BIOTECHNOLOGICAL APPROACH FOR THE REMOVAL

OF

GREEN PIGMENTS FROM CANOLA OIL

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirement of the degree of Master of Science

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McGill University

Montreal, Canada

January, 2003

Your file Votre référence
ISBN: 0-612-88156-3
Our file Notre référence
ISBN: 0-612-88156-3

SHORT TITLE

BIOCATALYSIS OF CHLOROPHYLLASE IN CANOLA OIL

ABSTRACT

M.Sc. Marianne Bitar

Optimization of the culture conditions for the biomass production of the alga *Phaeodactylum tricornutum* was investigated in relation to chlorophyllase activity. To obtain the highest total chlorophyllase activity, the biomass of *P. tricornutum* was harvested after a period of 7 days of incubation during which the incubation temperature was maintained at 18°C for 18 h during the day and 10°C for 6 h during the night. During culture incubation, illumination was provided by fluorescent lamps projecting an incident intensity of 330 $\mu mol/m^2.s$ and the pH of the culture was maintained at 8.4, adjusted by a stream flow of CO_2. The hydrolytic activity of a partially purified chlorophyllase extract, obtained from the fresh biomass of *P. tricornutum*, was investigated in an aqueous/miscible organic solvent system containing refined bleached deodorized (RBD) canola oil, and chlorophyll or pheophytin as substrate. The effect of a wide range of oil contents, chlorophyll and pheophytin concentrations, acetone concentrations, incubation temperatures and agitation speeds on the enzyme activity was studied. The optimum reaction conditions for chlorophyllase biocatalysis were determined to consist of 20 % oil, 10 % acetone and a 200 rpm agitation speed with optimum temperatures and substrate concentrations of 35°C and 12.6 μM for chlorophyll, and 30°C and 9.3 μM for pheophytin. The presence of RBD canola oil showed an inhibitory effect on chlorophyllase activity whereas acetone acted as an activator at low concentrations and an inhibitor at higher ones. Moreover, chlorophyllase showed a limited affinity towards pheophytin as substrate compared to that obtained for chlorophyll. Selected samples of crude commercial canola oil were analyzed for their green pigment content using high-performance liquid chromatography and chlorophyllase biocatalysis was investigated on eight varieties of crude commercial canola oil. The chlorophyllase activity was lower in the presence of crude commercial canola oil compared to that obtained in the presence of RBD canola oil, due to impurities found in crude canola oil.

RÉSUMÉ

M.Sc. Marianne Bitar

Nous avons optimisé les conditions de la culture de l'algue *Phaeodactylum tricornutum* en termes de biomasse et d`activité chlorophyllase. Pour obtenir une activité chlorophyllase maximale, l`algue *P. tricornutum* a été cultivée pendant 7 jours à une température de 18°C pendant le jour (18 heures) et de 10°C pendant le soir (6 heures) et en présence d`une illumination par des lampes fluorescentes projetant une intensité d'incident de 330 μmol/$m^2$.sec. Le pH du milieu de culture a été regulé à 8,4 par le $CO_2$. L'activité chlorophyllase de l`extrait brut preparé à partir de la biomasse fraîche a été déterminée sur la chlorophylle ou sur la phéophytine en présence d`un solvant organique miscible (acetone) et de l`huile de canola raffinée et désodorisée (RBD). Nous avons étudié l'effet du pourcentage d`huile additionné, de la concentration de chlorophylle et phéophytine, de la concentration d'acétone, de la température d'incubation et de la vitesse d'agitation sur l'activité chlorophyllase. Les conditions optimales retenues pour la biocatalyse de la chlorophylle et la phéophytine sont les suivantes: en présence de 20 % d'huile de canola RBD et de 10 % d'acétone, sous une agitation de 200 rpm, à une température et une concentration optimales respectivement de 35°C et 540 μM pour la chlorophylle et 30°C et 400 μM pour la phéophytine. La présence d'huile de canola RBD a montré un effet inhibiteur sur la chlorophyllase tandis que l'acétone a agit en tant qu'activateur à des faibles concentrations et en tant qu`inhibiteur à des fortes concentrations. La chlorophyllase a montré une affinité faible pour la phéophytine en comparaison avec la chlorophylle. Des échantillons d'huile de canola brute provenant de différentes variétés ont été analysés par HPLC. Ces analyses ont montré la présence d`un taux élevé de pigments verts dans ces échantillons en comparaison avec les huiles provenants d`autres sources. L`hydrolyse des pigments verts présents dans ces huiles par la chlorophyllase a été étudiée. La chlorophyllase a montré une activité hydrolytique faible en présence de l`huile brute de canola en comparaison avec l`huile de canola RBD, cela est certainement dû aux impuretés présentes dans l`huile brute.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my supervisor, Dr. Selim Kermasha, for his guidance, encouragement, friendship and patience during my graduate studies. For this, I will always be grateful.

I wish to extend a special word of appreciation to Dr. Salwa Karboune and Dr. Lamia L'Hocine for their guidance, advice, help, patience and time. I truly appreciate it.

