ABSTRACT

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Conventionally used as animal feeds, microalgae are now cultivated for products such as omega-3 fatty acids, resulting in a high amount of biomass as by-product. The biomass obtained after the extraction of DHA from *Crypthecodinium cohnii* is called 'algal biomeal'. Being nutritionally rich, the biomeal has potential to be used as a value-added ingredient in human food and animal feeds. Evaluation of the biomeal properties resulted in the development of a water-based sauce formulation which was analyzed for its proximate composition, textural attributes and microbial stability. The sauce was rich in carbohydrate and protein with low fat and ash content. It was microbiologically and texturally stable under refrigeration. This research shows that development of a shelf-stable palatability enhancer using algal biomeal offers a new ingredient for the food and feed industries, whereas the ability to produce a value-added ingredient also offers a viable option for algal biomeal.

CHARACTERIZATION OF ALGAL BIOMEAL FOR APPLICATIONS IN FOOD

By

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Chapter 1: Introduction

Algae are a large group of simple-plant like organisms typically classified into two main size-classes as macro- and microalgae (Hein et al., 1995). Apart from the toxin-releasing species such as *Chattonella marina*, *Karlodinium micrum*, *Prorocentrum minimum*, and *Pfiesteria piscicida* that cause harmful algal blooms (HABs) (Wang, 2004), algae have a long history as food and source of nutrients in different cultures (Barsanti & Gualtieri, 2006; Borowitzka & Borowitzka, 1988). Industrially, macroalgae harvested from natural habitats or cultivated at seashore areas have been employed for the production of hydrocolloids, including agar, alginate, and carrageenan that are used extensively as thickening and stabilizing agents in the food, chemical, and pharmaceutical industries (Carlsson et al., 2007; Radmer, 1996). Microalgae, on the other hand, received tremendous industrial attention in the last two decades due to their metabolic diversity (Radmer & Parker, 1994) alongside the advancements in algal biotechnology (Borowitzka, 1999; Chen, 1996; Apt & Behrens, 1999), enabling large-scale cultivation of microalgae for specific compounds.

Excellent reviews exist on the physiological and taxonomical characteristics of macro- and microalgae (Carte, 1996; Radmer, 1996; Pulz & Gross, 2004), the production of high-value molecules, animal feed, proteins (Spolaore et al., 2006; Fan & Chen, 2007; Jensen, 1993; Borowitzka, 1995; Becker 2007; Rogers & Hori, 1993), algae for soil fertility (Shields & Durrell, 1964; Pulz & Gross, 2004) and the design

and performance of various cultivation systems (Borowitzka, 1999; Chen, 1996; Ryther et al., 1981, Richmond, 2004). Most of the microalgae are rich sources of nutrients including essential fatty acids like docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Among the microalgae, dinoflagellates such as C*rypthecodinium cohnii* have been cultured industrially for the extraction of DHA. Microalgal DHA has many nutritional benefits and is used in a variety of products such as infant formula, poultry feed etc.

The large scale culturing of microalgae for DHA extraction results in a substantial amount of biomass obtained as by-product. This algal biomass has been termed ‘algal biomeal’. The biomeal is nutritionally rich and still contains a significant amount of DHA. This makes it ideal for use as a value added food ingredient. A hindrance to this is the fact that the properties of the biomeal remain unknown. The biomeal obtained from *Crypthecodinium cohnii* has potential applications in several food products such as pet food, flavor enhancers, etc. For this purpose, its characteristics have to be studied and its use as a value added ingredient needs to be investigated. The main objective of this study is to characterize the properties of the biomeal and explore its various applications as a food ingredient in several products such as pet food.

Chapter 2: Literature Review

2.1 Algae: Classifications

Algae are a large, diverse group of organisms that are similar to plants but differ in the level of differentiation and structural features. Algae can exist in various forms such as microscopic single cells, macroscopic multicellular conglomerations, matted or branched colonies or complex leafy forms (Barsanti and Gualtieri, 2006). Algae produce many different and unusual biochemical compounds, including fats, sugars, pigments, and bioactive compounds. They can be classified on the basis of pigment composition, storage products and a variety of ultra structural features. On the basis of pigment composition they are mainly classified as Blue-green algae, Red algae, Green algae, Euglenoids, Dinoflagellates, Cryptophytes, Golden algae, Haptophytes, Diatoms, Yellow-green algae and Brown algae (Radmer, 1996).

2.1.1 Macroalgae and Microalgae

Algae can be broadly classified as macroalgae and microalgae on the basis of the cell size and methods of cultivation (Table 2.1). Macroalgae are represented by a few species of Rhodophyta and Phaeophyta. They have been used traditionally in the production of phycocolloids like agar-agar, alginates or carrageenan. Macroalgal biotechnology represents a world market of U.S. $6 billion per year and more than 7.5 million tons a year macroalgae are harvested (Pulz and Gross, 2004). Microalgae, also known as phytoplankton are major primary food producers. Majority of natural product investigations have concentrated on two of the microalgal divisions-blue-

green algae and dinoflagellates (Carte, 1996). The microalgal biomass market has a size of 5000 t/year of dry matter (Pulz and Gross, 2004).

Table 2.1: Characteristics of Macro and Micro algae

CHARACTERISTIC	MACROALGAE	MICROALGAE	REFERENCE
Size	Large cell size upto 10 m in length	Small with diameter of 3-30 µm	Carlssson et.al,2007; Radmer, 1996
Cultivation	Harvested from natural habitats or cultivated at sea-shore areas	Cultivated in artificial systems such as open ponds or photobioreactors	Pulz and Gross, 2004
Nitrogen uptake	Slower	Faster	Hein et. al, 1995
Efficiency of photon capture per unit mass	Lower	Higher	Hein et. al, 1995
Broad classes	Chlorophyta, Phaeophyta, Rhodophyta	Cyanophyta, Chlorophyta, Bacillariophyta, Chyrsophyta	Carlsson et.al, 2007; Carte,1996

2.2 Conventional and Current Applications of Algae (Table 2.2 & 2.3)

Algal biotechnology has made major advances in the last few decades and several algae and algal products are produced commercially. Macroalgae are used traditionally as food and for the production of hydrocolloids which have a wide range of applications. Microalgae are cultivated for food, feed and for their biologically active compound (Borowitzka, 1992). Several macroalgal species have specific

requirements in terms of living environments and this limits their large-scale cultivation (Cralsson et.al, 2007). The total volume of seaweeds used in food is considerably larger than the sum of industrial applications, in weight and in value (Jensen, 1993). These seaweeds can be further explored through genetic improvement of the algal strains, developing newer applications and improvement in the culturing systems. The use of microalgae is increasing for the production of the bioactive components and the resulting biomass has wide potential for use in animal feeds and food products. Algae are also being looked at for environmental purposes such as biodiesel production and CO_2 sequestration.

2.2.1 Applications of Algae in Food Industry

A large number of algal species are used as food or food ingredients as a result of their availability locally and/or their nutritional contents. Macroalgae are used for a number of food products and the biomass for these products is obtained from wild, managed or cultivated stands of macroalgae that undergo a minimal amount of processing after harvest (Radmer, 1996). The most cultivated macroalgae is the kelp *Laminaria japonica*, which accounts for over 60% of the total cultured macroalgal production (Barsanti and Gualtieri, 2006). Macroalgae are cultivated mainly in the Asian countries, China, Japan and Korea as food due to their nutrient contents, especially vitamins, minerals and amino acids. The use of these seaweeds can also be attributed to their taste and texture (Jensen, 1993).

Another major application of seaweeds in the food industry is the use of hydrocolloids, mainly agars, alginates and carrageenans. These are mainly used for their gel-forming, suspending, water-retaining and stabilizing properties. Apart from their major applications in food industry, these hydrocolloids also find use in the textile and pharmaceutical industry (Barsanti and Gualtieri, 2006; Carlsson et. al, 2007).

Some of the *Nostoc* species are regionally being used as food and herbal ingredients. Also, *Arthrospira* has a history of human consumption, which can be located essentially in Mexico and Africa (Barsanti and Gualtieri, 2006). In spite of a high protein content, their use as a protein substitute is limited due to their strong fishy odor, color, powder-like consistency and high production costs (Becker, 2007).

2.2.2 Dietary Supplements

Extracts from several macroalgae may prove to be a source of effective anti-viral agents and antioxidants. Fucoxanthin, a carotenoid in brown algae is a potent drug candidate and acts as an antioxidant and inhibits GOTO cells of neuroblastoma and colon cancer cells (Barsanti and Gualtieri, 2006).

Spirulina is considered as some as a health food, a protein source, vitamin supplement, diet pill and as a treatment for anemia in humans (Campanella et al, 1999). *Arthrospira* is also used in human nutrition due to its protein content. *Chlorella* is a source of β-glucan which is an active immunostimulator, reducer of

blood lipids and a free radical scavenger. *Chlorella* is valued because of its supposed health promoting effects such as efficacy on gastric ulcers, wounds, constipation, antitumor action and preventive action against atherosclerosis. (Spolaore et. al, 2006). β-carotene is produced primarily from the green alga *Dunaliella* (Spolaore et. al., 2006; Radmer, 1996). Astaxanthin is obtained from the *Haematococcus pluvalis* and its concentration can reach 1.5 to 3% of dry weight (Spolaore et. al, 2006). Phycobiliproteins, phycocyanin and phycoerythrin are unique to algae and preparations are being developed for food and cosmetics (Pulz and Gross, 2004). Omega-3 and omega-6 fatty acids are essential fatty acids. Docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (AA) confer flexibility, fluidity and selective permeability properties to cellular membranes and are vital to brain development and beneficial to cardiovascular system (Carlsson et. al, 2007). A number of algal groups have been identified that produce these essential fatty acids in substantial quantities (Ward & Singh, 2005; Medina et. al., 1998).

2.2.3 Applications in Feed Industry

Arthrospira is primarily used as an adjunct for animal feed. Algae provide natural vitamins, minerals, essential fatty acids, improved immune response and fertility and better weight control. Microalgal biomass of the species *Chlorella, Scenedesmus and Spirulina* can affect the physiology of the animals (Spolaore et. al., 2006; Pulz and Gross, 2004).

Mass-cultured microalgae are the primary food source for larval and juvenile bivalves and for the larvae of some crustacean and fish species in mariculture. They also play a

role in enhancing the quality of the animal species cultured (Borowitzka, 1997; Brown et. al., 1997). Aquaculture feeds also include pigment-rich algal species to enhance the color of organisms such as salmon and trout (Spolaore et. al, 2006).

2.2.4 Applications in Other Industries

Extracts of macroalgae are often found as ingredients in face, hand, body creams or lotions, but the use of algae themselves, rather than extracts, is limited. The main microalgae established in the skin care market include *Arthrospira and Chlorella*. (Spolaore et. al., 2006). Microalgae are sources of stable isotopically labeled compounds, mainly sugars such as glucose, xylose, galactose. They are easily handled, cultured and photosynthesis allows them to incorporate labeled C, H, N (Apt and Behrens, 1999; Radmer and Parker, 1994). Macroalgal extracts when applied to fruit, vegetable, and crops, have resulted in higher yields, increased uptake of soil, improved seed germination and more resistance to frost (Barsanti and Gualtieri, 2006). The use of microalgal products with biological activity against plant diseases caused by bacteria or viruses seems to be a future trend (Pulz and Gross, 2004).

Aquatic biomass could be used as a raw material for co-firing to produce electricity, for liquid fuel production via pyrolysis or for biomethane generation through fermentation. Currently, production costs of biomass are too high to enable their use for solely energy purposes (Carlsson et. al, 2007). Algal cultures are also used for waste-water treatment and CO_2 sequestration and remediation. Removal of

atmospheric CO_2 requires marine sequestration and macroalgae have great potential for the same due to their high productivities (Gao and McKinley, 1994). Similarly microalgal cultures are used for tertiary waste-water treatment but have the drawbacks of high cost and slow generation time for the cultures (de la Noüe et. al, 1992). Microalgae can be used for the production of liquid fuel or bio-oil by pyrolysis or thermochemical liquefaction. Green algae produce hydrogen under certain conditions, which can be used as a source of energy. The handling of hydrogen and the cost of production are the major issues (Carlsson et. al, 2007).

2.3 Microalgae as a Source of Omega-3 Fatty Acids

Omega-3 fatty acids are essential fatty acids. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are used for atherosclerosis, hyperlipemia, schizophrenia and certain cancers (Ward and Singh, 2005). These fatty acids have traditionally been obtained from fish and fish oils. But safety issues have arisen because of the accumulation of toxins in fish. A number of algal groups have been identified that produce PUFA in substantial quantities. *Nitzschia, Porphyridium* and other species are being considered for EPA production. Most algae do not accumulate large amounts of EPA, limiting their commercial use. DHA is mainly obtained from *Crypthecodinium cohnii* and *Schizochyrium* (Spolaore et. al., 2006; Apt and Behrens, 1999). The use of microalgae for fatty acid production is advantageous because there is no seasonal limitation to production and they contain relatively simple fatty acid profiles with a high level of the desired fatty acid. This simplifies purification and reduces unpleasant flavors which may be caused due to impurities (Fan and Chen,

2007). Of all the essential fatty acids, DHA is very important since it is a crucial part of the cellular membranes, particularly of the brain and the retina. It's essential to the growth and development of infant brains and it is needed to maintain normal brain function in adults (Brown, 2001). DHA also improves the external appearance of animals and is required for larval growth and survival. It has been used to increase the ω-3 fatty acid content in chicken eggs by supplementing the feed with DHA (Apt and Behrens, 1999). Thus, DHA finds applications in infant formula (Kyle, 1994); dietary supplements for adults, pregnant and nursing women; animal feeds and maricultural products. DHA and other PUFA containing products have been approved by the FDA as GRAS (Ward and Singh, 2005). Therefore, DHA has wide applications and there is an increasing need to boost the production of DHA. Microalgae provide the solution to this. Also, DHA from microalgae can be considered to be a vegetarian source of DHA.

2.3.1 Cultivation of Microalgae for DHA Production

Open ponds are the oldest systems used for microalgal cultivation. They possess the advantages of minimum construction cost, utilization of land unsuitable for agriculture, etc. They also face the disadvantages of difficulty in maintaining monocultures, environmental contamination, and control of environmental parameters and high cost of recovery due to low cell density. Enclosed photobioreactor systems offer advantages over the open systems including better control of culture environment, protection from ambient contamination, higher cell densities to name a

few (Chen, 1996). Both these culture systems rely on light and photoautotrophic cultivation.

Heterotrophic cultivation eliminates the requirement for light and offers the possibility of greatly increasing cell concentration and volumetric productivity. Among the various culturing systems being used for microalgae, heterotrophic systems have the advantage that they are well-understood and high cell densities of between 20 and 100 g/l can be achieved (Borowitzka, 1999). Heterotrophic culturing also faces several problems such as limited species of heterotrophic algae, potential contamination by bacteria and inhibition of growth by soluble substrates. After extensive screening, *Crypthecodinium cohnii* was identified as a good producer of the ω-3 fatty acid DHA that can be cultured using heterotrophic systems (Chen, 1996)). This marine dinoflagellate has lipid content greater than 20% dry weight and is known for its ability to accumulate fatty acids with a high fraction of DHA with no other PUFA being present (de Swaaf et al, 2001; Jiang et al, 1999; Henderson et al, 1988). The culture components for *C. cohnii* include a carbon source, yeast extract and sea salt. Cultivation is carried out at 27°C and at a pH of 6.5 (de Swaaf et. al., 1999; Ratledge et. al, 2005). Glucose is the common carbon source used for *C. cohnii* but it has been shown that the algae prefers acetic acid above glucose as a carbon source and it produces a relatively higher level of DHA (Ratledge et. al, 2005). For economically feasible industrial cultivations of *C. cohnii,* high cell densities are required. High biomass densities (up to 109 g/L) and DHA concentrations of ~20 g/L have been achieved in carbon fed batch cultures of *C. cohnii* though high incubation

periods (400 h) were required. It has been demonstrated that DHA productivities of 1-1.5 g/ (L day) are achievable with this strain (Ward and Singh, 2005). Successful cultivation of these microalgae to produce DHA oil has been achieved by Martek Biosciences, Maryland, USA (Sijtsma & de Swaaf, 2004, Kyle 1996). A number of methods to extract DHA from microalgae and its various forms have been described by Glaude and Behrens (2002). Also, the production of DHA by microalgal biotechnology used by the Martek Company (USA) and Nutrinova (Germany) has been depicted by Pulz and Gross (2004).

2.3.2 Algal Biomeal

C. cohnii is therefore used primarily as a means for DHA production. For higher yields of DHA, higher biomass concentrations are desired. This results in a high amount of biomass that is obtained as a by-product from the lipid extraction industry. This biomass, that is known as biomeal (Fig 2.1) is currently used as animal feed and discarded in landfills. But in spite of its rich nutritional status, the use of biomeal as a food ingredient has not yet been explored. The process of obtaining biomeal is depicted in Fig. 2.2. The biomeal thus obtained is rich in nutrients. The biomeal also contains 18 of the 20 amino acids, vitamins from the B group and a number of minerals such as K, P, B, Ca, Fe, Mg, Mn. It contains a significant amount of DHA (2-4 %). Because the DHA is present in the bodies of the algae, it cannot be oxidized or denatured even if it is heated or mixed with a weak acid or alkali. Also, since the vital actions of the algae are stopped, the dried biomeal exhibits excellent

preservation stability (Iizuka et. al, 1996). This makes the biomeal valuable as a food ingredient and also as a source of DHA.

