline, paste, gel, fluid, viscous, volatile, homogeneous, heterogeneous, transparent, opaque and milky. The sample condition may be described as normal, with no change or slightly separated, precipitated, turbid, always depending of the product kind. (Brasil, 2004)

6.1.3.2 Color and Odor

In general, color and odor may be unaltered over time. But when there are natural compounds in the formulation, it's natural the occurance of changes, which are generally accepted by consumers. The sample to be evaluated is compared with the pattern, conditioned in the same flask kind, at same conditions. (Brasil, 2004)

6.1.3.3 pH

A cosmetic formulation to the skin may normally have a pH range between 5.5 and 7.0. The variation over the time can indicate some problem, such as undesirable reactions between the cream's components, between the cream and the package or could indicate some microbial contamination. Moreover, pH increase could be related to volatile aldehydes oxidation to carboxylic acids or lipid enzymatic hydrolysis, what releases free fatty acids. (Wajda, 1985; Masmoudi et al., 2005).

6.1.3.4 Water Activity

The water in a cosmetic product is crucial to its rheological properties, texture, consistency, viscosity and shelf time. The presence of water occurs in terms of water activity and bounded water, resulting in total water content, expressed as humidity.

The bound water is directly linked to the product molecules, and can't be removed or used for any kind of reaction. The no bounded water is available for physical (evaporation), chemical (darkening) and microbiological reactions and is measured with the name of water activity (A_w), which can vary from A_w=0,000 (when there is no available water) to A_w=1,000 (pure water).

A pure liquid, when in contact with air, loss molecules by evaporation. On the other hand, if the liquid is kept in a closed container in presence of air, the molecules come off of the liquid medium by evaporation until an equilibrium point. From this point, a compensation phenomenon occurs: for each molecule evaporated, another one condenses, exerting a vapor pressure.

By adding a solute in a liquid (what occurs during the preparation of an emulsion, for example) less molecules evaporation is observed; therefore the vapor pressure is reduced. Based on these concepts, the water activity (A_w) of a solution or

product is defined as the ratio between the solution vapor pressure (p) and the pure solvent vapor pressure (p_o), usually pure water (= 1) at the same temperature (equation 6.1).

$$a_w \equiv p/p_0 \qquad [6.1]$$

For cosmetics and hygiene products for topical use, it is important to measure water activity since it is known that higher water activity provides "freshness" and tied water is responsible for the delivery of the active principle. It is also a parameter entirely linked to the product moisture capacity, as shown in the Chapter 8, and together with temperature, pH and oxygen, is the factor that influences the most in the stability of cosmetics and food products.

6.1.3.5 Volatile Compounds

The volatile compound can be classified as phenols, aldehydes, ketones, alcohols, ethers, hydrocarbons, organic acids, anhydrides, esters, lactones, amines and compounds containing sulfur atoms. The origin of each compound is listed in table 6.2.

Volatile Product	Origin	Author
Aldehydes	Lipids auto-oxidation (unsaturated fatty acids).	Dart et al, 1985
Ketones	Lipids auto-oxidation (particularly unsaturated fatty acids).	Dart et al, 1985
Alcohols	Secondary products of lipids oxidative degradation; carotenoids break.	Shibamoto et al, 1992
Hydrocarbons	Lipids oxidative degradation.	Clifford et al, 1985
Carboxylic acids	Carbohydrates degradation; lipids degradation; thermic degradation, aldehydes auto-oxidation.	Feldman et al, 1969, Kawanda et al, 1985, May et al, 1985
Esters	Alcohol degradation; Microbial Production.	Guyot, 1982
Sulfur Compounds	Aminoacid degradation or it interactions with carbohydrates; oxidation reactions.	Dart et al, 1985, Kallio et al, 1990

Table 6.2: Biochemical origin of different volatiles.

6.1.3.6 Spreadability

Product spreadability can be defined as the ease with which a substance can be expanded in a surface, in a determined time under determined pressure. The major part of spreadability determination methods measures its deformation resistance. It can be evaluated in sensorial tests, but it only is used to classify the consumer's

acceptance. So, there are some methodologies to quantify this property (Borghetti and Knorst, 1996).

In these tests, the spreadability is the total area reached by the scattering of a given cream quantity. It's calculated using the equation:

$$E_i = (d^2 . \pi)/4 \quad [6.3]$$

where:

E_i: spreadability of the sample for a given weight (i) (cm^2)

d: average diameter (cm).

6.1.3.7 Microbiology

Microbiological contamination of a cosmetic product is measured through colony counting in Petry dishes. In some cases, they affect the preservative structure, influencing product stability. It's ideal that no contamination is found; however it's acceptable low colony counting. The presence of water and organic components in the formulation supports microorganism growth.

According to the "Good Manufacturing Practice", the cosmetic microbiological quality may not rely solely on its preservative system, however it cannot be dispensed. So, the preservative choice should be adequate (effective). Furthermore, it must be considered that the preservatives can partially or entirely inactivated, leaving the product without the expected protection. Thus, preservatives effectiveness tests are an essential part of products cosmetics security. These tests aim to determine the preservative type and effective concentration, and the minimum necessary to ensure satisfactory protection of the product from the manufacture to the final users.