I would also like to thank Dr. Wijdan Madani for her presence and her help as well. Special thanks also to all my colleagues and friends especially to Carolyn, Jacinthe, Galien, Abzal, Tamy, and Leila.

A special thanks to the staff members of the Food Science Department who created a friendly atmosphere in this department.

I am grateful to Daniel Proulx and Roxane Tremblay, from the University of Laval at Quebec, who provided the alga biomass culture used in this study.

Finally, I am most grateful to my family for supporting me in my most difficult times, for being always present, and for making me the person I am today. Your continuous guidance and patience was the key to my success. A special thanks to my three bothers, Jad, Elia and Nicolas, their love and faith in me kept me going all these two years.

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1. INTRODUCTION

Chlorophylls are green pigments distributed within living plant cells, higher plants, and many alga (Terpstra *et al.*, 1986). Loss of the central magnesium ion in chlorophyll results in the formation of pheophytin and subsequent removal of the carboxymethyl group gives pyropheophytin, while loss of the phytol group in chlorophyll and pheophytin yields chlorophyllide and pheophorbide, respectively. Chlorophylls, pheophytins, and pyropheophytins are considered to be lipophilic compounds due to the presence of the phytol group, whereas chlorophyllides and pheophorbides are hydrophilic ones (Schoch and Brown, 1987).

The chlorophyll content in edible oil is used as a measure of oil quality. During extraction, a portion of chlorophyll remains in the oil. The presence of high levels of chlorophyll is not only organoleptically unacceptable but also reduces the oxidative stability of oil due to the accelerated oxidation of methyl linoleate, thus increasing the oxidative rancidity and resulting in an undesirable color and taste in oil (Levadoux *et al.*, 1987).

Chlorophyllase (chlorophyll-chlorophyllido-hydrolase, EC 3.1.1.14) is an amphipathic intrinsic membrane glycoprotein enzyme (Terpstra *et al.*, 1986), which catalyzes the first step in the degradation of chlorophyll in fruits and vegetables during senescence or storage (Lambers *et al.*, 1984). The enzyme catalyzes, *in vitro*, the hydrolysis of chlorophyll into chlorophyllide and phytol in aqueous media (Moll *et al.*, 1978), and the synthesis of chlorophyll from chlorophyllide and phytol in organic solvent media (Shioi *et al.*, 1983).

Previous work in our laboratory was directed at the optimization of chlorophyllase extraction from the marine alga *Phaeodactylum tricornutum* (Khalyfa *et al.*, 1992). The development of organic solvent systems, including a water-miscible system, a biphasic system, and micellar systems containing polysorbates and spans (Khamessan *et al.*, 1993, 1994, 1995), was achieved for the enzymatic reaction of chlorophyllase using chlorophyll as substrate. Samaha and Kermasha (1997) studied the use of the chlorophyll derivative, pheophytin, as a substrate in a ternary micellar system

containing Span 85 as a surfactant. Furthermore, Gaffar and Kermasha (1999) investigated the biocatalysis of immobilized chlorophyllase, with chlorophyll and pheophytin as substrates, in a ternary micellar system containing Span 85.

Since many vegetable oils contain chlorophylls and carotenoids as natural pigments (Niewiadomski *et al.*, 1965), one of the potential applications of chlorophyllase in the food industry would be its utilization in the removal of edible green color. The level of chlorophyll content in canola oil is significantly higher than that found in other vegetable oils and is therefore one of the biggest quality impediments in the canola oil industry (Tautorus and Low, 1993). Bleaching of degummed green canola oil by means of enzymatic hydrolysis has been studied and shown that in a buffer/acetone solution, chlorophylls and pheophytins were hydrolyzed by chlorophyllase but in the presence of canola oil, only the chlorophylls and not the pheophytins were enzymatically hydrolyzed (Kalmokoff *et al.*, 1988).

The overall objective of this research work was to optimize the culture incubation conditions of the alga *P. tricornutum* to obtain optimal chlorophyllase activity and to investigate chlorophyllase biocatalysis in the removal of green pigments from canola oil by studying the effects of various parameters, including oil content, acetone content, enzyme concentration, incubation temperature, agitation speed, and substrate concentration.

In this research work, the alga *P. tricornutum* was used as a source of the chlorophyllase enzyme. The enzyme was extracted and partially purified according to the procedure developed in our laboratory (Kermasha *et al.*, 1992) with some slight modifications.

The specific objectives of this research were:

1. To characterize the endogenous green pigments in selected samples of crude commercial canola oil, using high-performance-liquid chromatography.

2. To optimize the biomass production of the marine alga *P. tricornutum* to obtain optimal chlorophyllase activity.

3. To optimize the biocatalysis of chlorophyllase and to investigate its activity in a reaction system containing refined bleached deodorized (RBD) canola oil, with chlorophyll and pheophytin as substrates.