Table 2.2: Applications of macro- and microalgal strains in the food, dietary supplement, and feed industries.

Industry	Strains	Uses/Products	Remarks	Ref.
Food	***Macroalgae***			
	Ahnfeltia, Chondrus, Eucheuma, Gigartina	Carrageenan	Principal source of the hydrocolloid	Barsanti & Gualtieri, 2006; Radmer, 1996;
	Ascophyllum, Laminaria, Macrocystis	Alginate	Seaweeds grow in cold and temperate waters, cultivated from wild, used in textile, pharmaceuticals	Barsanti & Gualtieri, 2006; Carlsson et al.,2007;
	Caulerpa lentillifera, Caulerpa racemosa	Edible green algae, used in salads	Known as green caviar/sea grapes	Barsanti & Gualtieri, 2006;
	Chondrus crispus	Thickening agent	Irish moss, sold as *hana nori*	Barsanti & Gualtieri, 2006;
	Cladosiphon okamuranus	Mozuku	Cultivated around Okinawa Island (Japan), grows at depth of 1-3 m	Barsanti & Gualtieri, 2006;
	Enteromorpha, Monostroma	Aonori; Green laver	Grows in bays and gulfs of south Japan	Barsanti & Gualtieri, 2006;
	Gelidium, Pterocladia	High quality agar	*Gelidium* harvested from wild; Demand is larger than available sources	Barsanti & Gualtieri, 2006; Jensen,1993
	Gracilaria	Salad vegetable	Cultivated in Hawaii, high source of Vitamin A	Barsanti & Gualtieri, 2006;

Gracilaria, Hypnea	Lesser quality agar	*Gracilaria* cultivated in Chile, China, Indonesia	Barsanti & Gualtieri, 2006;
Hizika fusiforme	Hiziki	Popular in Japan and Korea, vitamins lost in processing, Higher Fe, Cu, Mn content than kombu	Barsanti & Gualtieri, 2006;
Laminaria	Haidai; Kombu	High β-carotene (2.99 mg/100 g dw) and iodine content (130 mg/100 g dw), native to Japan and Korea	Barsanti & Gualtieri, 2006; Radmer, 1996;
Palmaria palmate	Dulse	High in iron, minerals and vitamins	Barsanti & Gualtieri, 2006
Porphyra	Nori	Harvesting and preparation of sea weed is exacting and time-intensive; Cultivated in Japan, Korea and China; Source of red pigment *r*- phycoerythrin; used as tag in medical diagnostic industry	Barsanti & Gualtieri, 2006; Jensen,1993; Radmer, 1996;
Undaria	Wakame	High β-carotene (1.30 mg/100 g dw) and iodine content (26 mg/100 g dw)	Barsanti & Gualtieri, 2006; Radmer, 1996;
Ulva, Enteromorpha	Ulvan	Potential source of rare sugar precursors, oligosaccharides	Carlsson et al.,2007;

	Microalgae			
	Arthrospira, Nostoc, Chlorella	Food; herbal ingredients; Dihé; protein source	Cultivated in China and India (*Nostoc*), Africa and Mexico (*Arthrospira*); consumed because of taste, protein content and nutrients; algae have not gained importance as protein source due to texture, color, odor	Barsanti & Gualtieri, 2006; Becker, 2007; Spolaore et al., 2006
Dietary Supplements	***Macroalgae***			
	Laminaria religiosa, Undaria pinnatifida	Anti-viral agents, fucoxanthin	Few trials extended to human subjects; large scale trials underway to test against HIV	Barsanti & Gualtieri, 2006
	Microalgae			
	Arthrospira, Chlorella, Spirulina,Euglena	Tablets, capsules, powders, liquids	Antioxidants, protein source, vitamin supplement, efficacy on gastric ulcers, wounds, antitumor actions, source of β glucans; polyunsaturated fatty acids, bottleneck is low productivity of culture in terms of biomass and product formation	Barsanti & Gualtieri, 2006; Campanella et al., 1999; Spolaore et al., 2006; Otles & Pire, 2001; Piñero-Estrada et al., 2001

	Arthrospira, Dunaliella, Haematococcus pluvalis	β-carotene, astaxanthin, lutein, bixin, lycopene, phycobiliproteins	Used as natural pigments, have antioxidant activity; high production cost for *Haematococcus*, cells of *Dunaleilla* easily damaged causing oxidation of β-carotene	Borowitzka, 1992; Carlsson et al., 2007; Pulz & Gross, 2004; Spolaore et al., 2006
	Crypthecodinium, Nitzchia, Navicula, Porphyridium	Omega-3 fatty acids: DHA, EPA, AA	Alternative to fish sources; deficiency associated with fetal alcohol syndrome, cystic fibrosis, Folling's disease; isolation of PUFAs difficult due to their presence in lipids other than triglycerides	Apt & Behrens, 1999; Brown 2001; Carlsson et al., 2007; Spolaore et al., 2006; Jiang et al., 1999
Feed	***Microalgae***			
	Amphora, Chlorella, Dunaliella, Isochrysis Navicula, Tetraselmis	Aquaculture feed and feed additives	Primary food for larval and juvenile bivalves, enhance quality of fish species cultured; high cost of microalgal production for aquaculture	Borowitzka, 1997; Brown et al., 1997
	Arthrospira, Chlorella, Scenedesmus, Spirulina	Animal feed supplements	Provide nutrients and affect physiology of animals	Pulz & Gross, 2004; Spolaore et al., 2006

Table 2.3: Applications of macro- and microalgal strains in cosmetics, chemicals, environmental treatments, and biofuels.

Industry	Strains	Uses/Products	Remarks	Ref.
Cosmetics	***Macroalgae***			
	Nonspecified	Face, hand, body creams/lotions	Thalassotherapy in France for rheumatism and osteoporosis	Barsanti & Gualtieri, 2006
	Microalgae			
	Arthrospira, Chlorella	Skin care, sun protection and hair care	Extracts of the algae instead of the algae are used	Spolaore et al., 2006
Chemical	***Microalgae***			
	Chlamydomonas, Dunaliella, Neochloris	Stable-isotopically labeled compounds: glucose, galactose, xylose	Microalgae easily handled, cultured; photosynthesis allows them to incorporate labeled C, H, and N; requires closed system of production	Apt & Behrens, 1999; Radmer & Parker, 1994
Environmental	***Macroalgae***			
	Ascophyllum, Ecklonia, Fucus	Soil additives, fertilizers, conditioners	Higher yields; increased uptake of soil nutrients; increased resistance to some pests	Barsanti & Gualtieri, 2006
	Brown and red algae	Waste-water treatment, biofilters for fishpond effluents, CO_2 sequestration; heavy metal biosorption	Macroalgae show higher productivity than sugarcane, can uptake inorganic N and P; macroalgal productivity affected by environmental factors and nutrients	Gao & McKinley, 1994; Davis et al., 2003

	Microalgae			
	Anabaena, Nostoc	Nitrogen fixation, water holding	Microalgal polymers and bio-active compounds beneficial	Pulz & Gross, 2004; Shields & Durrell, 1964
	Mixed cultures	Tertiary waste-water treatment, CO_2 sequestration	Remove inorganic N, P, heavy metals and toxic organic compounds; algal systems have long generation times; difficult and costly harvesting; increasing CO_2 decrease algal growth	Carlsson et al., 2007; de la Noüe et al., 1992; Lembi & Waaland, 1988
Biofuel	***Macroalgae***			
	Gracilaria, Macrocystis	Biomethane production	Highest yield of methane, high production costs, not yet commercialized	Carlsson et al., 2007
	Microalgae			
	Dunaliella, Hantzschia, Scenedesmus	Bio-oil via pyrolysis, biodiesel	Bio-oils have high oxygen content that lowers quality; biodiesel has low selling price	Carlsson et al., 2007; Chisti, 2007; Chisti, 2008; Patil et al., 2008; Behzadi & Farid, 2007; Rosenberg et al., 2008
	Green algae eg. C*hlamydomonas reinhardtii*	Biohydrogen	Hydrogen produced difficult to store and transport, cost could be an issue	Carlsson et al.,2007; Wu,2000; Melis & Melnicki, 2006

Figure 2.1: Algal Biomeal

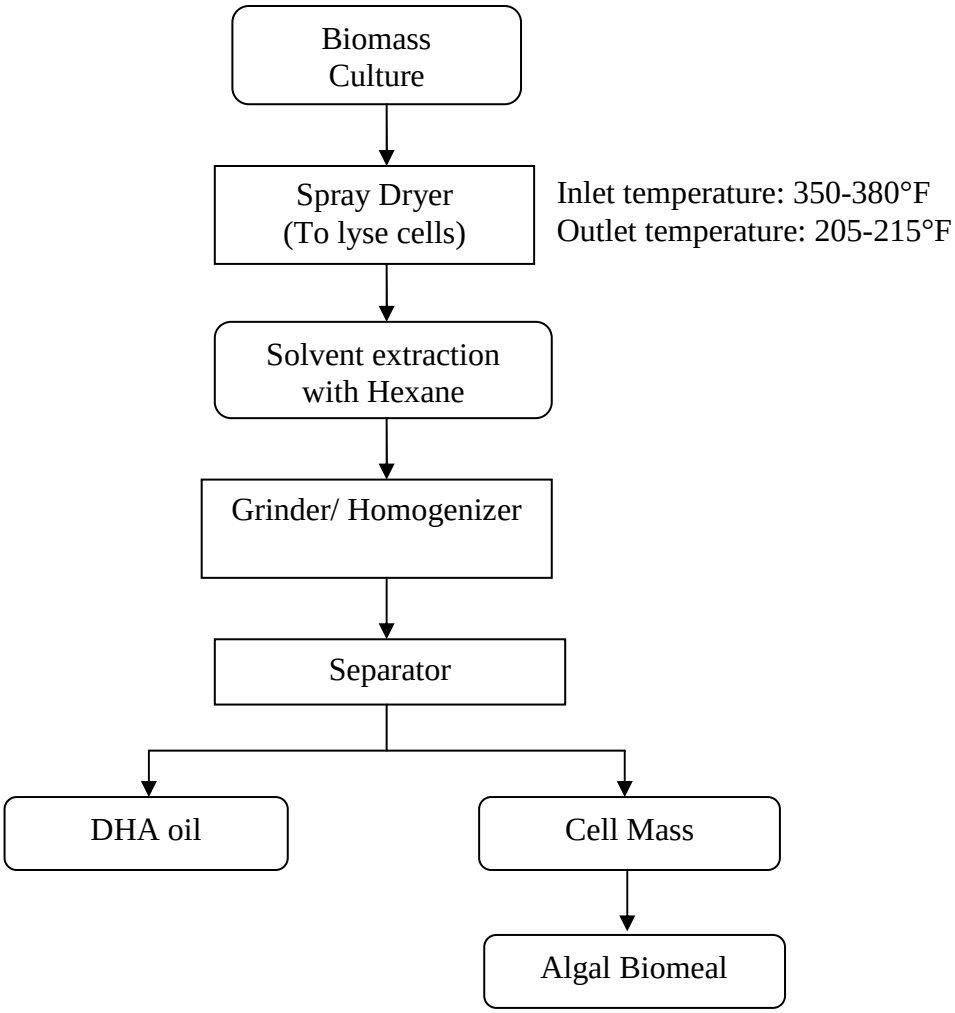


Figure 2.2: Flowchart for Biomeal Production

2.4 Potential Applications of Biomeal

The use of microalgae to produce polyunsaturated fatty acids results in a large amount of biomass as by-product. DHA is a very important product extracted from algae, *Crypthecodinium cohnii* being the microalgae used extensively for this purpose. The biomeal obtained after DHA extraction has excellent nutritional quality and is also a source of DHA. Apart from its nutritional

quality the biomeal also has a strong, unique flavor which could be appealing to humans and animals alike. Similar to other microalgae, the biomeal can be used as an ingredient to develop a variety of products for human and animal consumption. Being abundantly available and having no other applications, this waste product is currently disposed off as feed and for landfills. The abundance and nutritional quality makes the biomeal ideal for use as a value-added ingredient. Therefore, the objective of this research is to find new applications for this by-product in food and feed.

Chapter 3: Research Objectives

The goal of this project was to identify new value-added applications for algal biomeal obtained as a by-product of oil extraction from microalgae. In order to fulfill this goal, there were three specific objectives:

- To characterize the properties of the biomeal to enable its use as an ingredient
- To develop novel formulations taking advantage of the properties studied
- To evaluate the quality and shelf-stability of the products developed

Chapter 4: Materials and Methods

4.1 Materials

The algal biomeal used for this project was provided by Martek Biosciences, Columbia, MD in two lots of 4 kgs and 2 kgs respectively. The product name for the same is DHASCO® Biomass. It is the dried mass of the algae *Crypthecodinium cohnii* from docosahexaenoic acid single cell oil production. The biomeal is obtained by spray drying lysed algal cells. The biomeal has a particle size of 5 microns to 500 microns. The biomeal used for analysis was sieved through an ASTM 140 mesh sieve. It was stored under refrigeration.

TIC Pretested® Pre-Hydrated® Ticaxan® Xanthan gum, TIC Pretested® gum Arabic FT, TIC Pretested® gum guar and TIC Pretested® TICA-algin HG 400 (alginate) was supplied by TIC gums (Belcamp, MD, USA). Sodium citrate and anhydrous citric acid was obtained from Archer Daniels Midland (Decatur, IL, USA). Sodium hydroxide pellets and calcium chloride dihydrate was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Pure potassium sorbate (>99.0%) and glacial acetic acid (99+% pure) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Food grade pigment FD&C Yellow 6 (sunset yellow) was used.

4.2 Methods

4.2.1 Analysis of Biomeal Properties

Determining the moisture isotherm

The water activity of the biomeal sample was determined at room temperature using the Decagon water activity meter (Decagon Devices, Pullman, WA). The moisture content was determined by the oven method at 105°C. The moisture content of the biomeal was increased by step-wise addition of a known amount of water, followed by immediate measurements of the sample's water activity and its corresponding moisture content. For the desorption isotherm, biomeal with high moisture content was placed in a desiccator at room temperature. Samples in duplicate were withdrawn at regular intervals and subjected to moisture content and water activity measurements.

Determining the biomeal solubility

The solubility of the biomeal was determined at room temperature in each of the solvents investigated, namely water, chloroform, acetone, and ethanol. Solubility measurements were conducted in triplicate by adding 2 g of biomeal in 100 ml of respective solvent under constant stirring, then filtered through Whatmann Paper #1 before weighing the retaining residues on the filter paper.

Rheological testing of biomeal solutions

General rheological measurements were carried out using the TA Advanced Rheometer 2000 (TA instruments, New Castle, DE) with a 40 mm stainless steel parallel plate at 20°C with zero normal force and a shear stress of 2 Pa. Viscosity measurements were conducted to see the

changes in viscosity as shear rate increased for solutions of 10% biomeal with increasing concentrations (0-0.6%) of xanthan gum, sodium alginate, guar gum, and gum arabic.

Surface properties of biomeal solutions

Surface tension measurements were carried out using the KRÜSS Digital tensiometer K10T (KRÜSS, Hamburg, Germany) with the ring probe. Surface tension was measured for solutions with varying xanthan gum concentrations (0.1%-0.25%) with and without the addition of 10% biomeal (w/v). Similar measurements were conducted using a 30:70 oil-in-water emulsion with varying concentrations of xanthan gum with and without the addition of biomeal. In order to test the stability of an emulsion over time, surface tension measurements were taken for a 30:70 oil-in-water emulsion with 0.1% xanthan gum over a period of 180 min, with and without the addition of biomeal. All measurements were performed in triplicates and standard deviation was calculated.

Compatibility of biomeal with coagulating agents

In order to determine the optimum concentration of coagulating agents sodium citrate and calcium chloride compatible with the biomeal, general rheological measurements were carried out using the TA Advanced Rheometer 2000 with a 40 mm stainless steel parallel plate at 20°C with zero normal force and a shear stress of 2 Pa. Different combinations of 12 ml of 20% and 10% each sodium citrate and calcium chloride were mixed with 5 g biomeal and the mixtures were tested for viscosity. Biomeal pellets were formed by transporting the mixtures onto a water-absorbing paper towel and left to air dry at room temperature. Hardness of the pellets was determined over a period of 180 min using the TA.XT2*i*Texture Analyzer (Texture Technologies

Corp., Scarsdale, N.Y., U.S.A.) using a 25mm Perspex cylinder probe (50 kg load cell). The test was conducted using a distance of 5 mm, a pre test speed of 5 mm/sec and a post- test speed of 2mm/ sec. All the measurements were obtained in triplicates, at room temperature (20-22˚C) and moisture content of each of the samples was also estimated along with the textural properties.

Flavor profile analysis of the biomeal

To measure the flavor profile, 0.5 gm of the biomeal in a vial was placed in a water bath at 50°C for 30 min. A SPME fiber (Divinylbenzene/Carboxen/Polydimethylsiloxane; DVB/CAR/PDMS) was inserted through the film lining exposing 2 cm of the fiber to the volatiles for a total of 10 min absorption time. The SPME fiber was then desorbed for 10 min on the GC-MS equipment. The desorbed volatiles were transferred to the GC-MS with a 30 m capillary column (0.32 mm I.D.). The temperature was programmed to start at 40°C with a hold time of 5 min followed by an increase to 200°C at a rate of 5°C/min. The injector was set at 250°C and the column flow rate was 2 ml/min. A mass spectrometer with a scan range of 35 to 350 Da was used to identify the volatiles.