6.1.3.7.1 Sterilization

The product contamination can be due to raw materials contamination. When extracts or natural products are added in a formulation, some decontamination process can be carried, decreasing its microbial charge. Some industrial usual procedures are ultraviolet light exposure, autoclaving and pasteurization.

6.1.3.7.1.1 UV

Irradiation is the process of applying radiant energy to any target, such as a food, cosmetic or a package (Ingram & Roberts, 1980). It is used in different applications such as air sterilization, equipment surfaces and in food packaging. Ultraviolet irradiation is effective microbicide if used with intensity and sufficient

exposure time. Wavelengths below 200 nm are inefficient, since they are quickly absorbed by oxygen and water. The ultraviolet radiation in the range of 210 and 330 nm are more effective as germicides because they are absorbed by proteins and nucleic acids, causing chromosome disruption, genetic mutations and enzymes inactivation, then hencing cell death (Cardoso, 2007).

UV radiation is composed of radiation bands with specific characteristics and practical applications. The UV-A represents the bulk of the UV rays emitted by the sun, responsible for the skin bronzing. It is not harmful and is used in medical treatment of certain skin diseases. The UV-B, a small part of UV radiation from sunlight, is dangerous but is mostly absorbed by the ozone layer. Prolonged exposure to UV-B results in skin cancer and eye cataracts. The UV-C, germicidal, also known as "short wave UV", cause skin reddening and eye irritation, but does not cause skin cancer (Rouessac, 2000).

The wavelength of UV-C, which inactivates the DNA / RNA, the genetic material of microorganisms, is between 250 to 270 micrometers. In general, ultraviolet radiation has been shown to be the fastest, reliable, effective, economical and environmentally safe in the treatment of surfaces and liquids. Moreover, the combined use of ultraviolet radiation and chemical disinfectants has presented a synergistic effect in microorganisms control, especially in cases where the residual chemical agent should be controlled at minimum levels or absent (Abreu & Faria, 2004; Siemens Water Technologies, 2007).

6.1.3.7.1.2 Autoclave

The sterilization procedure that offers greater security is saturated steam under pressure, held in autoclaves. In this equipment, the microbes are destroyed by the combined action of temperature, pressure and humidity, which promote thermal coagulation and protein denaturation of cellular genetic structure. The saturated steam process has advantageous characteristics and other limiting factors. The advantages consist in the rapid heating and rapid penetration into textile fabrics or cotton, microbial spore destruction in a short exposure period, easy quality control and mortality, toxic materials absence and be economical. The constraints consist of the incomplete air removal from the inner chamber, not allowing the spread and steam expansion to achieve sterilization and equipment misuse, which can lead to overheating the steam, reducing the microbicide power, and don't sterilize powders and oils. (Godoy et al, 2000)

NBR ISO 11134 specifies requirements for the use of moist heat in sterilization process development, validation of the sterilization process and control of routine

sterilization. It involves all processes in wet heat, including steam and saturated steam-air mixture, is applicable to all industrial manufacturing processes and all others who perform sterilization by moist heat. The principles outlined may be useful in the use of moist heat sterilization in establishments other than hospital products industries.

6.1.3.7.1.3 Pasteurization

The method of pasteurization was created by Louis Pasteur, who tested a possibility of microorganism inactivation in wine through the application of heat. Pasteurization's main objective is the destruction of pathogenic organisms of a given product. A secondary objective is to increase its shelf life, reducing the rates of microbiological and enzymatic changes. Pasteurized products may encore contain many living organisms, which limits their shelf life. Thus, pasteurization is often combined with other preservation methods and many pasteurized products are stored under refrigeration (Potter & Hotchkiss, 1995). There are three types of pasteurization:

• Slow Pasteurization: lower temperatures for longer time intervals. The most commonly used set temperature is 65 ˚ C for thirty minutes.

• Fast Pasteurization: high temperatures for short time intervals. The most commonly used temperature is 75 ˚ C for 15 to 20 seconds. This type of pasteurization is known by HTST (High Temperature and Short Time).

• Very Fast Pasteurization: temperatures ranging from 130 ˚ C to 150 ˚ C for three to five seconds. It is known as UHT (Ultra High Temperature) or long life.

6.2 Materials and Methods

It was prepared 1kg of base cream, which was divided into two parts: the first 500g received 0,5% (w/w) *Spirulina platensis* EPS and 2ml of a water biomass extract (10% v/w) and the other 500g didn't received any other addictive. Each cream amount was divided in 5 plastic flasks, with capacity to 120g, each one with 100g of cream. The flasks were distributed to evaluate the response against some conditions. One Spirulina's Cream flask and one Base Cream were left in a paper closed box, at room temperature. The same was made in the refrigerator, in the incubator, in the shaker and in the illuminated shelf.

These creams were subjected to different types of stress to check its stability during 90 days (accelerated stability). The conditions tested were temperature (room temperature, incubator at 37°C, refrigerator at 5°C), light exposure (3500Lux, 12h/12h), and agitation (shaker 120 rpm, 27°C).