4. To study the biocatalysis of chlorophyllase with crude commercial canola oil containing endogenous green pigments.

2. LITERATURE REVIEW

2.1. Introduction

Increasing the world population and gradually improving living standards have stimulated the growth of the oil seed industry. As a result, oil crops have become the second most valuable commodity in world trade.

Rapeseed oil obtained from immature seeds has a high distinct greenish color due to its high content of green pigments (Usuki *et al.*, 1984). In the 1970's, Canadian plant breeders produced canola by genetically altering rapeseed in two ways. First they reduced the levels of glucosinolates (which contribute to the sharp taste in mustard), and then they decreased the levels of licosenic and erucic acids (two fatty acids not essential for human growth). "Canola" refers worldwide to varieties with less than two percent of erucic acid in the oil and 30 μmoles per gram or less of the normally measured glucosinolates in the meal. The presence of chlorophyll and its oxidized products in canola oil is of significant commercial impact within the canola industry. Crude oils having chlorophyll content of about 20-30 μg/mL are considered to be "difficult to refine oil" whereas oils exceeding that level undergo special refining oil treatments to remove oxidative products (Suzuki and Nishioka, 1993).

The development of a biotechnological enzymatic process, involving the hydrolytic activity of the enzyme chlorophyllase, may be considered as an alternative technique to the adsorptive bleaching technique in vegetable oil industry, especially for canola oil (Levadoux *et al.*, 1987). This breakthrough is feasible by the establishment of a new model for the chlorophyllase catalyzed hydrolytic reaction in organic solvent media containing crude canola oil.

2.2. Economical Figures

2.2.1. World Canola Production

The high erucic acid content in the oil seed caused various processing problems for the oil component, which lead to the development of a rapeseed variety with a low

erucic acid content of less than two percent. Utilization in Canada of the low erucic acid oil varieties "called canola oil", increased rapidly.

Leading producers of rapeseed are China, European Community, Canada and India. World production of rapeseed has increased faster over the past two decades than that of any other oilseed. Over the last fifteen years, rapeseed production has achieved an annual average growth rate of seven percent while soybean output grew annually at only four percent. World trade in rapeseed and its products, oil and meal, has also achieved spectacular growth. Rapeseed exports are now the second largest volume oilseed traded following soybeans.

Oil world annual 1987 reported that the rapeseed/canola seeds increased for a total increase of 85.6 % within the year 1978/1979 or an average increase of 9.5 % per year. The world fats and oil output had increased in the early nineties to 53.3 million metric ton (mmt). Soybeans being the leader with 29 % followed by palm 16 %, rapeseed/canola 13 % and others 36 %. As compared to the previous world fat and oil percentages, canola oil was considered within the "other oil" categories that had a total of 34 % (Mag, 1993).

2.2.2. Canadian Canola Market

Canada is the world leader in the production of canola oil, which is in demand in nutrition-conscious markets such as North America; largely because of its low level of saturated fats. At the present time almost 63 % of all the edible oils consumed in Canada are from low erucic acid rapeseed oil (LEAR), or canola seed oil (Mag, 1983).

Canadian canola production is forecast to decline by 19 %. In spite of this decline, total supplies are projected to be 9.3 mmt, only 220,000 mt lower than a year earlier due to the extremely high opening stock levels. Although world consumption of oil seeds continues to rise, it is not sufficient to offset the rise in production. The oil world September 2000 publication indicated that Canadian canola producers and exporters are enjoying increased demand due to less competition from Europe and also the outlook of a considerably smaller crop in Australia. In 1999/2000, the world canola/rapeseed market saw relatively high production in comparison to usage. Most of the major exporting

countries were able to rid themselves of their surpluses with the exception of Canada. The green seed problem has traditionally meant more expense for the crusher and subsequently reduced payment for the farmer.

Canadian producers did not like the prices offered and opted to store their canola. This situation meant that Canada had very high stock levels, which had limited prices. In 1999-2000, Canadian exports of canola were estimated to be 0.2-0.4 mt below the potential, as Canadian canola remained $10-25 per ton overpriced vis-à-vis Australia and the EU.

For the year 2000-2001, production is estimated to decrease significantly due to the decrease in seeded area and lower yields. However, supplies are expected to only fall slightly due to high carry-in stocks. Exports are forecast to increase slightly due to reduced competition from the EU and Australia. Carryout stocks are projected to decline significantly, but still remain the second largest on record, whereas canola prices are expected to decline by 5-10 %.

2.3. Canola Oil

2.3.1. Chemical Composition

Canola oil has a complement of fatty acids that makes it one of the healthiest edible oils with low erucic acid content, (compared to all other edible oils available on the market, canola oil has the lowest level of saturated fat at six percent); as for the livestock feed concern canola meal has no glucosinolates therefore it does not cause a growth inhibition. Canola oil is now recognized as one of the healthiest oils available to consumers because of the combination of its low saturated and high monounsaturated fatty acid profile as well as its unique level of the ω-3 fatty acid, α-linolenic acid.