4.2.2 Formulation of Products

Biomeal-alginate gels

One variation of biomeal products developed in the present study included the use of cross-linking agents such as sodium alginate and calcium chloride. Twenty-five ml of 1.5 % (w/v) sodium alginate in water was added to 5 g of the biomeal and the solution was stirred for 10 min. In order to enable flavor release from the biomeal, the mixture was heated in a water bath for 10 min at 50-60°C .This mixture was then added wells containing 10 % (w/v) calcium chloride in

water to form biomeal-alginate gels. These gels were then dried at room temperature till desired hardness was reached.

Biomeal pellets

Coagulated algal products have been made with fresh algae as mentioned by Kitahara (1987). Similar products were formulated using biomeal. Thirty ml of 20 %(w/v) sodium citrate in water was added to 10 g algal biomeal and the mixture was constantly stirred for 10-15 min in a water bath at 60-70°C. It was cooled to 40°C before 30 ml of 10% (w/v) calcium chloride in water was added. This mixture was then stirred for additional 30 min. Pellets were formed on a water absorbing paper towel and they were air dried for 6-7 hrs or till the desired hardness was achieved.

Biomeal sauce

To formulate a sauce product containing the biomeal, 10 g of biomeal was mixed with 100 ml water containing 0.2 g salt and 0.5 g sugar. The pH of the mixture was adjusted to 8.1 to facilitate browning by adding 1N NaOH. The mixture was then heated to 110°C in an oil bath for 20 min. After cooling to 70°C, 0.275 g xanthan gum and 0.2 g color pigment Yellow 6 were added to achieve the desirable consistency and color.

4.2.3 Analysis of Products

Alginate gels

The alginate gels formulated were tested for textural attributes such as springiness and firmness using the TA.XT2*i*Texture Analyzer using the Gummy Confectionary program with a 25mm Perspex cylinder probe and a 5 kg load cell. The test was conducted with a pre- test, test and post-test speed of 1 mm/sec. The test was performed using the distance mode with a target distance of 2.5 mm and a trigger force of 5 g. These attributes were measured over 300 min at different drying temperatures of 25°C, 35°C and 45°C. Three replicates were performed for each temperature. Similar measurements were obtained over a period of 5 weeks for gels stored at room temperature in air-lock bags. Two replicates were performed for the experiment and two samples were analyzed for each replicate.

Biomeal pellets

Hardness of the pellets was determined using the TA.XT2*i*Texture Analyzer using a 25 mm Perspex cylinder probe with a 50 kg load cell. The test was conducted with a pre- test, test and post-test speed of 1 mm/sec. The test was performed using the distance mode with a target distance of 5 mm and a trigger force of 5 g. Hardness was determined over a drying time of 480 min at drying temperatures of 25°C, 35°C and 45°C. Water activity of the pellets was also determined at room temperature at different drying times. Three replicates were performed for each temperature. Textural stability of the pellets over time was also studied. The pellets were stored at room temperature in air-lock bags and hardness of the pellets was evaluated at intervals of 1 week for 10 weeks. Two replicates were performed for the experiment and two samples were analyzed for each replicate.

All texture measurements were obtained at room temperature (20-22˚C) and moisture content of the samples was also estimated along with the textural properties.

Biomeal sauce

The loss of water or syneresis of the sauce was evaluated over a period of 8 weeks at three different temperatures- refrigeration (4°C), room temperature (22-25°C) and 35°C. Ten ml of samples stored in centrifuge tubes were centrifuged at a speed of 2,200 rpm ($707 \times g$) for 15 min in the Beckman Model TJ-6 centrifuge (Williams et. al., 2009). The volume of water exuded was determined from the graduated tube. Percent water loss was calculated as:

$$\frac{volume\ of\ water\ exuded}{total\ volume\ of\ sample} \times 100$$

The effect of combination of gums on syneresis was studied. In the sauce formulation, 50% of xanthan gum was replaced by an equal weight of curdlan gum and syneresis testing was done as described above. In a second study, to the original formulation, an additional 0.1375 % curdlan gum was added increasing the total concentration of gums. The experiment was replicated three times.

Stability of the color of the sauce was studied over 5 weeks. Samples were stored in glass bottles at refrigeration (4°C), room temperature (22-254°C), 35°C and 45°C. The effect of light on the color at room temperature was also studied for which samples were stored in glass bottles covered with aluminum foil at room temperature. All samples were analyzed for color using the HunterLab ColorFlex Spectrophotometer 45°/0° (HunterLab, Reston, VA). The color measurements were performed in the CIELAB color scale using Setup 1 (D65 illuminant, 10°

standard observer). Ten ml of a sample was used for color measurement using a standard glass sampling cup. All measurements were taken at room temperature.

Statistical analysis

Two replications of each sample were performed during color analysis and two samples were analyzed for each of the storage times and temperatures. The results were analyzed for statistical significance using MINITAB 1513 software with ANOVA followed by Dunnett's test ($p<0.05$) for mean comparison to control at time 0. To investigate the effect of light, ANOVA followed by Tukey's test ($p<0.05$) for mean separation was done. Complete statistical analysis can be found in Appendix A.

Microbial counts for total aerobic plate count (TPC) and yeasts and molds were carried out weekly to evaluate the shelf-stability of the sauce. Biomeal sauce samples were prepared and stored in glass bottles at 4 different storage temperatures- refrigeration (4°C), room temperature (22-25°C), 35°C and 45°C. Serial dilutions for the counts were done using DI water and plating was done using 3M™ Petrifilm™ Aerobic Count Plates and Yeast and Mold Count Plates (3M, St. Paul, MN) followed by incubation at 35±2°C for 48 hrs and at 20±2°C for 5 days for TPC and yeast and mold count respectively. All microbial counts were reported as colony forming units/ml (cfu/ml).

The effect of acetic acid (at 0.1%, 1%, 2%), citric acid (at 0.1%, 1%, 2%) and potassium sorbate (at 0.1%) as preservatives was studied. Sauce samples with added preservatives were stored at room temperature and were analyzed for TPC and yeast and mold count weekly.

Two replicates were performed for each storage temperature and level of preservative and two samples were analyzed from each replicate,

Proximate analysis of the sauce was performed to obtain the approximate moisture, ash, protein, fat and carbohydrate content. The analysis methods followed were according to the standard procedures outlined by Nielsen (2003).

The moisture content was determined using the oven method at 105°C. Percent moisture was calculated as:

$$\frac{weight\ of\ water\ lost}{total\ weight\ of\ sample} \times 100$$

Moisture-free samples were ashed in a muffle furnace at 600°C. Percent ash was calculated as:

$$\frac{\text{weight of sample after ashing}}{\text{dry weight of sample}} \times 100$$

Fat was analyzed using the Soxhlet procedure using petroleum ether as a solvent.

Protein was analyzed using the Bio-Rad microassay using Phosphate Saline Buffer (PSB) as the extracting solvent.

Total carbohydrate content was calculated as difference as outlined by the nutritional labeling requirements of the U.S. Food and Drug Administration (Nielsen, 2003; Anonymous, 1997).

Chapter 5: Results and Discussion

5.1 Biomeal Properties

5.1.1 Solubility of Biomeal

To enable the use of biomeal with solvents, its solubility in water, ethanol, chloroform and acetone was determined. The solubility as estimated is given in Table 5.1. Algal biomeal showed negligible solubility in all the solvents investigated including water. This limits the use of biomeal in a water-based application and necessitates the use of a suspending agent.

Table 5.1: Solubility of biomeal in solvents determined at room temperature as g/l

Solvent	**Solubility (g/l)**
Water	5.79± 0.08
Chloroform	3.41± 0.25
Acetone	2.40± 0.22
Ethanol	2.26± 0.15

n=3

5.1.2 Moisture Isotherm

The moisture isotherm of the biomeal was determined at room temperature (Fig. 5.1). Water activity of a food is important with respect to microbial growth, enzymatic and chemical activities of its constituents. Control of water migration during and post packaging remains crucial in the product's quality and shelf life. Moisture isotherm, which depicts the relationship

between the total moisture content and the water activity of food, at a constant temperature has been used by chemists, microbiologists and engineers as a guiding tool to understand and control water migration in the development of new food products. One important aspect of an isotherm is the hysteresis, the difference between adsorption and desorption isotherms that is related to the nature and state of components in a food. It reflects the structural and conformational rearrangement which may hinder or facilitate the movement of moisture, the irreversibility of the sorption process, as well as the effect on potential microbial and chemical deteriorations (Al-Muhtaseb et. al., 2002; Rockland & Beuchat, 1987; Ramaswamy & Marcotte, 2005).

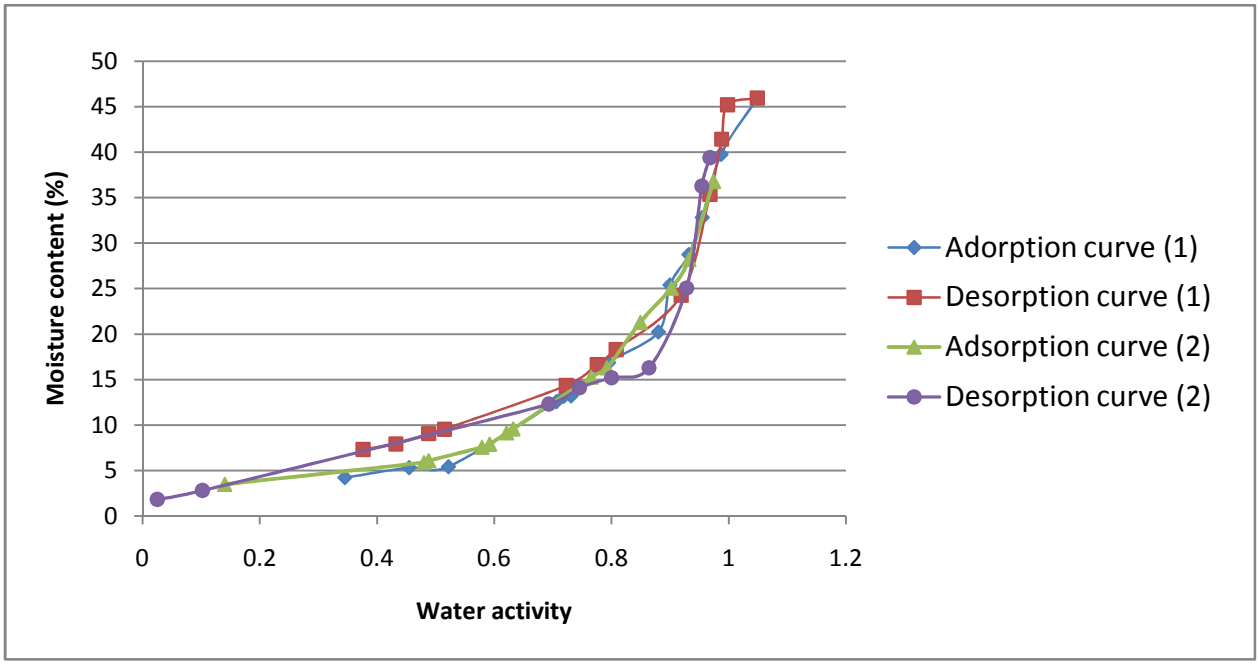


Figure 5.1: Moisture content (%) plotted vs. water activity of algal biomeal for adsorption and desorption determined at room temperature

The nature of the isotherm was similar to that showed by high-sugar and high-pectin foods (Al-Muhtaseb, 2002). The similar paths followed by the adsorption and desorption isotherms indicate a steady relationship between water activity and moisture content. Hysteresis is normally

attributed to capillary condensation taking place in mesopores (2-50 nm) present in the solid. The absence of hysteresis indicates a nonporous or macro porous adsorbent and unrestricted mono-layer adsorption (Delmelle et al., 2005, Coasne et al., 2002, Brantley & Mellott, 2000).

5.1.3 Surface Tension Measurements

In order to study the effect of biomeal on solutions and emulsions, surface tension measurements were performed. Since xanthan gum is known to stabilize emulsions (Papalamprou et. al., 2005; Mandala et. al., 2004) , surface tension measurements were obtained for solutions containing biomeal and xanthan gum as well as oil-in-water emulsions with increasing concentrations of xanthan gum (Fig. 5.2 a). As xanthan concentration increases, the corresponding surface tension was found to increase in a xanthan-only solution. Surface tension, a.k.a. the excess free interfacial free energy, is the free energy change associated with the isothermal, reversible formation of a surface. Surface tension is important while considering the stability of food foams and emulsions (Rao et. al, 2005). The combination of biomeal and xanthan showed relatively flat surface tension over a wide range of xanthan concentrations, indicating the ability of biomeal to stabilize xanthan solutions.

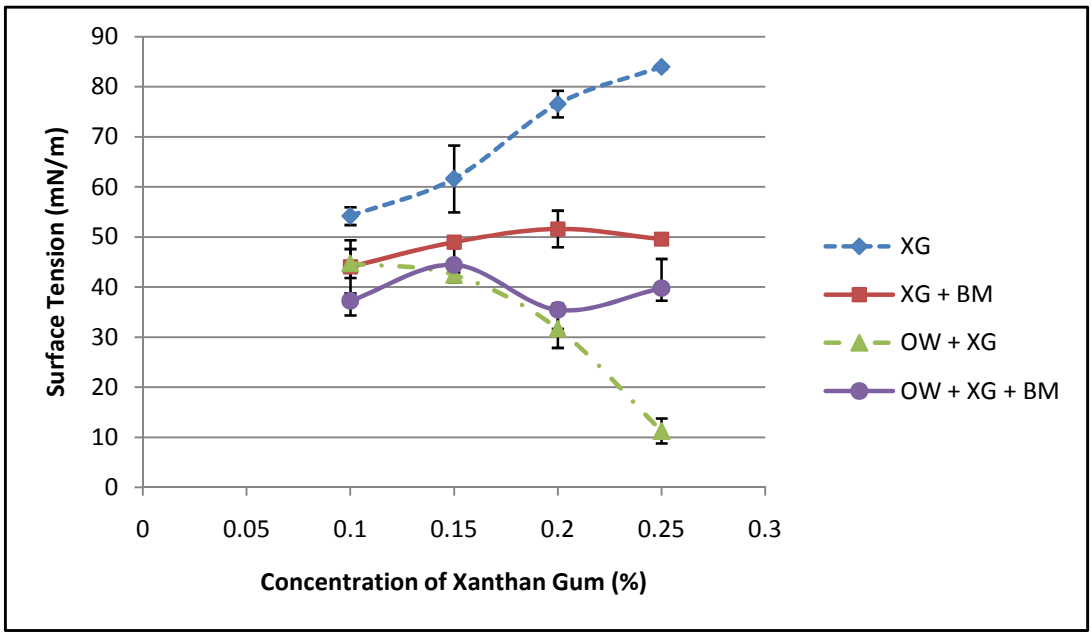


Figure 5.2 a: Change in surface tension of xanthan gum(XG) solutions with and without biomeal (BM), 10% (w/v);and 30:70 oil in water emulsions (OW) with xanthan gum, with and without biomeal (BM), 10% (w/v); n=3

For a 30:70 oil-in-water emulsion, surface tension decreased with increasing xanthan gum concentrations. This could be attributed to increased stabilization by higher concentrations of xanthan gum. The addition of biomeal to this emulsion caused a normalization of surface tension values. Over time (Fig. 5.2 b), surface tension of an oil-in-water emulsion with 0.1 % xanthan gum was found to decrease, indicating phase separations. Biomeal addition resulted in stable surface tension values over the experimental time of 200 min. The results indicate that the biomeal has a stabilizing effect on solutions and emulsions under conditions of this study, a property that can be used to improve the stability of multi-phase systems.