6.2.1 Preliminary Stability

Freeze-thaw and centrifugation were carried to evaluate the preliminary stability.

6.2.1.1 Freeze-Thaw

In appropriate plastic packaging, similar to that used for cosmetic products, about 20g of the sample was subjected to at least six extreme temperatures cycles. Cycles of alternate freezing and thawing included sample's exposure during 24 hours at high temperatures (30 ± 2 ºC) and 24 hours at low temperatures (–5 ± 2 ºC).

6.2.1.2 Centrifugation

In graduated conical centrifuge tubes 5g of sample was weighed in analytical balance and centrifuged (246xg) for thirty minutes at room temperature (Rieger, 1996, Morais, 2006). The non-occurrence of phase separation does not ensure its stability, but allows it to follow to a stability test. The creaming absence (oil phase floating) indicates that this emulsion, under normal gravity conditions, could be physically stable. (Tadros, 2004).

6.2.2 Accelerated Stability

Accelerated stability analysis was carried in days 1, 8, 15, 25, 40, 55, 70 and 90 after the moisturizers production. The recipients containing the samples were collected from each place (room temperature, refrigerator, incubator, shaker and under light) and 5g was collected for visual analysis of appearance, odor and color. After that, the water activity was measured, followed by pH measurement, volatiles acids and spreadability. Microbiology analysis was carried in days 1, 40 and 90).

6.2.2.1 Aspect, Color and Odor

The colors analyses were performed by visual comparison, under conditions of white light and also spectrophotometrically, as indicated by standard methods. Odor was evaluated by direct smelling (standard method). The appearance, odor and color were evaluated by comparison between the initial and final samples aspects of Spirulina Cream and the control (Base Cream).

6.2.2.2 pH

The pH determination was conducted in an aqueous dispersion of 10% (m/v) obtained with a dispersion of 0.5g of sample in freshly distilled water, homogenized

with a magnetic agitator using digital pH meter, in triplicate (Brasil, 2004).The electrode should be inserted directly into the dispersion water (Davis and Burbage, 1997; Isaac, 1998a; Brazilian Pharmacopoeia, 2001). pH values between 5.5 and 7.0 are compatible with cutaneous pH and should be used as a criteria of stability (Knorst, 1991; Milão, 2001).

6.2.2.3 Water Activity

The water activity measurement of 5 grams samples was performed in Aqua-Lab X. The equipment was periodically calibrated with appropriate standards.

6.2.2.4 Volatile Compounds

Certain amount of sample weighed analytically was dried at 100ºC to constant weight. The difference in weight was considered as volatile compounds (usually expressed in percentage).

6.2.2.5 Spreadability

To determine the spreadability, the methodology proposed by Isaac (1994) was employed. On a rectangular wooden board, a sheet of graph paper was fixed, and a rectangular glass plate (10 cm x 20 cm) putted above. The weighted sample (0.1g) was placed in the glass plate and another glass plate of known weight (325g) was placed on top. After three minutes, a weight of 250g was repeatedly added until reach a kilo (four weights, 250g each one).

To measure the area upon which the cream was able to spread, a device millimeter scale was used. Measurements were made in duplicates.

6.2.2.6 Microbiology

The creams microbiology was evaluated by successive dilution and inoculation into plates containing Nutrient Agar (to analyses the bacterial presence) and Potato Dextrose Agar – PDA (to fungi and yeast). To Type I Products (for use by children, for the eye area and products that come into contact with mucous membranes), the maximum count is 5 x 10^2 CFU/g or ml; while to Type II Products (other cosmetics, in which are body and face creams are included), the maximum count is ten times higher (Carturan, 1999).

6.2.2.6.1 Sterilization

The EPS and Biomass Sterilization were tested to foresee if the microbial count can be decreased. Sterilizations by UV exposure through 30 minutes, autoclaving through 15 minutes at 130°C and pasteurization at 65°C for 30 minutes, were performed. Aqueous extracts of the samples, 10% (m/v), were used. After the sterilization procedures, the aqueous extracts were diluted and plated in Nutrient Agar (NA) and Potato Dextrose Agar (PDA). NA plates were incubated at 30°C for 24h to NA and PDA at 37°C for 72h.

6.3 Results and Discussion

6.3.1 Freeze-Thaw

After 6 cycles of freeze-thaw (24h in freezer, -5°C, and 24h in incubator, 30°C), the cream's appearance was evaluated. Both Spirulina and Base Cream didn't show any modifications in odor and color, neither in the general cream aspect. There were no phase separations and any destabilization emulsion effect, such as creaming, floating, coalescence, etc.

6.3.2 Centrifugation

Centrifugation was performed as a preliminary analysis to determine any signs of instability indicative of the need for reform (Brasil, 2004). Any of the formulations, Base and Spirulina Cream, showed alterations in relation to its original appearance.

Considering this and the freeze-thaw test, it can be concluded that it's not necessary any reformulation, once problems was not observed when the samples were exposed to critical conditions. So, they can be analyzed for their response to the accelerated stability tests.

6.3.3 Color, Aspect and Odor

The organoleptic characteristics determine the parameters of the product acceptance by consumers. Those tests are intended to ensure that possible changes that may occur in the product will not be perceived by the senses and will not bring any loss of product benefit or safety.