Immature oil seeds have a green color, which is due to the presence of the photosynthetic pigment chlorophyll with its derivatives (Niewiadomski *et al.*, 1965). Residual chlorophyll content of up to 5 μg/mL is common. Canola oils, however, may contain up to 15-20 μg/mL under normal growing conditions (Mag, 1983). Under frost condition the crop must be harvested prematurely to minimize pod shatter and prevent

extensive seed loss, these seed would contain a chlorophyll content of around 50 μg/mL. Crude oils with chlorophyll content greater than 20-30 μg/mL are considered difficult to refine, oils that exceed this level require special treatment during refining to obtain a product with acceptable levels of color, free fatty acid content and oxidative flavor stability (Suzuki and Nishioka, 1993).

2.3.2. Refining of Oil

2.3.2.1. Definition

The objective of oil processing is to remove the impurities while maximizing the yield of neutral oil, and minimizing damage to its quality. In the oil processing various ingredients are added or subtracted at various stages.

The oils obtained directly from the crushing process are referred to as "crude oil". Crude oil contains various compounds, which must be removed to improve stability and shelf life. These compounds include phospholipids, mucilaginous gums, free fatty acids, color pigments and fine solid particles. Crude oil is not considered suitable for consumption because of its objectionable color and odor. The production of edible vegetable oils including canola oil involves two overall processes which are oil extraction and oil refining (Andersen, 1953).

2.3.2.2. Oil extraction

The first stage in processing crude canola oil is to roll or flake the seed that would result in cell ruptures and an easier oil extraction. Then the rolled seeds are cooked and subjected to a mild pressing process, which removes some of the oil and compress the seeds into large chunks called "cake fragments" which undergo further processing to remove most of the remaining oil. Finally the oil, which is extracted during each step, is combined (Andersen, 1953; Mag, 1983; Mustafa *et al.*, 2000).

2.3.2.2.1. Mechanical Extraction

The first extraction step is the mechanical pressing, which is the use of continuous expellers for the removal of the high oil content in the seed (40-45 % of oil).

2.3.2.2.2. Solvent Extraction

After performing the mechanical extraction, solvent extraction is used to extract the remaining oil found in the press cake (its oil content is below 20 %). The objective of solvent extraction is to remove as much oil as possible with a minimum solvent loss, because the oil is usually the most valuable component of the seed. Oil produced by this method has a high quality due to its low heat treatment requirements (Bell, 1967).

2.3.2.3. Degumming

The major oil refining step is the phospholipid removal (degumming), phospholipids have the propensity to form emulsions. Different degumming treatments are performed according to the nature of phospholipids.

2.3.2.3.1. Water Degumming

Phospholipids are converted to hydrated gums, which are insoluble in oil, and then separated, by centrifugal action. The oil is dried under vacuum to remove all water traces, which would cause further hydration and gum precipitation during storage.

2.3.2.3.2. Acid Degumming

The non-hydratable form is transformed to the hydratable form before centrifugation. Most of them can be precipitated to form the oil through the application of acid (the main acid used is phosphoric acid). Acids break the non-hydratable phospholipids, which are mainly present as calcium and iron based cations. Once the complex is broken, the phospholipids can be converted to hydratable gums (Lawson, 1995).

2.3.2.4. Physical Refining

The purpose of physical refining is to remove free fatty acids, odor, flavor, and some color from the oils, using steam distillation. Physical refining lower the loss of neutral oil in the by-products, and this would reduce the number of operations in the purification process.

2.3.2.5. Bleaching

The bleaching operation is used to remove most of the chlorophyll and carotenoid pigments from the oil by adsorption on acid-treated bentonite bleaching clay. Following water precipitation and/or organic acid processing, the oil still contains color compounds which are unattractive to many consumers and which also reduce the stability of the oil. These compounds are extracted through a process called "bleaching".

Bleaching is a process where the oil is passed over natural, diatomaceous clay, which removes color compounds and other by-products. The color pigments in the oil adsorb to the clay, which is then filtered from the oil by means of filter presses. The term bleaching is reserved for treatments designed to solely reduce the color of the oil. Beckman (1983) reported that during the oil extraction a portion of the chlorophyll remains, which is very difficult to remove by conventional bleaching methods. Although chlorophyll and pheophytin may be removed by the addition of bleaching clay, this process is very expensive since the bleaching clay also adsorbs valuable oil, equal to 1/3 to 3/4 of the weight used; leading to oil losses (Diosady, 1991).

2.3.2.6. Deodorization

"Deodorization" is the final step in the refining of all vegetable oils, including canola. Deodorization involves the use of steam distillation, which removes any residual volatile compound, which could impart adverse odors and tastes. The oil produced is referred to as "refined oil" and is ready to be packaged and sent to consumers. Other processing steps may be performed according to the purposes of the oil consumption.

2.3.2.6.1. Winterization

Winterization is the precipitation and the removal of waxes that may solidify under room temperature.

2.3.2.6.2. Hydrogenation

Hydrogenation is the addition of hydrogen to the unsaturated fatty acid double bonds in the neutral oil, thereby converting liquid oils into semi-solids suitable for the production of margarine and shortenings.