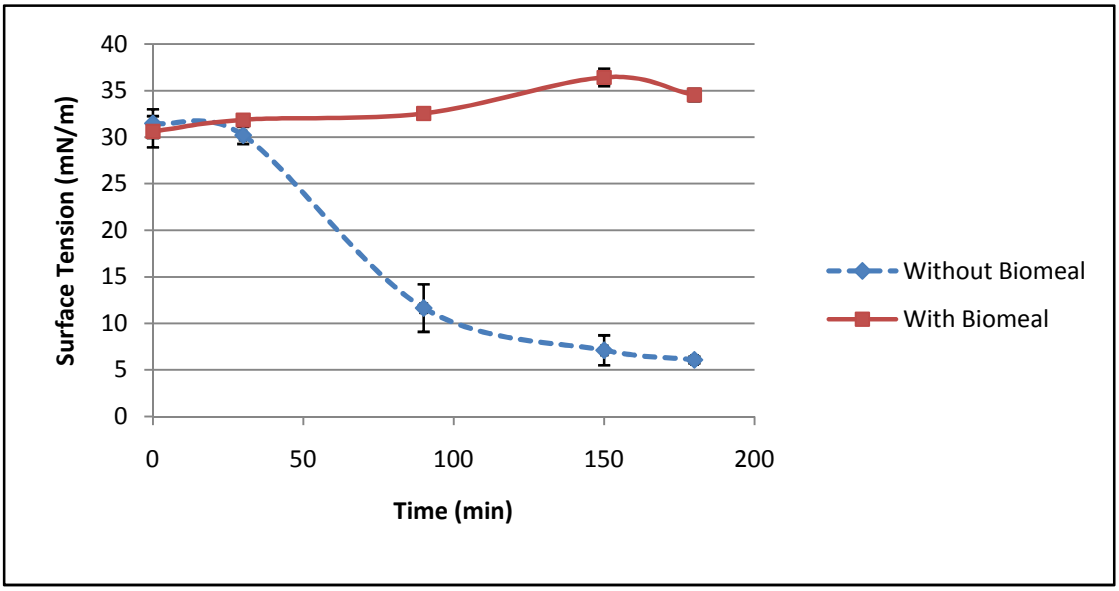


Figure 5.2 b: Change in surface tension with time of 30:70 oil in water emulsion with 0.1% Xanthan gum (w/v); without and with biomeal (10% w/v); n=3

5.1.4 Flavor Volatile Profile of Algal Biomeal

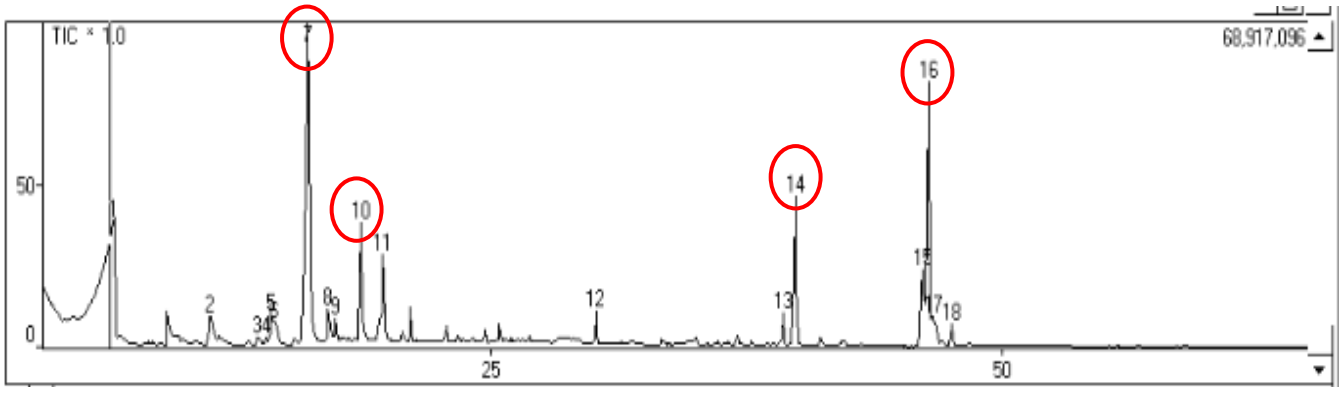


Figure 5.3: Mass spec for 0.5 gm of biomeal sample placed in a 50 °C water bath for 30 min, then 10 minute adsorption time using DVB/CAR/PDMS SPME fiber, then 10 minute desorption time at GC/MS.

The chromatogram obtained by the GC-MS analysis of the biomeal is depicted in figure 5.3.

The summary of the volatile peaks with their retention times and peak areas obtained from the analysis is given in Table 5.2a.

Table 5.2a: Volatile peaks with retention times and peak areas obtained from the mass spec of algal biomeal after 10 minute adsorption and desorption

Peak No	R.Time	Area	Height	%Total
1	6.517	885037496	24348708	27.7
2	11.244	67734742	5393718	2.12
3	13.52	20541624	1607782	0.64
4	13.952	13937477	1563691	0.44
5	14.228	42842105	6170374	1.34
6	14.392	59653728	4716011	1.87
7	16.022	856962620	66669090	26.82
8	17.009	61589045	6738849	1.93
9	17.392	23291055	4471518	0.73
10	18.604	143652363	23844468	4.5
11	19.709	108613210	17382045	3.4
12	30.133	24628806	6473169	0.77
13	39.317	42878996	6300441	1.34
14	39.894	153952465	28813627	4.82
15	46.123	134441510	15463430	4.21
16	46.452	442453532	54620863	13.85
17	46.658	76463603	5887689	2.39
18	47.572	36882588	4421915	1.15

Based on peak height and area, further identification was attempted for peak numbers 7, 10, 14 and 16. On comparison with library results, the top matches (scores of 88 and above) for the major peaks identified along with their match score are detailed in table 5.2b. Taking into consideration the first match on the list, the volatiles were tentatively identified as 3-methyl-2, 5-furandione; maltol; hexadecanoic acid, methyl ester and 11- octadecenoic acid, methyl ester respectively. Further research is necessary to confirm the presence of and quantify these volatiles in the algal biomeal.

Table 5.2b: Library identifications and their respective match scores for the peaks obtained from the mass spec of algal biomeal

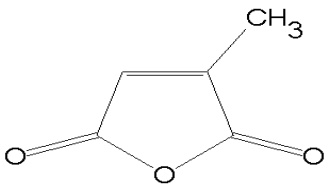
Peak No.	Match Score	Probable Molecule
7	88	2,5-Furandione, 3-methyl-
	88	2,5-Furandione, 3-methyl-
	88	2,5-Furandione,dihydro- 3-methylene-
10	91	Maltol
14	92	Hexadecanoic acid, methyl ester
	90	Hexadecanoic acid, methyl ester
	88	Hexadecanoic acid, methyl ester
16	92	11-octadecenoic acid, methyl ester
	89	5- octadecenoic acid, methyl ester

As seen in Fig. 5.4a, 3-methyl-2, 5-Furandione is a furan derivative, possibly arising from carbohydrate thermal degradation (Guillén & Manzanos, 2002). This compound has been detected in oak wood smoke, *Microcitrus inodora* (Australian wild lime) and in the fresh ripe fruits of *Mandragora autumnalis* (mandrake fruit). This compound along with other furan derivatives has a caramel, sweet, butterscotch, brandy, burnt, spicy and sugar notes and contributes to the odor and aroma of fresh fruits and also the smoky flavor of wood (Guillén & Manzanos, 2002; Shaw et al., 2000; Hanus et al., 2006).

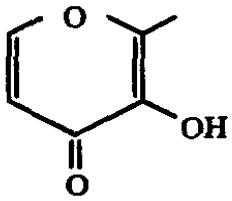
Maltol or 3-hydroxy-2-methyl-4H-pyran-4-one (Fig. 5.4b) naturally occurs in certain conifers and is a potent flavor enhancer (Portela et al., 1996). Maltol is known to have a sweet, caramel-like flavor with fruity overtones especially pineapple and strawberry flavors (Mussinan et al., 1979; Pittet et al., 1970). Owing to its flavor enhancing characteristic, it could be postulated that

maltol is one of the compounds responsible for the flavor enhancing properties attributed to algal biomeal.

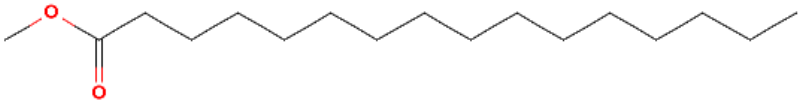
Methyl ester of hexadecanoic acid and methyl ester of 11-octadecenoic acid are esters of fatty acids also known as methyl palmitate and methyl vaccenate (Fig. 5.4 c and d). Free fatty acids and their esters contribute to the flavor of a variety of foods such as cheeses and fruit flavors (Carunchia Whetstine et al., 2003; Woo et al., 1984; Zabetakis & Holden, 1997). Since algal biomeal is rich in lipids, especially polyunsaturated fatty acids, degradation products of the same play a significant role in imparting the characteristic biomeal flavor.



a) 3-methyl-2, 5-Furandione
(Source: http://www.chemsynthesis.com/)



b) Maltol
(Source: Mussinan et al., 1979)



c) Methyl ester of hexadecanoic acid
(Source: http://sci-toys.com/scichem/jqp005/8181.html)

d) *cis*-11- octadecenoic acid, methyl ester
(Source:http://www.chemicalbook.com)

Figure 5.4 Structures of the major flavor volatiles isolated from algal biomeal

5.2 Biomeal Formulations

5.2.1 Compatibility with Hydrocolloids

Owing to the negligible solubility of biomeal in water, hydrocolloids can be used to stabilize biomeal solutions and act as suspending agents. To elucidate the flow behavior of various hydrocolloid-biomeal solutions, changes in the solutions' apparent viscosity $\eta_{a,}$ defined as the ratio of shear stress to shear rate (Rao et. al., 2005) were studied. Solutions of biomeal with xanthan gum, alginate, gum arabic and guar gum were tested (Fig. 5.5a). A typical shear-thinning behavior was observed for all solutions investigated, as apparent viscosity decreased with increasing shear rate. As expected, as total gum concentration increased, the viscosity of the solutions increased. As seen in Fig. 5.5a, guar gum and gum arabic failed to increase the solution viscosity appreciably, even at higher concentrations or to create a homogenous solution. Xanthan gum showed the highest viscosity at all values of shear rate for all concentrations studied. Xanthan gum was thus the most compatible with the biomeal and was used as the hydrocolloid to formulate a sauce using the biomeal (Figure 5.5b).

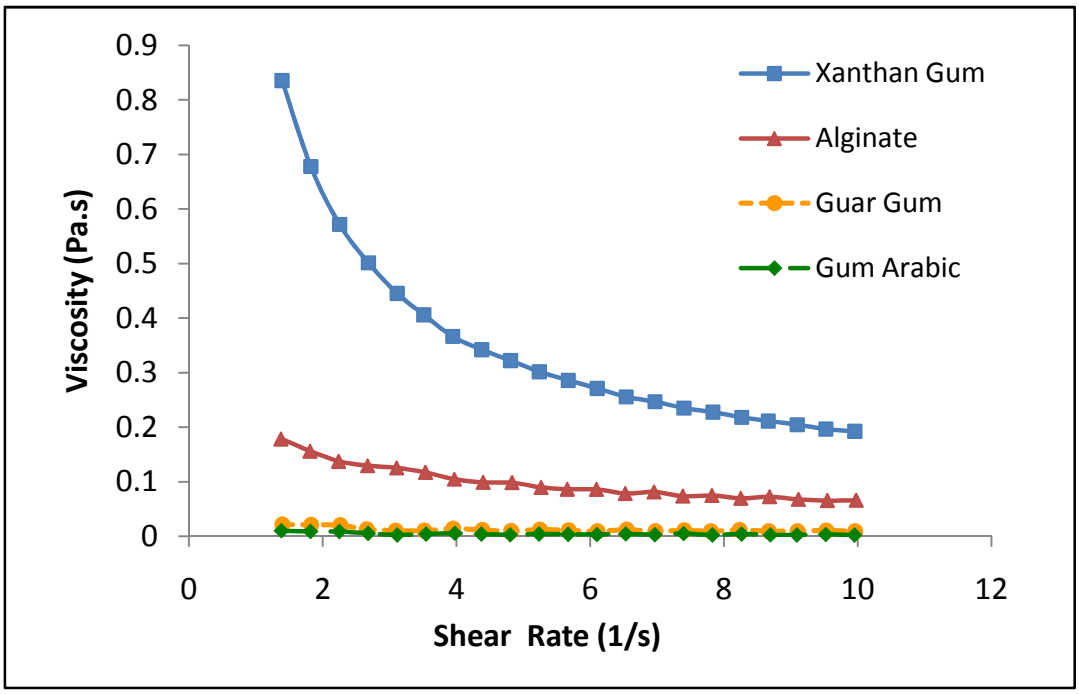


Figure 5.5a: Change in viscosity with shear rate of a 10% (w/v) biomeal solution with 0.2 % (w/v) of xanthan gum, alginate, guar gum or gum arabic

Figure 5.5b: Biomeal sauce created with 10% biomeal and 0.275% xanthan gum

5.2.2 Compatibility with Coagulating Agents

In order to determine the optimum concentrations of the coagulating agents, apparent viscosity measurements were obtained for the solutions with varying concentration of coagulating agents (Fig. 5.6a). Based on the brown algae products created by Kitahara (1987), sodium citrate and calcium chloride were chosen as coagulating agents. A relatively high viscosity of the combination of coagulating agents indicated a stable solution for the formation of coagulated biomeal products. In order to further investigate the stability of the solutions, biomeal pellets were formed and tested for hardness over a drying time of 180 min under room temperature (Fig. 5.6b). As expected, the hardness of pellets for each combination of coagulating agents increased with drying time. The combinations of 10% sodium citrate- 10% calcium chloride and 20% sodium citrate- 20% calcium chloride yielded very low product hardness over the drying period. On the other hand, 10% sodium citrate-20% calcium chloride and 20% sodium citrate- 10% calcium chloride gave higher hardness values over the drying period. At the end of 180 min, products obtained using the 20% sodium citrate- 10% calcium chloride combination gave the highest hardness value. Based on the two studies, 20% sodium citrate and 10% calcium chloride was the optimum combination of coagulants for the formation of biomeal pellets (Fig. 5.6c).

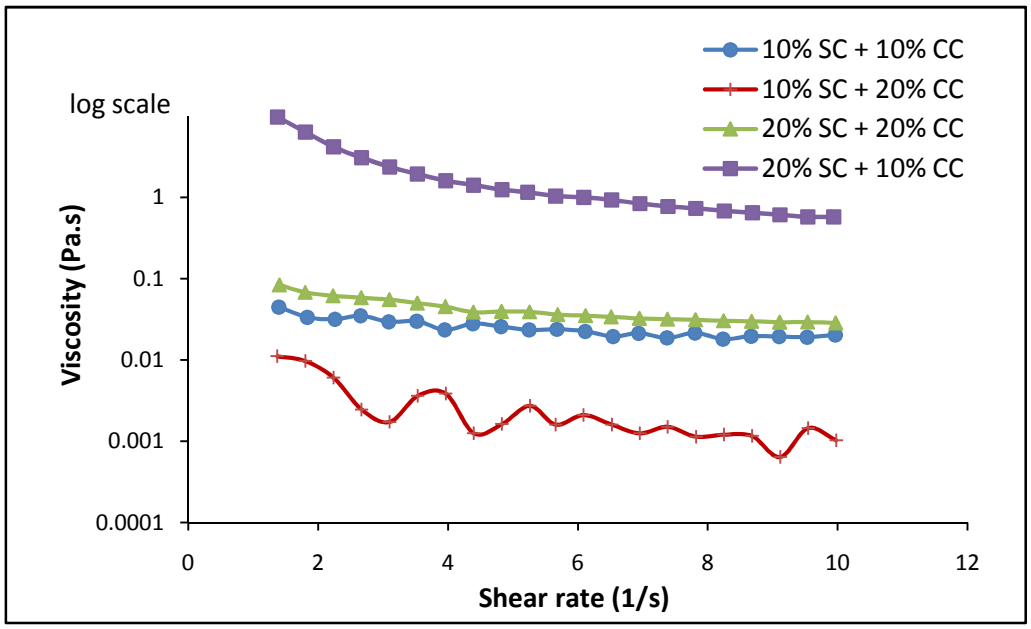


Figure 5.6a: Change in viscosity with shear rate of algal biomeal with varying combinations of coagulating agents: sodium citrate (SC) and calcium chloride (CC)

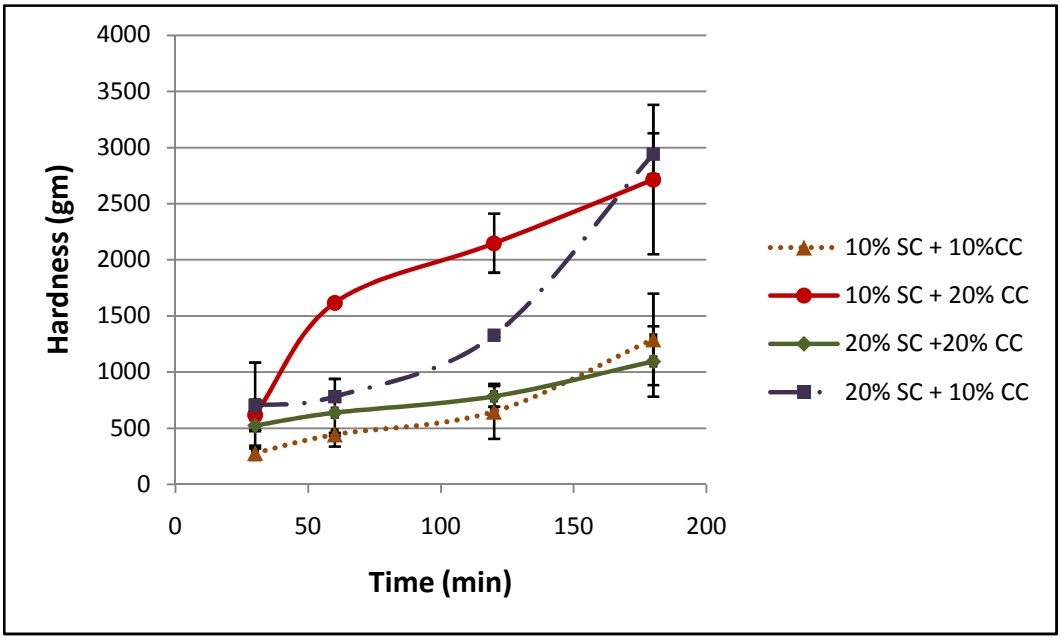


Figure 5.6b: Characterization of hardness in gms of the products obtained using various combinations of coagulating agents at different times during a drying period of 180 min; n=3

Figure 5.6c: Biomeal pellets created using 20% sodium citrate and 10% calcium chloride

5.2.3 Nutritional Profile of Biomeal Sauce

The nutritional content of the biomeal sauce as evaluated by standard methods is given in Table 5.3.

Table 5.3: Nutritional composition of biomeal sauce

Nutrient	**Content (%)**
Carbohydrate	7.83
Protein	0.93
Fat	0.26
Moisture	89.88
Ash	1.09

5.2.4 Color Analysis

The CIELAB system that objectively measures the color of foods in terms of light reflected from the surface was used to determine the L*, a* and b* coordinates for fresh biomeal sauce at room temperature (Table 5.4). Color is a major attribute that influences consumers and it plays an important part in any purchase. Color is often associated with product freshness and influences the price the consumer will be willing to pay for the same (Side, 2002; Judd, 1952). Thus, it is important to know the color attributes of a product and a standard measuring system for the same.