The results obtained for general aspects (color, odor and aspect) are summarized in the table 6.3. It was noted that the Spirulina Cream was more susceptible to the extreme conditions to which it was subjected, in relation to the control. Probably, this is a consequence of microbiological contamination, a consequence of EPS addition to the cream, which it can act as carbon source.

		Creams					
		Base			*Spirulina*		
Condition	**Time (days)**	Aspect	Color	Odor	Aspect	Color	Odor
Room temperature (dark)	*1*	N	N	N	N	N	N
	8	N	N	N	N	N	N
	15	N	N	N	N	N	N
	25	N	N	N	N	N	N
	40	N	N	N	N	N	**L**
	55	N	N	**L**	N	N	**L**
	70	N	N	**L**	N	N	**L**
	90	N	N	**L**	N	N	**L**
Incubator (37°C)	*1*	N	N	N	N	N	N
	8	N	N	N	N	N	N
	15	N	N	N	N	N	N
	25	**L**	N	N	**L**	N	N
	40	**L**	N	N	**L**	N	**L**
	55	**L**	N	**L**	**L**	N	**L**
	70	**M**	N	**L**	**M**	N	**L**
	90	**M**	N	**L**	**M**	N	**M**
Refrigerator (4°C)	*1*	N	N	N	N	N	N
	8	N	N	N	N	N	N
	15	N	N	N	N	N	N
	25	N	N	N	N	N	N
	40	N	N	N	N	N	N
	55	N	N	N	N	N	N
	70	N	N	N	N	N	N
	90	N	N	N	N	N	N
Shaker (27°C, 120rpm)	*1*	N	N	N	N	N	N
	8	N	N	N	N	N	N
	15	N	N	N	N	N	N
	25	N	N	N	N	N	N
	40	N	N	N	N	N	**L**
	55	N	N	**L**	N	N	**L**
	70	N	N	**L**	N	N	**L**
	90	N	N	**L**	N	N	**L**
Under Light	*1*	N	N	N	N	N	N
	8	N	N	N	N	N	N
	15	N	N	N	N	N	N
	25	N	N	N	N	N	N
	40	N	N	N	N	N	N
	55	N	N	N	N	N	**L**
	70	N	N	**L**	N	N	**L**
	90	N	N	**L**	N	N	**L**

Table 6.3: Aspect, color and odor analysis.

Aspect	N – normal, without alteration L - lightly modified M – modified I - intensely modified	**Color**	N – normal, without alteration L - lightly modified M – modified I - intensely modified	**Odor**	N – normal, without alteration L - lightly modified M – modified I - intensely modified

It's important to note that low temperature didn't showed effect in creams stability. Changes were noted when the creams were subjected to higher temperature (50ºC in incubator). Creams surface became very dry and brittle, general viscosity was altered and little phase separation was noted (untied water in the middle of the dry cream). So, high temperature was the only condition that affects cream composition.

In terms of color, any changes was noted in the conditions tested, what was not observed to the odor. Cream's odor was classified as "Modified" only when subjected to high temperature (50ºC). In conditions in which lightly changes were observed, both creams were altered. In this case, changes in Spirulina Cream started before than the Base Cream, probably due to cream manipulation in no sterile place (to simulate the normal consumer's manipulation). It may have left the cream susceptible to microbiological contamination, and microorganisms growth may be a possible cause to odor generation. Also, the additives (Biomass extract and EPS) can bring some microbial charge, what will be discussed.

(a)

(b)

(c)

Fig. 6.1: Flasks used for the Accelerated Stability Test (a): the flasks in the left column contained the Base Cream, and in the right the Spirulina Cream. (Code: A→ Ambient; E→ Incubator; G→ Refrigerator; S→ Shaker; Glass Flask with color cover→ light exposure. The modified aspects of the creams in Incubator (b), in comparison with a normal aspect (refrigerator) (c) are shown.

6.3.4 pH

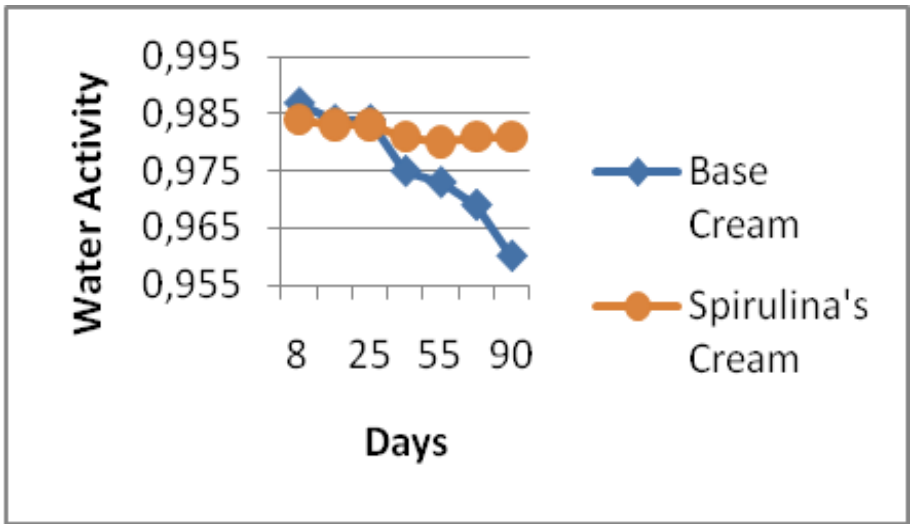
Both creams maintained their pH values almost constant trough the time. The inicial and final pH are shown in table 6.4. The intermediate data are not shown, but were always between 6.4 and 6.9, wich are acceptable values for skin products, not causing skin problems. The little pH modification is an indicative of no occurance of any critical reaction between the cream's componentsand no critical microbian contamination.