2.3.2.6.3. Interesterification

Interesterification is the modification of the triglyceride structure of the oil thus leading to a change in the melting characteristic. Mixing the oil with other edible oils more solid by nature does this process; it is considered as an alternative for the hydrogenation process (Andersen, 1953).

2.4. Chlorophyll

2.4.1. Definition

Chlorophyll was discovered by Pelletier and Caventou (1818); the word chlorophyll is derived from ancient Greek roots indicating the green color of leaves (Strain *et al.*, 1971). Chlorophylls are green pigments responsible for the color of the leaves in vegetables and fruits. In higher plants and alga, the chlorophylls occur in sub-cellular organelles, the chloroplast, but in photosynthetic bacteria they are located in special membrane structures, called chromatophores (Svec, 1991).

2.4.2. Chemistry and Degradation of Chlorophylls

Chlorophylls are tetrapyrrolic pigments with a magnesium ion fixed at the center of the pyrol ring (Vernon and Seely, 1966). Chlorophylls are insoluble in water due to the phytol ring located at Carbon 18 (Fig. 1), but they are soluble in organic solvents and detergent solutions (Strain *et al.*, 1971). Chlorophyll derivatives include chlorophyll *a*, and *b*, pheophytin *a,* and *b* and others. The ratio of chlorophyll *a* and *b* in higher plants is usually 3 to 1, respectively (Strain *et al.*, 1971; Goodwin, 1976). Chlorophyll *a* is found as the major pigment found in alga and spinach leaves (Strain and Svec, 1966). Chlorophylls yield pheophytins when the central magnesium ion is removed with acids (Strain *et al.*, 1971), whereas the removal of the phytol group yields chlorophyllide or pheophorbide. Chlorophyll, pheophytin, and pyropheophytin are lipophilic compounds whereas their phytol free derivatives chlorophyllide, pheophorbide and pyropheophorbide are hydrophilic compounds.

The extent of pigment degradation in fruits and vegetables can be influenced by storage conditions such as light, temperature, relative humidity and volatile substances

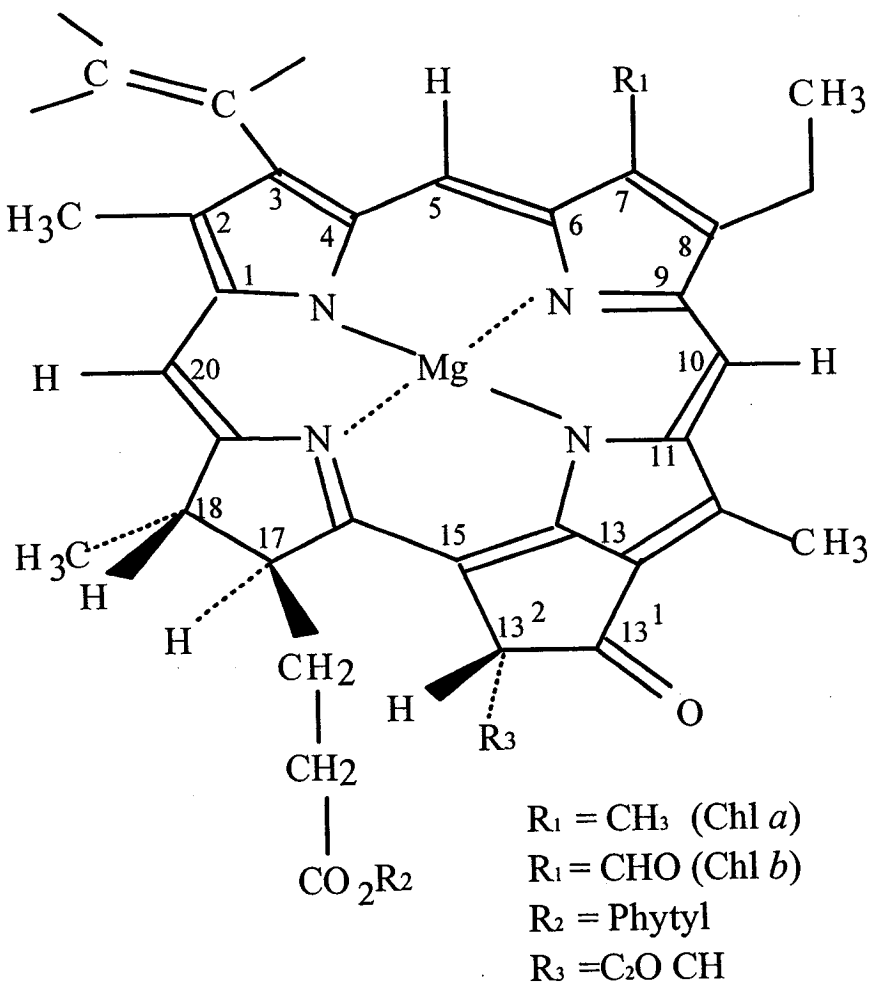

Figure 1. Molecular structure of chlorophyll and its derivatives.

such as ethylene (Francis, 1985). Chlorophylls are decomposed by acids, alkalies, oxidizing reagents, hydrolytic enzymes, and oxidative enzymes (Strain *et al.*, 1966). The phytol group may be replaced in chlorophylls, chemically, or enzymatically, to yield the corresponding chlorophyllides (Vernon and Seely, 1966; Hendry *et al.*, 1987). The loss of Mg^{+2} ion might be due to an enzymatic reaction (Owens and Falkowski, 1982) or a chemical reaction (Hendry *et al.*, 1987).