The CIELAB space can be visualized as a three-dimensional space and the location of any color is determined by its color coordinates: L*(lightness), a* (the red/green coordinate with +a* indicating red and –a* indicating green) and b* (the yellow/blue coordinate with +b* indicating yellow and –b* indicating blue) (Hui et. al, 2004). The finished biomeal sauce showed L, a, b values at 20.06, 19.72, 26.91, indicating a brown-yellowish color that is similar to a barbeque sauce, which should be more appealing to consumers than the greenish tone inherent from the microalgae. The consistent color scores acquired from four different sauce samples also suggest that the sauce is relatively homogeneous with minimal color variations.

Table 5.4: CIELAB color attributes, brightness (L*), red component (a*), yellow component (b*) of biomeal sauce

Sample	L*	a*	b*
1	20.21	19.54	26.59
2	20.13	19.56	26.68
3	19.99	19.84	27.33
4	19.99	19.92	27.05

5.3 Quality and Stability of Products

5.3.1 Biomeal-Alginate Gels

For the biomeal gel products (Fig. 5.7a), the main characteristics of hardness/firmness and springiness were determined using the TA.XT2*i*Texture Analyzer. Hardness, cohesiveness, elasticity/springiness, are common texture attributes evaluated for gel products (Konstance, 1993; Andrew and Morrison, 2001). Figure 5.7b shows a typical texture analyzer plot obtained by compressing biomeal pellets at two different drying times. The height of the first peak gives the hardness in terms of the force required to compress the sample. The firmness (Fig. 5.7c) and springiness (Fig. 5.7d) of the gels were determined while drying at different temperatures. Higher drying temperatures resulted in higher values for firmness and springiness at each drying time indicating faster drying. Drying was done until the texture testing resulted in overload of the force. Overload indicated that the texture attribute has exceeded the measurable value which could be co-related to acceptable hardness and springiness attributes. Monitoring these parameters assisted in the determination of the adequate drying time for each temperature to ensure optimum textural characteristics for the products.

Figure 5.7a: Biomeal-alginate gels formulated using 1.5% sodium alginate and 10% calcium chloride

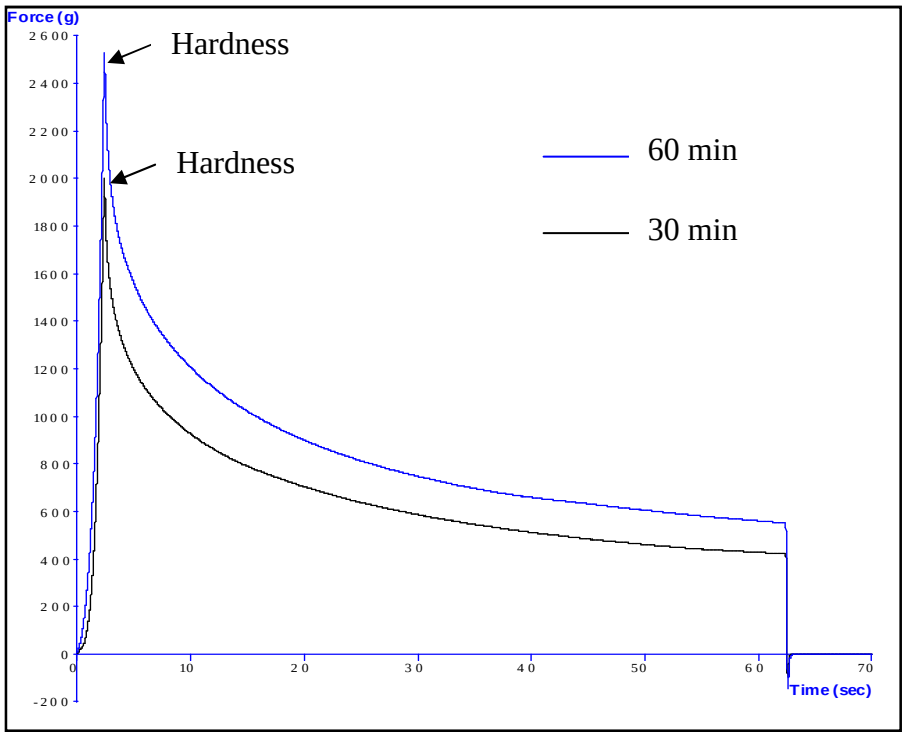


Figure 5.7b: Graph of force vs. time obtained using the Texture Analyzer, in compression mode, after drying alginate samples for 30 and 60 min with the highest peak force indicating hardness

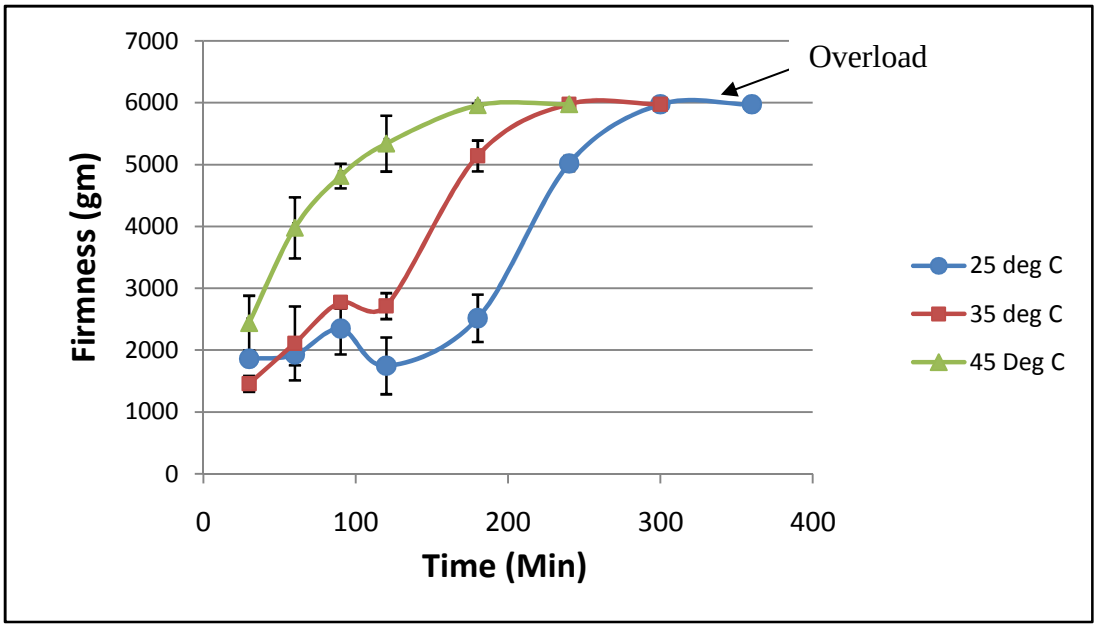


Figure 5.7 c: Change in firmness in gms of biomeal and alginate gel products with time during drying at temperatures of 25°C, 35°C and 45°C; n=3

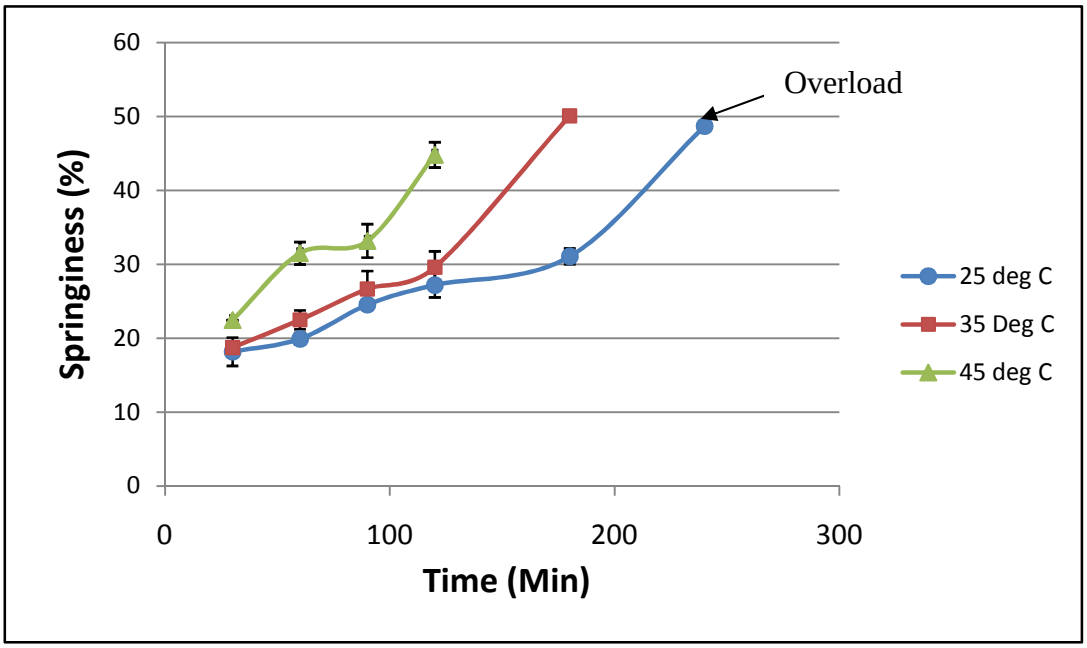


Figure 5.7d: Change in springiness of biomeal and alginate gel products with time during drying at temperatures of 25°C, 35°C and 45°C; n=3

In order to evaluate the textural shelf stability of the alginate gels, the firmness and springiness measurements were recorded over a period of 4 weeks, at the end of which excessive moisture loss caused an increase in firmness resulting in overload on the texture analysis test (Fig. 5.8). Visual observation indicated change in the texture of the gels (Fig 5.9). There was a prominent whitening of the gel surface and shrinkage of the gel products. This indicated that the biomeal-alginate gels were not shelf–stable.

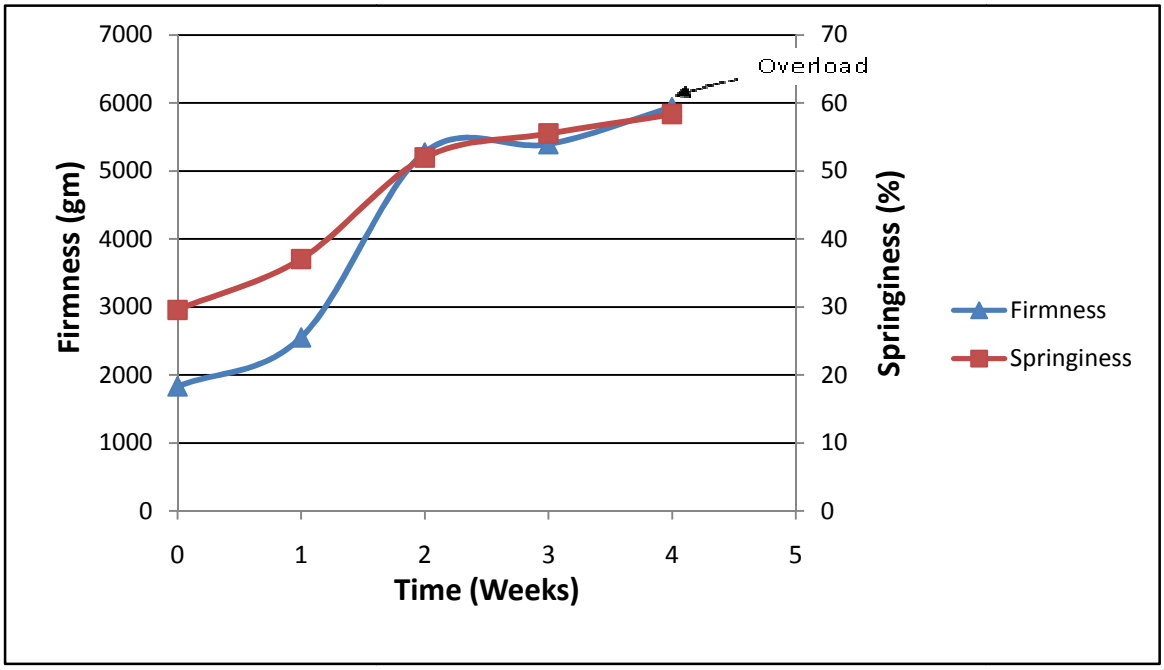


Figure 5.8: Change in firmness and springiness of alginate gels over a storage period of 4 weeks at room temperature

Week 0

Week 1

Week 2

Week 3

Week 4

Figure 5.9: Change in textural appearance of biomeal-alginate gels over a storage period of 4 weeks

5.3.2 Biomeal Pellets

Based on the aforementioned compatibility study of the biomeal with different crosslinking agents, the biomeal pellets were developed. For kibbles and cookie-like products, the most commonly measured textural attributes are their hardness and fracturability (Swanson et. al, 1999; Townsend et. al, 2005). The hardness (Fig. 5.10) of the pellets was determined while drying at three different temperatures. Higher drying temperatures resulted in higher values for hardness. Air-drying was done until the texture testing resulted in overload of the force. It was observed that a drying time of about 400 min was required for the pellets at 25°C.

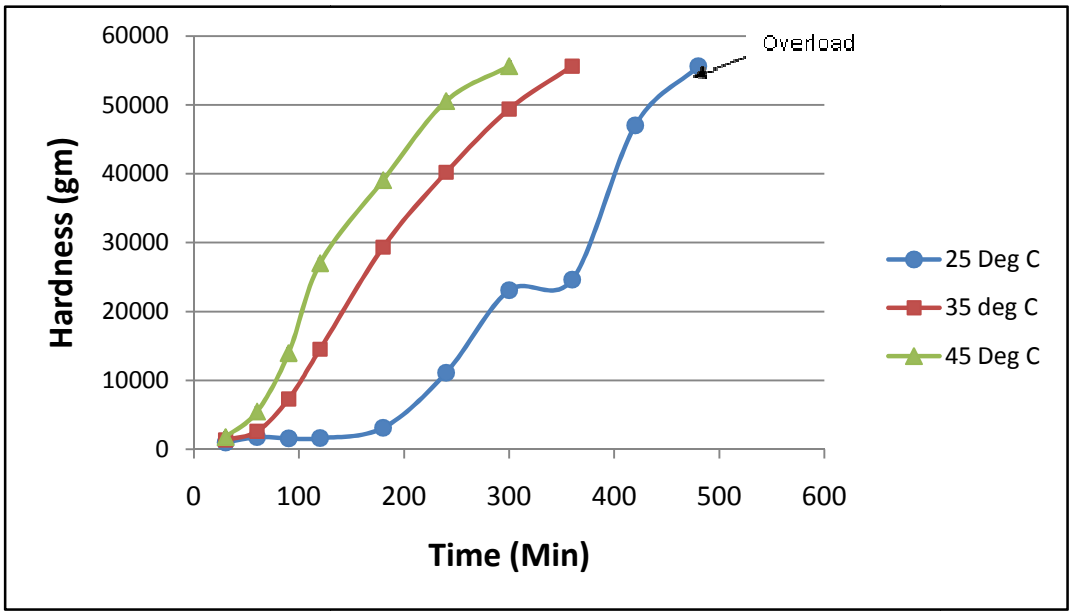


Figure 5.10: Change in hardness of biomeal pellets with time during drying at different temperatures

Along with textural attributes, water activity measurements were performed to determine drying time to ensure a sufficiently low water activity (Fig. 5.11). At room temperature, a drying time of about 10 hours was required to reduce the pellet water activity to less than 0.87, which is slightly higher than the desired water activity inhibitory of microbial growth (<0.85) (Russell & Gould,

2003). During storage, the moisture content of biomeal pellets continued to decline (Fig. 5.12), leading to pellet water activity lower than 0.85. However, such excessive moisture loss also resulted in significant increase of hardness. The pellets were found to fracture after eight weeks of storage when the hardness reached higher than 50K gm. Based on these findings, biomeal pellet is not a feasible product for further development because it lacks consistent textural attributes while the quality and shelf life stability remains a significant concern.