	Initial pH	**Final pH**
Base Cream		
Room Temperature	6.56±0.02	6.79±0.03
Incubator	6.80±0.03	6.45±0.01
Refrigerator	6.50±0.01	6.46±0.02
Shaker	6.40±0.02	6.76±0.01
Under Light	6.65±0.02	6.67±0.02
Spirulina's Cream		
Room Temperature	6.53±0.03	6.87±0.01
Incubator	6.71±0.01	6.90±0.03
Refrigerator	6.32±0.01	6.70±0.01
Shaker	6.49±0.02	6.82±0.02
Under Light	6.78±0.01	6.85±0.03

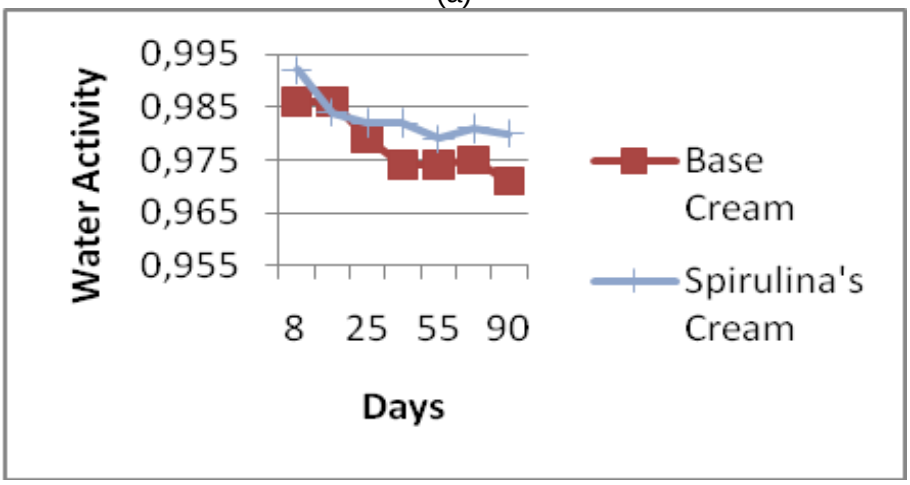
Table 6.4: pH values inicial and final (t=1 and t=90 days)

6.3.5 Water Activity

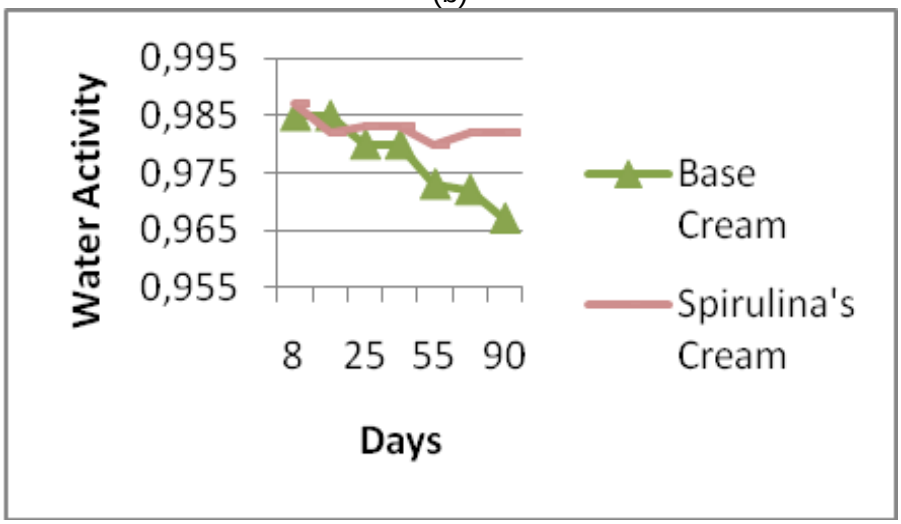
Water activity measurements showed that in all cases the loss of water was smaller in the Spirulina Cream than in the Base Cream (Figure 6.2). It indicates that the EPS increases the free water content. It's also related with a bigger moisturizer power, as discussed in Chapter 8. It's possible to see that, through time, water activity was always equal or higher than the Spirulina Cream. Moreover, water activity lost was much lower in Spirulina Cream (0.2% maximum decrease, while the base cream decreased 2.0% in average). It seems to be a small difference, but the EPS addition prevented 10 times the free water product loss.