2.4.3. Chlorophyll in Canola Oil

The level of chlorophyll content in crude canola oil is significantly higher than that found in other vegetable oils; this is the biggest quality impediment in canola oil industry (Tautorus and Low, 1993). The exact quantity depends to a large extent on the degree of maturity (Daun, 1982). If the seed is damaged by early frost, it remains immature and green, and its chlorophyll content is high. The conventional hexane extraction process extracts not only the triglyceride oil, but chlorophyll is also readily extracted and it imparts a greenish color to the crude oil. If this oil is then processed by the conventional refining techniques the chlorophyll is converted to the pigment pheophytin, which gives oil a dark undesirable color, and contributes to an off-flavor. The presence of high content of green pigments is not only organoleptically unacceptable in a food product but also reduces the oxidative stability of oils (Usuki *et al.*, 1984). Unsaturated fatty acids, an integral component of oilseeds, are highly reactive with oxygen, producing undesirable flavors and odors during storage. The chemical process that results in these odors and flavors is known as oxidative rancidity. Canola oil has been known to develop a strong unpleasant "room odor" when heated to frying temperatures (Nakai, 1991). This specific odor is apparently due to the presence of oxidation products of linolenic acid (Mounts, 1979). Usuki *et al.* (1984) reported that the exposure to light is largely responsible for the oxidation of fats and oils, especially in the presence of chlorophyll.

High levels of chlorophyll in the oil are detrimental to the quality of the product, resulting in economic losses to both the growers and processors of canola oil. The removal of chlorophyll from oils requires large quantities of bleaching clays, resulting in

high processing cost and in significant losses of oil through its adherence to the clays (Levadoux *et al.*, 1987).

2.5. Approaches Undertaken

2.5.1. Genetic Approach

Biotechnology is enhancing the ability of plant breeders to introduce new varieties of oilseeds with particular applications for both edible and industrial purposes.

Researchers found a way to reduce green color in canola oil by the genetic modifications of the genes responsible for the formation of chlorophyll. The best way to reduce the green pigment content was to destabilize the chlorophyll A/B (CAB) complex. The CAB proteins bind to the chlorophyll and are the base for the biosynthesis process to occur.

The technique used involves transgenic, which is to insert a new gene into a plant. A complement gene is used to suppress production of CAB, which leads to the promotion of chlorophyll degradation. The level of pigment in the transgenic line seed was almost the same as the non-frozen control plants. A critical aspect for this study was to assure that this gene would act only on the seed and not on the whole plant. Otherwise photosynthesis in the green leaf would not occur, due to the lack of chlorophyll pigments.

2.5.2. Extraction Approach

The green pigment removal from canola oil was done either by altering the extraction process to minimize chlorophyll extraction with oil or by the removal of chlorophyll from crude oil during the refining operations. The aim was to disperse a source of phosphoric acid in canola oil to form a mixture having a moisture content of less than 0.1 % by weight at a certain temperature until a precipitate containing chlorophyll color impurities is formed.

2.5.2.1. Ultrafiltration

Chlorophyll and its derivatives have been known to be found in the oil in a true solution, but some work still under study, have showed that chlorophyll may be found in

a suspension of tiny particles, probably colloidal in size. That would lead to the removal of chlorophyll through the use of membrane filtration, or even ceramic microfiltration. Magnesium atom of chlorophyll may be exchanged with hydrogen, and later with other metals under slightly acidic conditions in an ion-exchange reaction. Once magnesium is removed in the form of an ion (Mg^{+2} ion), the rest of the molecule may form an exchangeable ion of opposite (negative) charge, and may be removed by ion exchange.

2.5.2.2. Supercritical Fluids Extraction

Supercritical fluids, such as carbon dioxide are excellent solvents for a wide variety of non-polar compounds, including oils, essential oils and many compounds responsible for food flavor (Rizvi, 1994). Although this technique was considered to be an efficient method to extract chlorophyll from edible oils, it is considered to be non-feasible in the economical perception. Chlorophyll is expected to be much less soluble in supercritical carbon dioxide than the triglycerides at lower supercritical pressures, and thus it may be possible to extract most of the oil, leaving the chlorophyll and other impurities as a residue. Recently Professor Rizvi (Cornell University) indicated that chlorophyll is practically insoluble in supercritical carbon dioxide, which may lead to incomplete chlorophyll extraction using this method (Diosady, 1991).