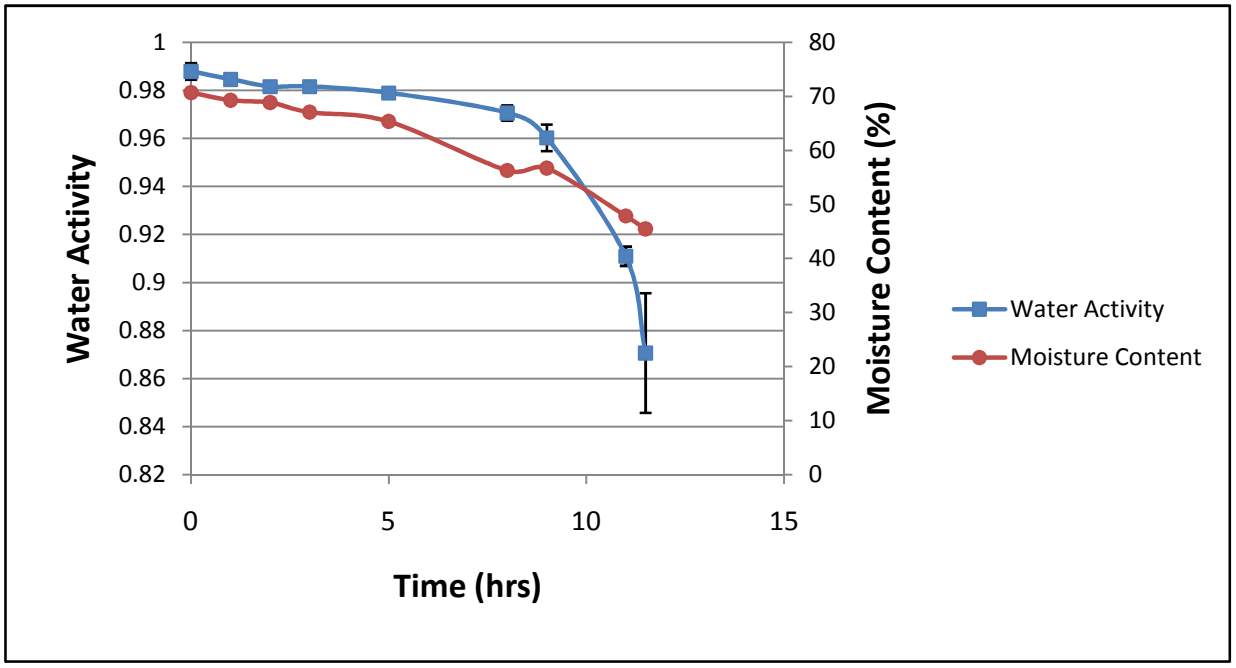


Figure 5.11: Change in water activity and moisture content of pellets over 10hrs of drying at room temperature; n=3

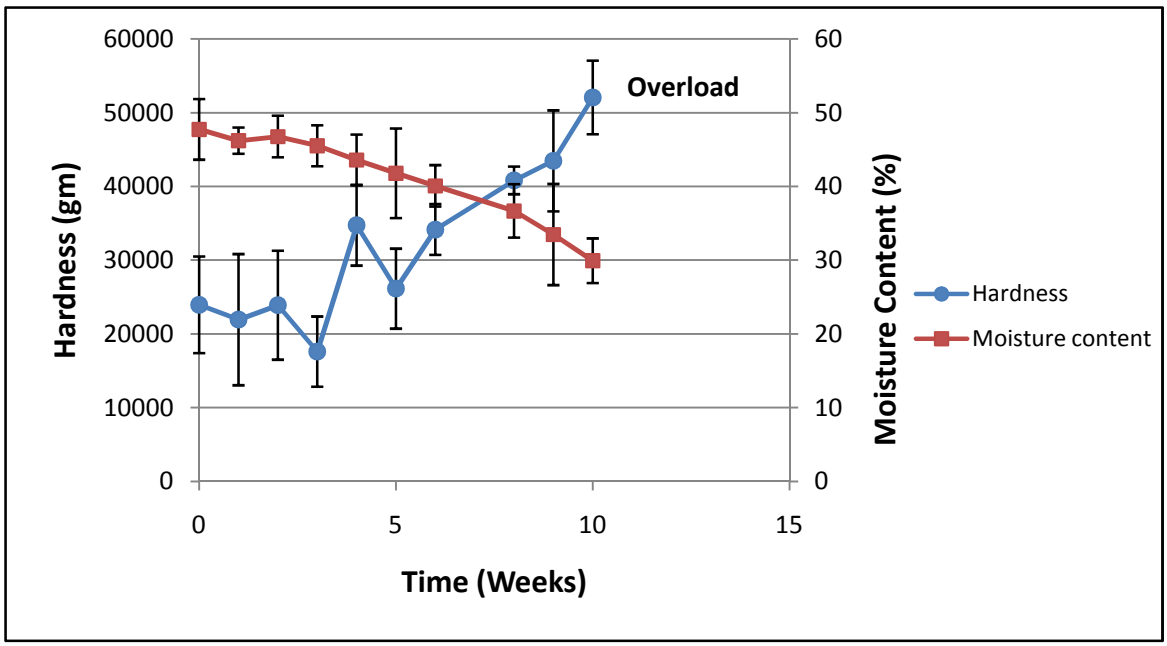


Figure 5.12: Stability of biomeal pellets in terms of hardness when stored at room temperature; n=3

5.3.3 Biomeal Sauce

Color stability

In order to evaluate the overall stability of the sauce, color measurements were done over a period of 5 weeks at different temperatures (Table 5.6). There was an increase over time in the lightness coordinate L* for the samples stored at room temperature and at higher temperatures of 35°C and 45°C. Redness coordinate a* showed a decrease in value for all samples except the samples stored at refrigeration temperature. Yellowness coordinate b* showed a slight increase in value for all samples except the samples stored at refrigeration temperature. Thus, storage at all temperatures except refrigeration temperatures caused an increase in lightness (L*), decrease in redness (a*) and increase in yellowness (b*) (Fig. 13). The pigment used in the sauce formulation was FD&C Yellow 6 (sunset yellow). This pigment has been shown to have good stability under conditions of light, pH, moderate stability in the presence of prolonged exposure

to heat and poor stability to ascorbic acid. Oxidizing, reducing agents, acids, alkalis, heat and light are few of the factors that affect the stability of color in a system. Also, the growth of micro-organisms especially molds and reducing bacteria can cause severe fading in the color of pigments (Francis, 1999). Since the biomeal sauce has exhibited microbial growth at all storage conditions except refrigeration (data indicated later), the change in color under the same conditions could be attributed to microbial contamination. This is further supported by the fact that at room temperature, biomeal sauce stabilized by the addition of 1-2% preservatives showed no significant change in color over time (Table 5.5).

Table 5.5: CIELAB color attributes, brightness (L*), red component (a*), yellow component (b*) of biomeal sauce with acetic acid before and after storage

Level of acetic acid (%)	CIELAB attribute	Week 0	Week 8
0.1	L	22.78	18.46
	a	21.26	11.13
	b	31.03	20.58
1	L	28.04	28.17
	a	23.53	22.79
	b	36.04	33.06
2	L	28.48	28.49
	a	23.53	23.32
	b	36.29	35.46

Table 5.6: CIELAB color attributes, brightness (L*), red component (a*), yellow component (b*) of biomeal sauce at different storage temperatures over a storage period of 4 weeks

Storage Temperature	CIELAB attribute	Week 0	Week 1	Week 2	Week 3	Week 4
Refrigeration temperature	L	20.08 ± 0.12^{a}	21.34 ± 0.31^{b}	20.82 ± 0.31^{c}	20.30 ± 0.16^{a}	22.26 ± 0.43^{d}
	a	19.71 ± 0.19^{a}	18.49 ± 0.38^{b}	19.20 ± 0.29^{c}	19.49 ± 0.20^{a}	18.33 ± 0.17^{d}
	b	26.91 ± 0.34^{a}	25.25 ± 0.52^{b}	26.17 ± 0.71^{a}	27.59 ± 0.25^{a}	22.63 ± 0.71^{c}
Room temperature	L	20.08 ± 0.12^{a} (20.08±0.12)	$26.3\pm0.0.49^{b}$ (25.11±1.49)	29.69 ± 0.63^{c} (28.64±1.83)	29.26 ± 0.65^{d} (28.37±1.47)	28.02 ± 1.08^{e} (27.18±1.27)
	a	19.71 ± 0.19^{a} (19.71±0.19)	17.66 ± 0.68^{b} (18.43±1.29)	13.82 ± 0.22^{c} (14.86±1.98)	14.30 ± 0.52^{d} (15.54±1.89)	15.12 ± 0.36^{e} (15.98±1.62)
	b	26.91 ± 0.34^{a} (26.91±0.34)	29.73 ± 0.66^{b} (29.34±0.54)	27.76 ± 0.79^{a} (28.12±0.72)	27.76 ± 0.79^{a} (28.30±0.67)	27.43 ± 0.58^{a} (24.56±1.58)
35°C	L	20.08 ± 0.12^{a}	28.00 ± 0.69^{b}	27.55 ± 1.66^{c}	26.48 ± 1.25^{d}	24.52 ± 0.20^{e}
	a	19.71 ± 0.19^{a}	16.34 ± 1.66^{b}	14.73 ± 1.23^{c}	14.65 ± 0.76^{d}	14.53 ± 0.12^{e}
	b	26.91 ± 0.34^{a}	29.26 ± 0.42^{b}	28.06 ± 0.54^{c}	28.11 ± 0.65^{c}	26.88 ± 0.22^{d}
45°C	L	20.08 ± 0.12^{a}	27.64 ± 0.68^{b}	28.68 ± 1.13^{c}	26.76 ± 1.85^{d}	24.45 ± 1.15^{e}
	a	19.71 ± 0.19^{a}	15.17 ± 0.41^{b}	13.46 ± 0.64^{c}	14.73 ± 0.41^{d}	14.66 ± 0.05^{e}
	b	26.91 ± 0.34^{a}	28.34 ± 0.69^{b}	27.96 ± 0.91^{a}	29.26 ± 0.95^{c}	27.18 ± 0.54^{a}

Values with the same superscript in a row are not significantly different from the control ($p>0.05$); n=4
Values in parentheses denote CIELAB attributes for samples stored in darkness at room temperature

The effect of light on the change in color was also studied. CIELAB attributes indicated that there was no significant effect of light on the color of the biomeal sauce. Samples stored in light showed similar trends in color change as the samples stored in darkness, both at room temperature.

Figure 5.13: Color of biomeal sauce after storage for 4 weeks; 1) Refrigeration Temperature 2) Room Temperature 3) Room Temperature (without light) 4) 35°C 5) 45°C

Syneresis study

The biomeal sauce showed significant syneresis of almost 50% after 2 weeks of storage at room temperature and 35°C. At refrigeration temperature, syneresis observed was lower and increased to about 45% after 3 weeks (Table 5.7). The compatibility of xanthan with biomeal and its inherent ability to impart a high viscosity and a good mouthfeel (Imeson, 1997) to the product made it the ideal hydrocolloid for use in the formulation. However, xanthan alone apparently could not prevent syneresis from occurring in the biomeal sauce. In an attempt to reduce syneresis, the use of curdlan gum was investigated. By keeping the total concentration of gums in the formulation constant, 50% of xanthan gum by weight was substituted by an equal weight of curdlan gum (ratio of xanthan gum to curdlan gum being 1:1). This complex caused a reduction in syneresis at all storage temperatures, with syneresis being the lowest at refrigeration temperature. The interaction of curdlan-xanthan complex has been shown to eliminate syneresis in food systems undergoing multiple freeze-thaw cycles (Williams et. al., 2009). The results obtained from the present study indicate that curdlan-xanthan combination could be an adequate stabilizing agent to significantly reduce syneresis.

Table 5.7: Syneresis of biomeal sauce evaluated over time at refrigeration temp, room temp and 35°C with total 0.275% hydrocolloid (w/v)

Storage Time (Weeks)	Hydrocolloid		% Water Loss		
	Xanthan gum (%)	Curdlan gum (%)	Refrigeration temperature	Room Temperature	35°C
1	0.275	0	33.33±2.88	25.00±5.00	41.66±2.88
	0.137	0.137	25.00±0.00	33.33±2.88	40.00±0.00
2	0.275	0	31.66±2.88	45.00±5.00	50.00±0.00
	0.137	0.137	25.00±0.00	36.66±2.88	31.66±7.64
3	0.275	0	41.66±2.88	51.66±2.88	50.00±0.00
	0.137	0.137	26.66±2.88	33.33±2.88	38.33±2.88
4	0.275	0	43.33±2.88	50.00±0.00	51.66±2.88
	0.137	0.137	25.00±0.00	30.00±0.00	35.00±0.00
5	0.275	0	46.66±2.88	51.66±2.88	55.00±0.00
	0.137	0.137	-	-	-
6	0.275	0	43.33±2.88	55.00±5.00	53.33±2.88
	0.137	0.137	25.00±0.00	33.33±2.88	38.33±2.88

To further lower syneresis, the overall concentration of gums in the system was increased with xanthan: curdlan concentration being 2:1 (Fig. 5.14). Since refrigeration was found most ideal for storage of the biomeal sauce product, syneresis was studied under refrigeration conditions. This complex showed a significant reduction in syneresis with only 2% water loss observed after 3 weeks of storage. The viscosity of the sauce was evaluated for each polymer combination and the use of 2:1 xanthan-curdlan combination showed a similar viscosity profile as when only xanthan gum was used in the formulation (Fig. 5.15).

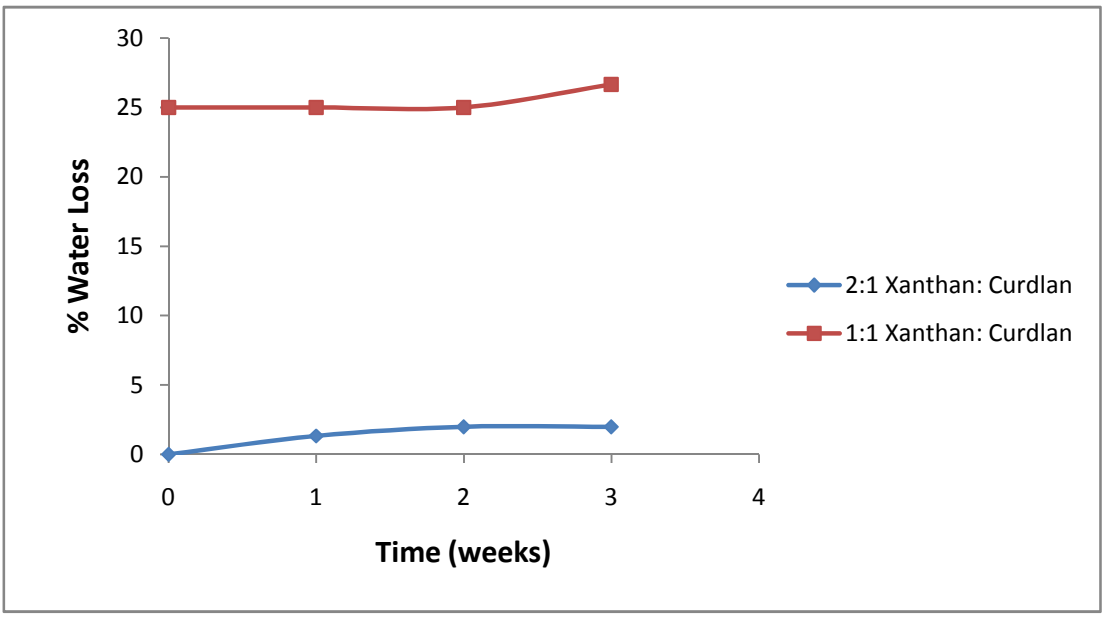


Figure 5.14: Syneresis of biomeal sauce with 1:1 xanthan-curdlan complex (0.1375% of each gum) and 2:1 xanthan-curdlan complex (0.275% xanthan and 0.1375% curdlan) at refrigeration temperature

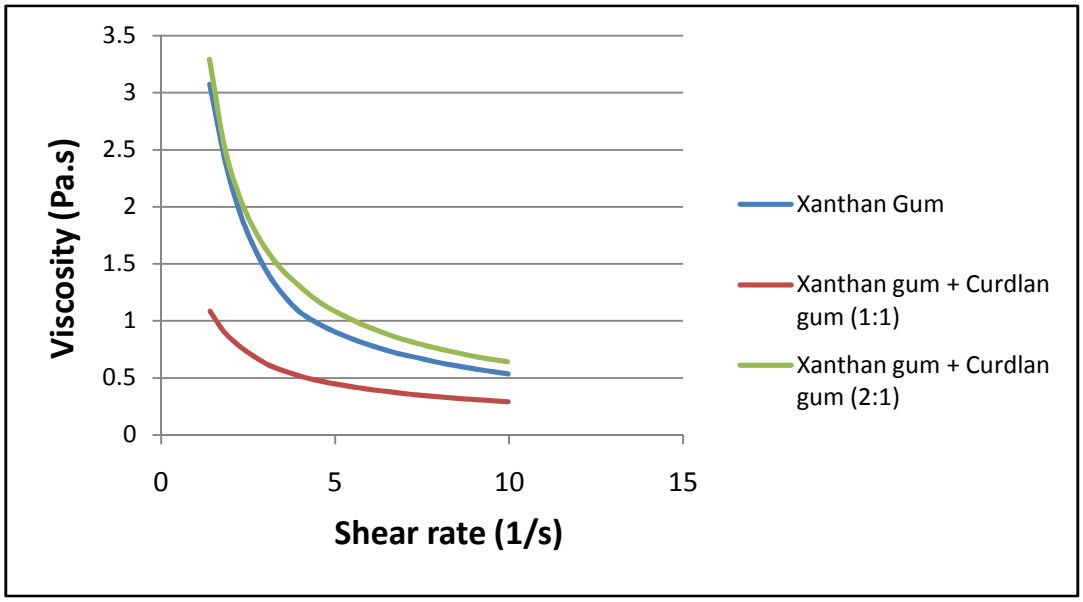


Figure 5.15: Change in viscosity with shear rate of the biomeal sauce with xanthan gum, 1:1 xanthan-curdlan complex (0.1375% of each gum) and 2:1 xanthan-curdlan complex (0.275% xanthan and 0.1375% curdlan)

Microbial analysis

Microbiological analysis of the sauce was conducted at 4 different temperatures to evaluate its stability without the addition of external preservatives. The extent of microbial growth in terms of total plate counts obtained over a period of 11 weeks at the 4 different temperatures are given in Table 5.8. There was no significant yeast and mold growth observed during the period. As the results indicated, the biomeal sauce was microbiologically unstable under all storage conditions except refrigeration. At room temperature, the sauce had a shelf–life of 2 weeks after which it showed increased microbiological activity. At higher temperatures, the sauce was stable only for a week. The low stability of the sauce could be attributed to the high pH and water activity which makes it susceptible to bacteria as well as yeast and mold spoilage (Jay et. al, 2005).