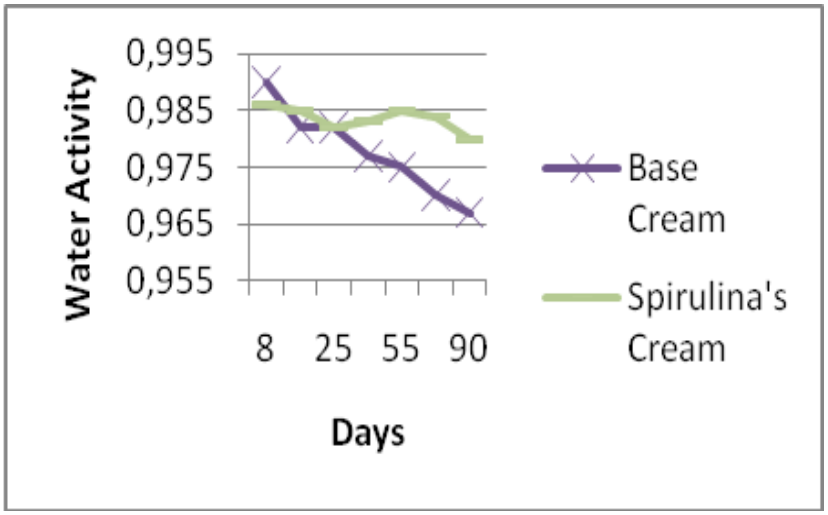
(a)



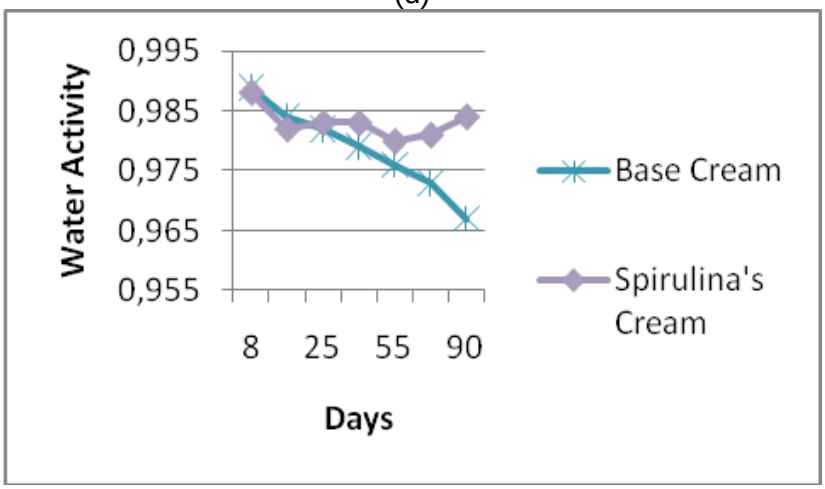
(b)



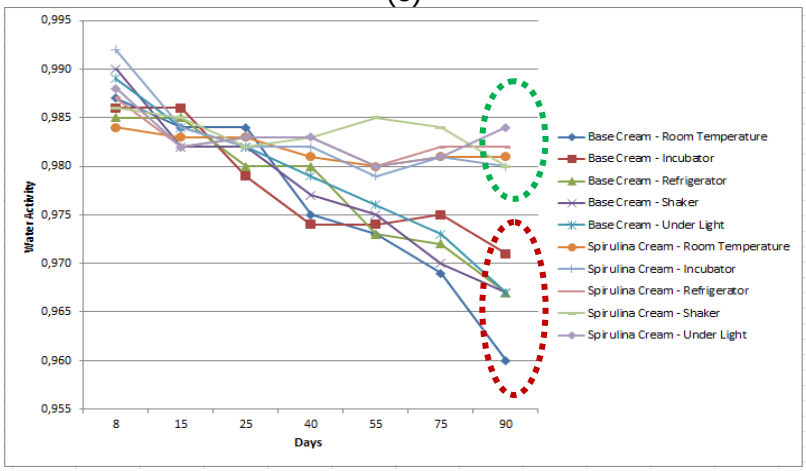
(c)



(d)



(e)



(f)

Fig. 6.2: Water activity profile for Base Cream and Spirulina Cream, at Room Temperature (a), Incubator (b), Refrigerator (c), Shaker (d) and Under Light (e). Resume of all creams and conditions, showing the best performance of Spirulina Cream, against Base Cream (f).

6.3.6 Volatile Compounds

The volatile compounds were measured in days 1, 8, 15, 25, 40, 55, 70 and 90, and results are shown in Table 6.5. Through time, no significant changes were observed, which indicates no occurrence of intrinsic reactions neither an expressive microbial contamination.

	Volatile Compounds (%)
Base Cream	
Room Temperature	2,89±0,17
Incubator	2,68±0,02
Refrigerator	2,91±0,18
Shaker	2,74±0,06
Under Light	2,44±0,15
Spirulina's Cream	
Room Temperature	2,66±0,01
Incubator	2,58±0,05
Refrigerator	2,47±0,13
Shaker	2,26±0,28
Under Light	2,23±0,30

Table 6.5: Volatiles compounds measured.