2.5.2.3. Organic Solvents

Chlorophyll is reported to be soluble in a number of organic solvents, such as low molecular weight alkanols. Chlorophyll will be preferentially extracted into the methanol phase during the process, reducing the chlorophyll content of the resulting oil. As a consequence this would result in the oil recovery from the non-polar hexane phase, while the polar phase would contain the dissolved waste materials (Diosady, 1991).

2.5.3. Chlorophyllase Approach

2.5.3.1. pH Dependence

Since all enzymatic reactions in aqueous solutions are strongly pH dependent, one of the most intriguing aspects of the enzymatic catalysis in organic solvents is that of the pH in a nearly anhydrous organic medium. The enzyme chlorophyllase can be roughly

classified into three groups as a function of pH value: enzyme with an acidic optimum pH, such as *Alianthus* (pH 4.5; McFeeter *et al.*, 1971), tea leaves (pH 5.8; Ogura, 1972) and Heracleum leaf meal (pH 6.0; Mayer, 1930); enzyme with a neutral optimum pH, such as *C. vulgaris* (pH 7.2-7.3; Moll *et al*, 1978), sugar beet (pH 7.1; Bacon and Holden, 1970) and tobacco (pH 7.0-7.5; Shimizu and Tanaki, 1963); and alkali optimum pH, such as *Phaeodactylum tricornutum* (pH 8.5; Khalyfa, 1992). For an acetone dried powder chlorophyllase, obtained from sugar beet leaves (Holden, 1961), the chlorophyllase activity increased as the pH of the suspension was raised to 9.0, but for a purified enzyme in acetone, the optimum pH was 7.7. Klein and Vishniac (1961) found that the activity of rye chlorophyllase on pheophytin solubilized by Triton X-100 was constant at pH between 6.0 and 8.0 and there was no activity below 6.0.

The optimum pH value of chlorophyllase from *P. tricornutum*, investigated in different organic solvent systems, including water/miscible, biphasic, and micellar ternary was reported to be around 8.0 (Khamessan *et al.*, 1993, 1994, 1995; Khamessan and Kermasha, 1996).

2.5.3.2. Substrate Specificity

Substrate specificity of enzymes comes from their ability to utilize the free energy of bonding to the substrate in order to facilitate the reaction; the net binding energy is the difference between the binding energies of the substrate with the enzyme and water. Therefore, substrate specificity is the difference obtained when water is replaced by organic media (Zaks and Klibanov, 1986).

McFeeter indicated that the enzyme chlorophyllase has higher specificities towards chlorophylls *a* and *b* compared to those of methyl and ethyl chlorophyllides and pheophorbides. The analyses of substrate specificity data for chlorophyllase indicated that the presence of a 13-keto group and a methyl alcohol group esterified at the C_{17} position in chlorophyll derivatives resulted in maximum binding affinity for substrates (McFeeter, 1975). If the magnesium ion is removed from the tetrapyrrole ring, the decrease in the size of the alcohol group would improve the substrate binding; the data

also shows that the major effect of magnesium ion removal is to decrease the apparent value of K_m.

2.5.3.3. Progress in the Biocatalysis of Chlorophyllase in Organic Media

Biocatalysis of partially purified chlorophyllase from *P. tricornutum* was studied using chlorophyll as a substrate in a model system composed of a wide range of organic solvents including water/miscible, biphasic and a ternary micellar system containing polysorbates and spans (Khamessan *et al.*, 1994, 1995 and 1996). Chlorophyll and pheophytin were used as substrates with the free and immobilized form of chlorophyllase in a ternary micellar system having Span 85 (Samaha and Kermasha, 1997).

The hydrolytic activity was first determined in a water/miscible organic media with different mixtures of acetone and Tris-HCl buffer solutions. Chlorophyllase was stored in different environments containing variable concentrations of acetone to verify the stability of the enzyme. It was found that chlorophyllase had a higher stability in higher concentrations of acetone compared to its presence in a high aqueous media (Khamessan *et al.*, 1994).

In the biphasic system, the hydrolytic activity was studied under different mixtures of hexane and Tris-HCl buffer solutions. The optimum amount of hexane was 45 %, but the incubation time, enzyme content, and other kinetic parameters were not encouraging compared to the water/miscible system (Khamessan *et al.*, 1996).

Work in our laboratory (Khamessan and Kermasha, 1996) was carried out on the optimization of the use of the ternary micellar system for the biocatalysis of chlorophyllase using chlorophyll as a substrate. The surfactants used were polysorbates (20, 40, 60, 80, and 85) and spans (20, 40, 60, 80 and 85). The use of Polysorbate 80 increased the enzymatic activity, decreased the incubation time and decreased the enzyme content, compared to the use of the biphasic system. Moreover, the use of Span 85 surfactant increased the enzymatic activity, with a greater reduction in incubation time and enzyme content, compared to the use of Polysorbate 80.

Samaha and Kermasha (1997) studied the hydrolytic activity of chlorophyllase under the same conditions but using chlorophyll derivatives as substrates. The overall data illustrated that chlorophyllase had lower affinity for chlorophyll derivatives as substrates than that reported for chlorophyll.