To increase the stability of the sauce without significantly altering the product, reducing pH was considered the most suitable preservation method. Weak-acid preservatives have been used to inhibit micro-organisms in food for a long time and offer an alternative to chemical additives for consumers that prefer natural foodstuffs (Lambert & Stratford, 1999). The effect of citric acid and acetic acid were investigated. In order to estimate the minimum concentration of preservative required to inhibit microbial growth, 3 levels of each were tested, namely 0.1%, 1% and 2%. The results were expressed in terms of total plate counts (TPC) and yeast and mold counts (Tables 5.9 and 5.10, respectively).

Table 5.8: Microbial load (total plate counts (TPC) as cfu/ml) of the sauce samples at refrigeration temp, room temp, 35°C and 45°C over a period of 11 weeks

Storage Time (Weeks)	Refrigeration	Room Temperature	35°C	45°C
0	<10	<10	<10	<10
1	<10	<10	260000	1700000
2	<10	870000	1730000	1780000
3	<10	>6150000 est.	1930000	1050000
4	<10	4880000 est.	2030000	2640000 est.
5	<10	4550000 est.	>7000000 est.	>2900000 est.
7	<10	3613333 est.	>7000000 est.	>1573333 est.
9	<10	>7000000 est.	>7000000 est.	>7000000 est.
11	<10	>7000000 est.	>7000000 est.	>7000000 est.

Table 5.9: Total plate counts (cfu/ml) of the sauce samples at refrigeration temp, room temp, 35°C and 45°C over a period of 5 weeks with different levels of preservatives

Time	Citric acid			Acetic acid		
(weeks)	0.1%	1%	2%	0.1%	1%	2%
0	630	15	<10	180	53	<10
1	>6000000 est.	20	<10	1670000	<10	<10
2	>6000000 est.	<10	<10	3360000 est.	30	<10
3	>6000000 est.	20	<10	1850000	25	<10
5	>6000000 est.	40	<10	>7000000 est.	100	<10

Table 5.10: Microbial load (Yeast and Mold*) of the sauce samples with different levels of citric acid and acetic acid over a period of 5 weeks

Time	Citric acid			Acetic acid		
(weeks)	0.1%	1%	2%	0.1%	1%	2%
0	20	<10	<10	10	<10	<10
1	200	<10	<10	70	<10	<10
2	390000	<10	<10	>4000000 est.	<10	<10
3	>4000000 est.	<10	<10	>4000000 est.	<10	<10
5	>4000000 est.	<10	<10	>4000000 est.	<10	<10

* The counts presented are in terms of mold colonies since there was no yeast growth detected

Both citric acid and acetic acid prevented the growth of micro-organisms at concentrations higher than 1%. Acetic acid was more effective than citric acid as a preservative as it suppressed the microbial count at a level lower than that for citric acid.

The potency of potassium sorbate as a preservative for the biomeal sauce was also evaluated. Potassium sorbate at 0.1 % inhibited any form of microbial growth during the study period of 9 weeks (Table 5.11).

Table 5.11: Microbial load (TPC and yeast and mold count as cfu/ml) of the sauce samples stored at room temperature with 0.1% of potassium sorbate over a period of 9 weeks

Time (Weeks)	TPC	Yeast and Mold count
0	<10	<10
1	<10	<10
2	<10	<10
3	<10	<10
4	<10	<10
5	<10	<10\
7	<10	<10
9	<10	<10

Chapter 6: Conclusions

Algal biomeal was studied for its various properties. Biomeal showed negligible solubility in the solvents investigated including water which led to the evaluation of its compatibility with hydrocolloids. Xanthan gum was highly compatible with the biomeal. Chromatographic analysis of the biomeal helped in the identification of the potential contributors to the biomeal flavor, namely 3-methyl-2, 5-furandione, maltol, and methyl esters of fatty acids. The biomeal was shown to add to the surface stability of xanthan gum solutions and oil-in-water emulsions.

Based on the properties evaluated, a sauce based formulation was successfully developed using the biomeal in conjunction with xanthan gum and pigment Yellow 6. This formulation was microbiologically stable at refrigeration temperature, whereas at room temperature glacial acetic acid at 2% proved to be an effective preservative. The biomeal sauce showed increased syneresis at higher temperatures as compared to refrigeration temperature. The use of curdlan gum in combination with xanthan gum effectively reduced syneresis at all temperatures, almost eliminating it at refrigeration temperature.

Thus, the development of a shelf-stable palatability enhancer using algal biomeal offer a new ingredient for the food and feed industries to improve the palatability of dry or low-moisture products, the ability to produce a value-added ingredient also offers a viable option for algal biomeal. The limitation to the use of this ingredient is the lack of data on its possible toxicity and digestibility. Further studies would focus on these aspects of research and also increasing the stability of the products, especially dry biomeal products.

Appendix A: Statistical Analysis

Comparison of L-values by Dunnett's test for different temperatures:

One-way ANOVA: control-l-RT, wk1, wk2, wk3, wk4

Source DF SS MS F P

Factor 4 245.013 61.253 134.40 0.000
Error 15 6.836 0.456
Total 19 251.850

S = 0.6751 R-Sq = 97.29% R-Sq(adj) = 96.56%

Individual 95% CIs For Mean Based on Pooled StDev

```
Level        N    Mean  StDev -----+---------+---------+---------+----
control-l-RT 4  20.080  0.109  (-*-)
wk1          4  26.305  0.499              (--*-)
wk2          4  29.692  0.636                    (-*-)
wk3          4  29.265  0.659                   (--*-)
wk4          4  28.020  1.086                (-*--)
                                -----+---------+---------+---------+----
                                  21.0      24.0      27.0      30.0
```

Pooled StDev = 0.675

Dunnett's comparisons with a control

Family error rate = 0.05
Individual error rate = 0.0156

Critical value = 2.73
Control = control-l-RT
Intervals for treatment mean minus control mean

```
Level  Lower  Center  Upper -------+---------+---------+---------+--
wk1    4.923  6.225  7.527  (--------*-------)
wk2    8.311  9.613 10.914                (--------*--------)
wk3    7.883  9.185 10.487                (-------*--------)
wk4    6.638  7.940  9.242          (--------*--------)
                               -------+---------+---------+---------+--
                                   6.0       7.5       9.0      10.5
```

One-way ANOVA: control-l-RT, Lwk1-Ref T, ref wk2, ref wk3, ref wk4

Source	DF	SS	MS	F	P
Factor	4	12.3210	3.0802	36.32	0.000
Error	15	1.2721	0.0848		
Total	19	13.5931			

S = 0.2912 R-Sq = 90.64% R-Sq(adj) = 88.15%

Individual 95% CIs For Mean Based on Pooled StDev

Level	N	Mean	StDev	---+---------+---------+---------+------
control-l-RT	4	20.080	0.109	(---*---)
Lwk1-Ref T	4	21.340	0.317	(---*---)
ref wk2	4	20.820	0.314	(---*---)
ref wk3	4	20.303	0.164	(---*---)
ref wk4	4	22.267	0.432	(---*---)
				---+---------+---------+---------+------
				20.00 20.80 21.60 22.40

Pooled StDev = 0.291

Dunnett's comparisons with a control

Family error rate = 0.05
Individual error rate = 0.0156

Critical value = 2.73

Control = control-l-RT

Intervals for treatment mean minus control mean

Level	Lower	Center	Upper	----+---------+---------+---------+-----
Lwk1-Ref T	0.6984	1.2600	1.8216	(------*------)
ref wk2	0.1784	0.7400	1.3016	(------*------)
ref wk3	-0.3391	0.2225	0.7841	(------*------)
ref wk4	1.6259	2.1875	2.7491	(------*------)
				----+---------+---------+---------+-----
				0.00 0.80 1.60 2.40

One-way ANOVA: control-l-RT, L wk1 35, wk 2 35, wk 3 35, w 4 35

Source	DF	SS	MS	F	P
Factor	4	166.422	41.605	42.69	0.000
Error	15	14.619	0.975		
Total	19	181.040			

S = 0.9872 R-Sq = 91.93% R-Sq(adj) = 89.77%

Individual 95% CIs For Mean Based on Pooled StDev

Level	N	Mean	StDev	----------+---------+---------+---------+--
control-l-RT	4	20.080	0.109	(---*--)
L wk1 35	4	28.000	0.693	(--*---)
wk 2 35	4	27.553	1.662	(---*--)
wk 3 35	4	26.480	1.256	(--*---)
w 4 35	4	24.523	0.203	(---*--)

----------+---------+---------+---------+--
21.0 24.0 27.0 30.0

Pooled StDev = 0.987

Dunnett's comparisons with a control

Family error rate = 0.05
Individual error rate = 0.0156

Critical value = 2.73

Control = control-l-RT

Intervals for treatment mean minus control mean

Level	Lower	Center	Upper	----------+---------+---------+---------+--
L wk1 35	6.0161	7.9200	9.8239	(---------*---------)
wk 2 35	5.5686	7.4725	9.3764	(---------*---------)
wk 3 35	4.4961	6.4000	8.3039	(---------*---------)
w 4 35	2.5386	4.4425	6.3464	(---------*---------)

----------+---------+---------+---------+--
4.0 6.0 8.0 10.0

One-way ANOVA: control-l-RT, L wk 1 45, L wk 2 45, L wk3 45, L wk4 45

Source	DF	SS	MS	F	P
Factor	4	187.20	46.80	35.77	0.000
Error	15	19.62	1.31		
Total	19	206.82			

S = 1.144 R-Sq = 90.51% R-Sq(adj) = 87.98%

Individual 95% CIs For Mean Based on Pooled StDev

Level	N	Mean	StDev	----------+---------+---------+---------+--
control-l-RT	4	20.080	0.109	(---*---)
L wk 1 45	4	27.643	0.689	(---*---)
L wk 2 45	4	28.685	1.136	(---*---)
L wk3 45	4	26.765	1.855	(---*---)
L wk4 45	4	24.455	1.151	(----*---)

----------+---------+---------+---------+--
21.0 24.0 27.0 30.0

Pooled StDev = 1.144

Dunnett's comparisons with a control

Family error rate = 0.05
Individual error rate = 0.0156

Critical value = 2.73

Control = control-l-RT

Intervals for treatment mean minus control mean

Level	Lower	Center	Upper	-+---------+---------+---------+--------
L wk 1 45	5.357	7.563	9.768	(--------*--------)
L wk 2 45	6.399	8.605	10.811	(--------*--------)
L wk3 45	4.479	6.685	8.891	(--------*--------)
L wk4 45	2.169	4.375	6.581	(--------*--------)

-+---------+---------+---------+--------
2.5 5.0 7.5 10.0

Comparison of a-values by Dunnett's test for different temperatures:

One-way ANOVA: control-a, wk 1 Rt, wk 2 Rt, wk 3Rt, wk 4RT

Source	DF	SS	MS	F	P
Factor	4	99.425	24.856	130.29	0.000
Error	15	2.862	0.191		
Total	19	102.286			

S = 0.4368 R-Sq = 97.20% R-Sq(adj) = 96.46%

Individual 95% CIs For Mean Based on Pooled StDev

Level	N	Mean	StDev	---+---------+---------+---------+------
control-a	4	19.715	0.193	(--*-)
wk 1 Rt	4	17.663	0.681	(-*--)
wk 2 Rt	4	13.828	0.220	(-*-)
wk 3Rt	4	14.300	0.522	(--*-)
wk 4RT	4	15.128	0.363	(--*-)

---+---------+---------+---------+------
14.0 16.0 18.0 20.0

Pooled StDev = 0.437

Dunnett's comparisons with a control

Family error rate = 0.05
Individual error rate = 0.0156

Critical value = 2.73

Control = control-a

Intervals for treatment mean minus control mean

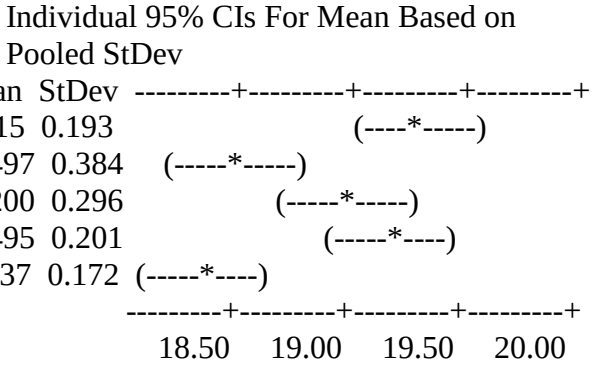
Level	Lower	Center	Upper	-----+---------+---------+---------+----
wk 1 Rt	-2.8949	-2.0525	-1.2101	(----*-----)
wk 2 Rt	-6.7299	-5.8875	-5.0451	(-----*----)
wk 3Rt	-6.2574	-5.4150	-4.5726	(-----*-----)
wk 4RT	-5.4299	-4.5875	-3.7451	(----*-----)

-----+---------+---------+---------+----
-6.0 -4.5 -3.0 -1.5

One-way ANOVA: control-a, wk 1 Ref, wk 2 Ref, wk 3 Ref, Wk 4 ref

Source	DF	SS	MS	F	P
Factor	4	5.9026	1.4757	21.52	0.000
Error	15	1.0284	0.0686		
Total	19	6.9310			

S = 0.2618 R-Sq = 85.16% R-Sq(adj) = 81.21%



Pooled StDev = 0.262

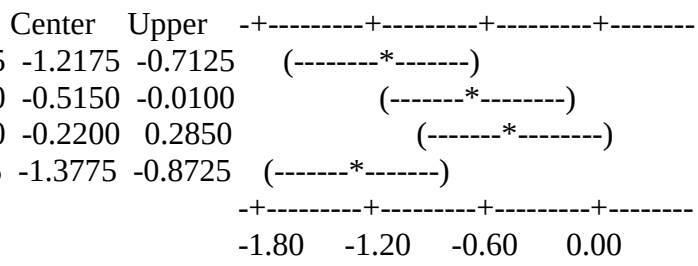
Dunnett's comparisons with a control

Family error rate = 0.05
Individual error rate = 0.0156

Critical value = 2.73

Control = control-a

Intervals for treatment mean minus control mean



One-way ANOVA: control-a, wk 1 35, wk 2 35, wk 3 35, w 4 35

Source	DF	SS	MS	F	P
Factor	4	77.984	19.496	19.72	0.000
Error	15	14.832	0.989		
Total	19	92.816			

S = 0.9944 R-Sq = 84.02% R-Sq(adj) = 79.76%

Individual 95% CIs For Mean Based on Pooled StDev

Level	N	Mean	StDev	---+---------+---------+---------+------
control-a	4	19.715	0.193	(-----*----)
wk 1 35	4	16.345	1.669	(-----*----)
wk 2 35	4	14.733	1.234	(-----*----)
wk 3 35	4	14.653	0.764	(----*-----)
w 4 35	4	14.533	0.126	(-----*----)

---+---------+---------+---------+------
14.0 16.0 18.0 20.0

Pooled StDev = 0.994

Dunnett's comparisons with a control

Family error rate = 0.05
Individual error rate = 0.0156

Critical value = 2.73

Control = control-a

Intervals for treatment mean minus control mean

Level	Lower	Center	Upper	-------+---------+---------+---------+--
wk 1 35	-5.2878	-3.3700	-1.4522	(----------*----------)
wk 2 35	-6.9003	-4.9825	-3.0647	(----------*----------)
wk 3 35	-6.9803	-5.0625	-3.1447	(----------*----------)
w 4 35	-7.1003	-5.1825	-3.2647	(----------*----------)

-------+---------+---------+---------+--
-6.0 -4.5 -3.0 -1.5

One-way ANOVA: control-a, wk 1 45, wk 2 45, wk 3 45, wk 4 45

Source	DF	SS	MS	F	P
Factor	4	93.170	23.293	144.48	0.000
Error	15	2.418	0.161		
Total	19	95.589			

S = 0.4015 R-Sq = 97.47% R-Sq(adj) = 96.80%

Individual 95% CIs For Mean Based on Pooled StDev

Level	N	Mean	StDev	-----+---------+---------+---------+----
control-a	4	19.715	0.193	(--*-)
wk 1 45	4	15.175	0.417	(-*-)
wk 2 45	4	13.463	0.647	(-*-)
wk 3 45	4	14.733	0.416	(-*-)
wk 4 45	4	14.668	0.054	(-*-)

-----+---------+---------+---------+----
14.0 16.0 18.0 20.0

Pooled StDev = 0.402

Dunnett's comparisons with a control

Family error rate = 0.05
Individual error rate = 0.0156

Critical value = 2.73

Control = control-a

Intervals for treatment mean minus control mean

Level	Lower	Center	Upper	+---------+---------+---------+---------
wk 1 45	-5.3143	-4.5400	-3.7657	(-------*------)
wk 2 45	-7.0268	-6.2525	-5.4782	(------*-------)
wk 3 45	-5.7568	-4.9825	-4.2082	(-------*-------)
wk 4 45	-5.8218	-5.0475	-4.2732	(-------*------)

+---------+---------+---------+---------
-7.0 -6.0 -5.0 -4.0

Comparison of b-values by Dunnett's test at different temperatures:

One-way ANOVA: control b, wk 1 Rt, wk 2 Rt, wk 3 Rt, wk 4 Rt

Source	DF	SS	MS	F	P
Factor	4	18.380	4.595	13.14	0.000
Error	15	5.246	0.350		
Total	19	23.627			