6.3.7 Spreadability

Spreadability was better to the Spirulina Cream then the Base Cream in all conditions tested (Figure 6.3). It's important to note that the average spreadability was much smaller to the samples placed in the incubator. It can be explained by the alteration in visual viscosity and the phase separation. In all other cases, spreadability didn't presented great variations, showing no interference of the different conditions tested. On the other hand, by comparing the Base and the Spirulina Creams, a big difference was noted. At room temperature, for example, spreadability of Spirulina Cream was almost twice of the Base Cream.

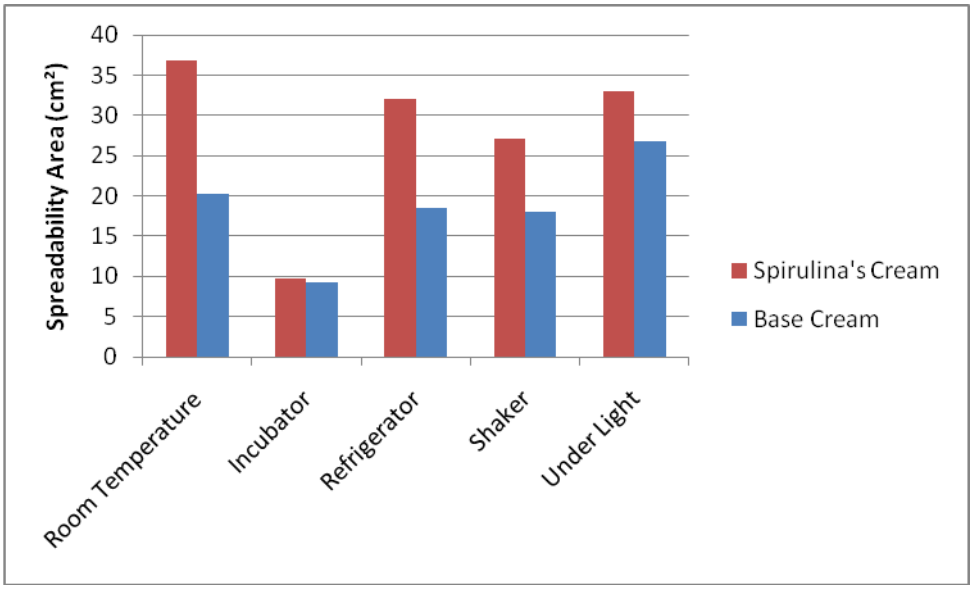


Fig. 6.3: Spreadability average comparision among all conditions tested.

Table 6.6 shows the diameter average values of each cream in each condition. It's clear the lower spreadability obtained for the samples placed in the incubator, showing that high temperatures promote great aqueous phase evaporation, changing the cream's general aspect and the parameters related to its viscocity(spreadability, for example). The average diameter values for the Base Cream, considering all conditions, were about 5cm, 1 cm lower than the average obtained with Spirulina Cream. So, using Equation 6.2, the area covered by a certain cream amount can be calculated (Table 6.6). Disconsidering the samples placed in the incubator, the Spirulina Cream spreadability was always higher than the Base Cream: 59,1% (under light), 63,7% (shaker), 70,2% (refrigerator) and 83,7% (room temperature).

	0,1g		
	Medium Diameter (cm)	**Area (cm²)**	**cm²/g**
Base Cream			
Room Temperature	5.09	20.33	203.26
Incubator	3.42	9.20	92.05
Refrigerator	4.94	19.12	191.24
Shaker	4.79	18.03	180.33
Under Light	5.14	20.75	207.51
Spirulina's Cream			
Room Temperature	6.90	37.34	373.35
Incubator	3.53	9.77	97.66
Refrigerator	6.44	32.56	325.57
Shaker	6.13	29.53	295.25
Under Light	6.49	33.02	330.21

Table 6.6 : Spreadability values to each cream and each condition; average considering 90-days experiment.

6.3.8 Microbiology

The microbiology contamination was evaluated in days 1, 40 and 90, . Fungi and yeast weren't found, while bacteria were. The total count is sumarized in table 7. The microbiological count was always below the maximun limit allowed (5×10^3). It was noted a little increase in the total microbial charge, but only after 3 months.

This unusual kind of contamination can be explained by the so-called "Phoenix Effect" (Maibach, 2001). This effect is generated by a intense stress at the moment of the product preparation causing most microrganisms death and the latent state in the remaining. Those who were in latent state begin to growth after some time. In the Creams preparation, after the EPS addition the pH went up (source of stress), being readly corrected by citric acid adding, probably giving conditions to the Phoenix effect.

Base Cream			
Day	1	40	90
Bacteria (UFC/g)	$1{,}10 \times 10^1$	$0{,}93 \times 10^2$	$1{,}36 \times 10^3$

Spirulina Cream			
Day	1	40	90
Bacteria (UFC/g)	$2{,}70 \times 10^1$	$1{,}13 \times 10^2$	$2{,}92 \times 10^3$

Table 6.7: Bacterial Count of Base Cream and Spirulina Cream, in the beginning, in the middle and in the end of the experiment.

6.3.8.1 Sterilization

One hypotesis to the microbial charge in the creams, higher in the Spirulina Cream, is the use of EPS and microalgal as carbon source for growth before the final product for ulation. To confirm this, three sterilization methods were tested.