S = 0.5914 R-Sq = 77.79% R-Sq(adj) = 71.87%

Individual 95% CIs For Mean Based on Pooled StDev

Level	N	Mean	StDev	-+---------+---------+---------+--------
control b	4	26.913	0.342	(----*-----)
wk 1 Rt	4	29.738	0.665	(----*----)
wk 2 Rt	4	27.768	0.795	(----*-----)
wk 3 Rt	4	27.802	0.463	(-----*----)
wk 4 Rt	4	27.430	0.586	(-----*----)

-+---------+---------+---------+--------
26.4 27.6 28.8 30.0

Pooled StDev = 0.591

Dunnett's comparisons with a control

Family error rate = 0.05
Individual error rate = 0.0156

Critical value = 2.73

Control = control b

Intervals for treatment mean minus control mean

Level	Lower	Center	Upper	-----+---------+---------+---------+----
wk 1 Rt	1.6844	2.8250	3.9656	(---------*--------)
wk 2 Rt	-0.2856	0.8550	1.9956	(--------*---------)
wk 3 Rt	-0.2506	0.8900	2.0306	(--------*---------)
wk 4 Rt	-0.6231	0.5175	1.6581	(--------*---------)

-----+---------+---------+---------+----
0.0 1.2 2.4 3.6

One-way ANOVA: control b, wk 1 ref, wk 2 ref, wk 3 ref, wk 4 ref

Source	DF	SS	MS	F	P
Factor	4	59.772	14.943	51.37	0.000
Error	15	4.363	0.291		
Total	19	64.135			

S = 0.5393 R-Sq = 93.20% R-Sq(adj) = 91.38%

Individual 95% CIs For Mean Based on Pooled StDev

Level	N	Mean	StDev	--+---------+---------+---------+-------
control b	4	26.913	0.342	(--*---)
wk 1 ref	4	25.248	0.526	(---*--)
wk 2 ref	4	26.170	0.714	(---*--)
wk 3 ref	4	27.608	0.211	(---*--)
wk 4 ref	4	22.633	0.712	(--*---)
				--+---------+---------+---------+-------
				22.4 24.0 25.6 27.2

Pooled StDev = 0.539

Dunnett's comparisons with a control

Family error rate = 0.05
Individual error rate = 0.0156

Critical value = 2.73

Control = control b

Intervals for treatment mean minus control mean

Level	Lower	Center	Upper	-------+---------+---------+---------+--
wk 1 ref	-2.7051	-1.6650	-0.6249	(-----*----)
wk 2 ref	-1.7826	-0.7425	0.2976	(----*----)
wk 3 ref	-0.3451	0.6950	1.7351	(----*-----)
wk 4 ref	-5.3201	-4.2800	-3.2399	(-----*----)
				-------+---------+---------+---------+--
				-4.0 -2.0 0.0 2.0

One-way ANOVA: control b, wk 1 35, wk 2 35, wk 3 35, wk 4 35

Source	DF	SS	MS	F	P
Factor	4	15.764	3.941	18.39	0.000
Error	15	3.215	0.214		
Total	19	18.978			

S = 0.4629 R-Sq = 83.06% R-Sq(adj) = 78.54%

Individual 95% CIs For Mean Based on Pooled StDev

Level	N	Mean	StDev	------+---------+---------+---------+---
control b	4	26.913	0.342	(----*----)
wk 1 35	4	29.265	0.420	(----*----)
wk 2 35	4	28.067	0.545	(----*----)
wk 3 35	4	28.118	0.658	(----*----)
wk 4 35	4	26.880	0.220	(----*----)
				------+---------+---------+---------+---
				27.0 28.0 29.0 30.0

Pooled StDev = 0.463

Dunnett's comparisons with a control

Family error rate = 0.05
Individual error rate = 0.0156

Critical value = 2.73

Control = control b

Intervals for treatment mean minus control mean

Level	Lower	Center	Upper	--------+---------+---------+---------+-
wk 1 35	1.4597	2.3525	3.2453	(-------*------)
wk 2 35	0.2622	1.1550	2.0478	(-------*------)
wk 3 35	0.3122	1.2050	2.0978	(------*------)
wk 4 35	-0.9253	-0.0325	0.8603	(-------*------)
				--------+---------+---------+---------+-
				0.0 1.2 2.4 3.6

One-way ANOVA: control b, wk 1 45, wk 2 45, wk 3 45, wk 4 45

Source	DF	SS	MS	F	P
Factor	4	14.203	3.551	6.73	0.003
Error	15	7.909	0.527		
Total	19	22.112			

S = 0.7261 R-Sq = 64.23% R-Sq(adj) = 54.69%

Individual 95% CIs For Mean Based on Pooled StDev

Level	N	Mean	StDev	---------+---------+---------+---------+
control b	4	26.913	0.342	(-------*-------)
wk 1 45	4	28.348	0.695	(------*-------)
wk 2 45	4	27.962	0.912	(-------*------)
wk 3 45	4	29.268	0.957	(-------*------)
wk 4 45	4	27.188	0.538	(-------*-------)
				---------+---------+---------+---------+
				27.0 28.0 29.0 30.0

Pooled StDev = 0.726

Dunnett's comparisons with a control

Family error rate = 0.05
Individual error rate = 0.0156

Critical value = 2.73

Control = control b

Intervals for treatment mean minus control mean

Level	Lower	Center	Upper	--------+---------+---------+---------+-
wk 1 45	0.0346	1.4350	2.8354	(---------*--------)
wk 2 45	-0.3504	1.0500	2.4504	(--------*--------)
wk 3 45	0.9546	2.3550	3.7554	(---------*--------)
wk 4 45	-1.1254	0.2750	1.6754	(---------*--------)
				--------+---------+---------+---------+-
				0.0 1.5 3.0 4.5

Comparison of L-values by Tukey's test to study the effect of light:

One-way ANOVA: wk1, wk1-l

Source DF SS MS F P
Factor 1 2.83 2.83 2.27 0.183
Error 6 7.49 1.25
Total 7 10.32

S = 1.117 R-Sq = 27.44% R-Sq(adj) = 15.34%

Individual 95% CIs For Mean Based on Pooled StDev

```
Level  N   Mean  StDev  ---+---------+---------+---------+------
wk1    4 26.305  0.499           (-------------*-------------)
wk1-l  4 25.115  1.499  (-------------*-------------)
                        ---+---------+---------+---------+------
                          24.0      25.0      26.0      27.0
```

Pooled StDev = 1.117

Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons

Individual confidence level = 95.00%

wk1 subtracted from:

```
      Lower  Center  Upper   -+---------+---------+---------+--------
wk1-l -3.123 -1.190  0.743   (------------*------------)
                             -+---------+---------+---------+--------
                               -3.0      -1.5       0.0       1.5
```

One-way ANOVA: wk2, wk2-l

Source DF SS MS F P
Factor 1 2.21 2.21 1.17 0.322
Error 6 11.34 1.89
Total 7 13.54

S = 1.375 R-Sq = 16.28% R-Sq(adj) = 2.33%

```
                    Individual 95% CIs For Mean Based on
                    Pooled StDev
Level  N    Mean  StDev  -----+---------+---------+---------+----
wk2    4  29.692  0.636         (-------------*-------------)
wk2-l  4  28.642  1.837  (-------------*-------------)
                          -----+---------+---------+---------+----
                             27.6      28.8      30.0      31.2
```

Pooled StDev = 1.375

Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons

Individual confidence level = 95.00%

wk2 subtracted from:

```
      Lower  Center  Upper  ---+---------+---------+---------+------
wk2-l -3.429  -1.050  1.329  (---------------*---------------)
                             ---+---------+---------+---------+------
                              -3.0      -1.5       0.0       1.5
```

One-way ANOVA: wk3, wk3-l

Source	DF	SS	MS	F	P
Factor	1	1.58	1.58	1.21	0.314
Error	6	7.86	1.31		
Total	7	9.44			

S = 1.144 R-Sq = 16.78% R-Sq(adj) = 2.91%

```
                    Individual 95% CIs For Mean Based on Pooled StDev
Level  N    Mean  StDev    +---------+---------+---------+---------
wk3    4  29.265  0.659           (-------------*-------------)
wk3-l  4  28.375  1.478    (-------------*-------------)
                           +---------+---------+---------+---------
                             27.0      28.0      29.0      30.0
```

Pooled StDev = 1.144

Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons

Individual confidence level = 95.00%

wk3 subtracted from:

```
      Lower  Center  Upper  ---------+---------+---------+---------+
wk3-l -2.870  -0.890  1.090  (------------*-----------)
                             ---------+---------+---------+---------+
                                 -1.5       0.0       1.5       3.0
```

One-way ANOVA: wk4, wk4-l

Source	DF	SS	MS	F	P
Factor	1	1.39	1.39	0.99	0.359
Error	6	8.44	1.41		
Total	7	9.83			

S = 1.186 R-Sq = 14.11% R-Sq(adj) = 0.00%

```
                    Individual 95% CIs For Mean Based on
                    Pooled StDev
Level  N    Mean  StDev  ---+---------+---------+---------+------
wk4    4  28.020  1.086          (-------------*--------------)
wk4-l  4  27.188  1.279  (--------------*-------------)
                          ---+---------+---------+---------+------
                            26.0      27.0      28.0      29.0
```

Pooled StDev = 1.186

Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons

Individual confidence level = 95.00%

wk4 subtracted from:

```
      Lower  Center  Upper  ---------+---------+---------+---------+
wk4-l -2.885  -0.832  1.220  (------------*-------------)
                             ---------+---------+---------+---------+
                                 -1.5       0.0       1.5       3.0
```

Comparison of a-values by Tukey's test to study the effect of light:

One-way ANOVA: wk 1 Rt, wk1-l

Source DF SS MS F P
Factor 1 1.19 1.19 1.11 0.332
Error 6 6.43 1.07
Total 7 7.62

S = 1.035 R-Sq = 15.66% R-Sq(adj) = 1.60%

Individual 95% CIs For Mean Based on Pooled StDev

```
Level    N   Mean  StDev ------+---------+---------+---------+---
wk 1 Rt  4  17.663  0.681  (-----------*-----------)
wk1-l    4  18.435  1.296        (-----------*-----------)
                          ------+---------+---------+---------+---
                             17.0      18.0      19.0      20.0
```

Pooled StDev = 1.035

Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons

Individual confidence level = 95.00%

wk 1 Rt subtracted from:

```
      Lower  Center  Upper  -----+---------+---------+---------+----
wk1-l -1.019   0.772  2.564        (-------------*--------------)
                               -----+---------+---------+---------+----
                                  -1.2       0.0       1.2       2.4
```

One-way ANOVA: wk 2 Rt, wk2-l

Source DF SS MS F P
Factor 1 2.14 2.14 1.07 0.341
Error 6 12.02 2.00
Total 7 14.16

S = 1.415 R-Sq = 15.13% R-Sq(adj) = 0.98%

Individual 95% CIs For Mean Based on Pooled StDev

```
Level    N    Mean  StDev  ---------+---------+---------+---------+
wk 2 Rt  4  13.828  0.220  (----------*----------)
wk2-l    4  14.863  1.990         (----------*----------)
                               ---------+---------+---------+---------+
                                    13.2      14.4      15.6      16.8
```

Pooled StDev = 1.415

Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons

Individual confidence level = 95.00%

wk 2 Rt subtracted from:

```
      Lower  Center  Upper  ------+---------+---------+---------+---
wk2-l -1.414   1.035  3.484        (---------------*---------------)
                               ------+---------+---------+---------+---
                                  -1.5       0.0       1.5       3.0
```

One-way ANOVA: wk 3Rt, wk3-l

Source	DF	SS	MS	F	P
Factor	1	3.08	3.08	1.60	0.253
Error	6	11.55	1.92		
Total	7	14.62			

S = 1.387 R-Sq = 21.03% R-Sq(adj) = 7.87%

Individual 95% CIs For Mean Based on Pooled StDev

```
Level    N    Mean  StDev  -----+---------+---------+---------+----
wk 3Rt   4  14.300  0.522  (----------*----------)
wk3-l    4  15.540  1.891         (----------*----------)
                               -----+---------+---------+---------+----
                                  13.2      14.4      15.6      16.8
```

Pooled StDev = 1.387

Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons

Individual confidence level = 95.00%

wk 3Rt subtracted from:

```
      Lower  Center  Upper  -----+---------+---------+---------+----
wk3-l -1.160  1.240  3.640         (--------------*--------------)
                                -----+---------+---------+---------+----
                                   -1.6       0.0       1.6       3.2
```

One-way ANOVA: wk 4RT, wk4-l

Source	DF	SS	MS	F	P
Factor	1	1.47	1.47	1.06	0.342
Error	6	8.31	1.38		
Total	7	9.78			

S = 1.177 R-Sq = 15.04% R-Sq(adj) = 0.88%

Individual 95% CIs For Mean Based on Pooled StDev

```
Level   N    Mean  StDev  ---+---------+---------+---------+------
wk 4RT  4  15.128  0.363  (-------------*--------------)
wk4-l   4  15.985  1.624         (--------------*-------------)
                           ---+---------+---------+---------+------
                             14.0      15.0      16.0      17.0
```

Pooled StDev = 1.177

Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons

Individual confidence level = 95.00%

wk 4RT subtracted from:

```
      Lower  Center  Upper  ----+---------+---------+---------+-----
wk4-l  -1.178   0.857  2.893       (-------------*------------)
                                ----+---------+---------+---------+-----
                                  -1.5       0.0       1.5       3.0
```

Comparison of b-values by Tukey's test to study the effect of light:

One-way ANOVA: wk 1 Rt, wk1-l

Source	DF	SS	MS	F	P
Factor	1	0.304	0.304	0.82	0.400
Error	6	2.228	0.371		
Total	7	2.532			

S = 0.6093 R-Sq = 12.02% R-Sq(adj) = 0.00%

```
                    Individual 95% CIs For Mean Based on
                    Pooled StDev
Level    N    Mean  StDev  --------+---------+---------+---------+-
wk 1 Rt  4  29.738  0.665         (--------------*--------------)
wk1-l    4  29.348  0.548  (--------------*--------------)
                           --------+---------+---------+---------+-
                                29.00     29.50     30.00     30.50
```

Pooled StDev = 0.609

Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons

Individual confidence level = 95.00%

wk 1 Rt subtracted from:

```
      Lower   Center   Upper   -+---------+---------+---------+--------
wk1-l  -1.4442  -0.3900  0.6642    (--------------*--------------)
                                -+---------+---------+---------+--------
                                  -1.40     -0.70      0.00      0.70
```

One-way ANOVA: wk 2 Rt, wk2-l

Source DF SS MS F P
Factor 1 0.252 0.252 0.44 0.533
Error 6 3.466 0.578
Total 7 3.718

S = 0.7600 R-Sq = 6.78% R-Sq(adj) = 0.00%

Individual 95% CIs For Mean Based on Pooled StDev

```
Level    N    Mean  StDev  ---+---------+---------+---------+------
wk 2 Rt  4  27.768  0.795  (---------------*--------------)
wk2-l    4  28.123  0.723        (---------------*--------------)
                             ---+---------+---------+---------+------
                             27.00     27.60     28.20     28.80
```

Pooled StDev = 0.760

Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons

Individual confidence level = 95.00%

wk 2 Rt subtracted from:

```
       Lower  Center   Upper  ------+---------+---------+---------+---
wk2-l  -0.9600  0.3550  1.6700      (---------------*----------------)
                                 ------+---------+---------+---------+---
                                   -0.80      0.00      0.80      1.60
```

One-way ANOVA: wk 3 Rt, wk3-l

Source DF SS MS F P
Factor 1 0.500 0.500 1.49 0.268
Error 6 2.012 0.335
Total 7 2.512

S = 0.5790 R-Sq = 19.91% R-Sq(adj) = 6.56%

```
                    Individual 95% CIs For Mean Based on
                    Pooled StDev
Level    N    Mean  StDev --------+---------+---------+---------+-
wk 3 Rt  4  27.802  0.463  (-------------*-------------)
wk3-l    4  28.303  0.676           (-------------*-------------)
                               --------+---------+---------+---------+-
                                  27.50     28.00     28.50     29.00
```

Pooled StDev = 0.579

Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons

Individual confidence level = 95.00%

wk 3 Rt subtracted from:

```
       Lower  Center   Upper  ----+---------+---------+---------+-----
wk3-l  -0.5019  0.5000  1.5019        (-------------*-------------)
                                ----+---------+---------+---------+-----
                                  -0.70      0.00      0.70      1.40
```

One-way ANOVA: wk 4 Rt, wk4-l

Source	DF	SS	MS	F	P
Factor	1	0.86	0.86	0.60	0.468
Error	6	8.60	1.43		
Total	7	9.46			

S = 1.197   R-Sq = 9.07%   R-Sq(adj) = 0.00%

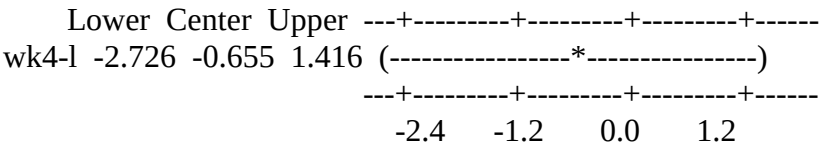
```
                    Individual 95% CIs For Mean Based on
                    Pooled StDev
Level    N    Mean  StDev -------+---------+---------+---------+--
wk 4 Rt  4  27.430  0.586        (-------------*-------------)
wk4-l    4  26.775  1.588  (-------------*-------------)
                               -------+---------+---------+---------+--
                                  26.0      27.0      28.0      29.0
```

Pooled StDev = 1.197

Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons

Individual confidence level = 95.00%

wk 4 Rt subtracted from:



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