Aqueous extracts (10% w/v) were prepared, both with Spirulina EPS and biomass. One part of this aqueous extracts was inoculated in Agar Nutrient and PDA plates without sterilization. The other part of the aqueous extracts was submited to one of the following sterilization methods: autoclaving, ultraviolet exposure and pasteurization and inoculated in Agar and PDA plates. After incubation, sterilization methods were evaluated through microbial count. UV exposure and Pasteurization promoted a decrease in the microbial charge, and by autoclavation promoted complete microbial charge removal (Table 6.8).

Considering allowed microbial count, any sterilization treatment is really necessary to the Spirulina Cream. In the accelerated stability test, after 90 days the total microbial count didn't reached the maximum value allowed . But, as the slight odor modification noted can be due to this small bacterial contamination, sterilization methods can be used to avoid odour modifications

	UFC/mL
Limit	5,00 x 10^3
Ambient	
EPS	3,15 x 10^2
Biomass	2,30 x 10^2
Ultraviolet	
EPS	5,10 x 10^1
Biomass	3,20 x 10^1
Pasteurized	
EPS	1,10 x 10^1
Biomass	2,00 x 10^1
Autoclaved	
EPS	0
Biomass	0

Table 6.8: Microbial count before and after sterilization tests.

6.4 Conclusions

- The Preliminar Stability Test showed that the Spirulina and Base cream were apparently stable, without the necessity to recast;
- Little sensorial modifications (in aspect and odor) were noted through the 90-days analysis, probably caused by microbial contamination;
- The Spirulina Cream had better water retention capacity, indicating that, besides sensorial improved properties, it can be more moisturizing (Chapter8);
- Both creams presented negative changes when incubated at high temperature, losing water, modifying the cream aspect and losing spreadability. For all the other conditions, the Spirulina Cream had best spreadability values than the Base Cream;
- Few contamination was observed in both creams; the total microbial count were always below the maximum limit allowed by legislation;
- The best sterilization method for both EPS and Biomass was autoclaving.

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General Conclusion

After this work, it was possible to conclude that it was possible to cultivate *Spirulina platensis* and obtain biomass and EPS at usual levels, with compositions similar to those described in the literature. Also, it was found that the *S. platensis* EPS has a protein-glucan nature.

Some properties were investigated to find some cosmetic application to this biomass extract and polysaccharide. The antioxidant capacity test demonstrated the potential of *S. platensis* biomass and EPS for cosmetics application, and, in this way, these products could replace or unless diminish the use of synthetic antioxidant substances, like BHA and BHT, by *S. platensis* biomass extracts. In other hand, the *S. platensis* EPS showed visual similarity to Kaolim, which is a mineral used in cosmetic industry to improve rheological and sensorial properties.

Nowadays, both the mondial and Brazilian cosmetic market have place to new and innovative products and, in this context, the microalgae are beginning to be intensively researched as source for new and innovative cosmetic products and stand out as one interesting alternative for the cosmetic industries. So, adding the important properties found in the microalgae products and this place in the market, it was decided to insert this material into emulsions and study the acceptability. So, it was found a cream formulation that, with the EPS and biomass extract addition, there were no emulsion problems in viscosity, aspect, pH or color.

All the creams tested (with or without Spirulina EPS addition) showed a non-Newtonian, pseudoplastic and viscoelastic behavior. All of them also showed thixotropic behavior. The more adequate hysteresis area was achieved with 0.5% *Spirulina platensis* EPS addition to the Base Cream. The 0.5% of *Spirulina platensis* EPS cream presented the best Minimum Apparent Viscosity, indicating its better spreadability;

The frequency and tension scans were useful to observe the G' and G" moduli behavior of each sample. The base cream and the 0.5% EPS cream showed the more constant pattern, what indicates best emulsion stability (distorted in the other creams probably by the great amount of solid particles in it).

The higher recovery was achieved for the Base and 0.5% EPS creams. The results achieved in these tests showed that the cream with 0.5% EPS presented general better results in relation to the Base Cream. The 0.5% EPS cream was chosen for the next tests. By analyzing the rheograms, some sensorial and physico-chemical stability improvements of the addition of 0.5% EPS could be predicted: it can make the cream smoother and improve spreadability.

After this preliminar formulation, it was necessary to perform Stability tests, to see if the active compounds could interact and destabilize the formula. The Preliminar Stability Test showed that the Spirulina and Base cream were apparently stable, without the necessity to recast. Little sensorial modifications (in aspect and odor) were noted through the 90-days analysis, probably caused by microbial contamination. The Spirulina Cream had better water retention capacity, indicating that, besides sensorial improved properties, it can be more moisturizing.

Despite this good results, both creams presented negative changes when incubated at high temperature, losing water, modifying the cream aspect and losing spreadability. For all the other conditions, the Spirulina Cream had best spreadability values than the Base Cream.

Looking this entire scenario, it's possible to conclude that the *Spirulina platensis* products can be an important biotechnological source of bioactive substances, improving the performance of a lot of anti-aging and related products, in a growing and increasingly critical market.