Gaffar and Kermasha (2000) studied the hydrolytic activity of immobilized chlorophyllase in a ternary micellar system using chlorophyll and its derivatives as substrates. The overall data illustrated that chlorophyllase showed the highest immobilization efficiency of 85 % on silica gel support. In addition the immobilized chlorophyllase had a higher affinity for chlorophyll than exhibited for pheophytin.

The chlorophyllase activity was improved by changing the environment of the enzymatic reaction. A biphasic organic system was used but it proved to have difficulties related to the hydrolytic activity of chlorophyllase since chlorophyll was poorly soluble in non-polar immiscible organic solvents. Chlorophyllase and chlorophyll affinities were greater in miscible organic solvents than in immiscible ones. A ternary micellar system with a hexane/aqueous media was used and it showed that the hydrolytic activity of chlorophyllase was higher when using the surfactant Span 85. As for the substrates, pheophytin was found to give the highest hydrolytic activity of chlorophyllase, and even more, the partially purified pheophytin showed best results. In the micellar ternary system the chlorophyllase content and the incubation time was lower compared to that of the biphasic organic system and the water/miscible organic solvent. Magnesium chloride addition was considered to be an activating agent to chlorophyllase. Membrane lipids affected positively the forward reaction by activating chlorophyll and it substrates.

Khamessan *et al.*, (1996) studied the effect of canola oil on chlorophyllase activity among different organic solvent media. The results demonstrated that the use of micellar ternary system containing Span 85 was the most appropriate one for the hydrolytic activity. However, the results confirmed that the increase in canola oil concentration in the reaction medium decreased the hydrolytic activity of chlorophyllase.

2.6. Chlorophyllase

2.6.1. Definition

The enzyme chlorophyllase (chlorophyll-chlorophyllido-hydrolase, EC 3.1.1.14) was discovered by Willstätter and Stoll (1913), it is an intrinsic membrane glycoprotein enzyme, due to its carbohydrate residue (Terpstra, 1978) as well as its high content in asparagines (Lambers *et al.*, 1986). The removal of the phytol group could be the first step in chlorophyll catabolism. Chlorophyllase has a relatively specific esterase activity for the phytol ester linkage in chlorophyll. The enzyme has a hydrophilic part, associated with a carbohydrate group as well as a hydrophobic part, which is tightly connected to membrane lipids (Terpstra *et al.*, 1986). Since the chlorophyll is a hydrophobic compound insoluble in aqueous solutions, an enzymatic approach requires the presence of an appropriate organic solvent (Michalski *et al.*, 1988).

Chlorophyllase may be the first enzyme discovered to retain its enzymatic activity in organic solvents. The poor solubility of chlorophyllase and its substrate chlorophyll in aqueous solutions precludes hydrolytic activity, but can be overcome by including an organic solvent in the reaction media (Khamessan *et al.*, 1993, 1994 and 1995).

2.6.2. Sources

Chlorophyllase was reported to be present in higher plants and alga photosynthetic membranes. The first soluble preparations were made from leaves, tissue cultures, and alga by a number of investigators (Barret and Jeffery, 1962; McFeeter *et al.*, 1971; Ellsworth *et al.*, 1976; Rodriguez *et al.*, 1987; Martinez *et al.*, 1995). Barret and Jeffrey (1962) and Kermasha *et al.* (1992) showed that the alga *P. tricornutum* is a rich source of the enzyme chlorophyllase among various marine alga sources.

2.6.3. Extraction and Partial Purification of Chlorophyllase

The solvents most suitable for the extraction of chlorophyllase from fresh plant material are those miscible with water, such as acetone, pyridine, methanol, and ethanol. The organic solvents used must be completely water miscible, unreacting with proteins and have a good precipitation effect. The two most widely used solvents are acetone and

ethanol; acetone has a lesser tendency to cause denaturation than ethanol. Chlorophyllase is present in an inactive form, called pro-chlorophyllase, and it becomes only active after cell disintegration of the isolated membrane fragments (Terpstra, 1978). Chlorophyllase has been partially purified from *P. tricornutum* using 90 % of acetone concentration (Lambers *et al.*, 1986; Kermasha *et al.*, 1992), this cause the breakage of the lipo-protein linkage of the enzyme and the membrane (Holden, 1963). Purified chlorophyllase fraction, extracted from *P. tricornutum*, was obtained from a combination of preparative electrophoresis and ion exchange liquid chromatography (Khalyfa *et al.*, 1992).

2.6.4. Enzyme Mechanism

Chlorophyllase catalyses, *in vitro*, either the hydrolysis of chlorophyll into chlorophyllide and phytol (Moll *et al.*, 1978) or the phytylation of chlorophyllide into chlorophyll (Montalbini and Buonaurio, 1986). The enzyme as well has the capability of transesterification of alcohols (Shioi *et al.*, 1983). It is not clear whether *in vivo* the enzyme catalyses the esterification or the hydrolytic reaction, or even both (Bacon and Holden, 1970).

The degree of purification of chlorophyll can greatly affect the enzyme activity. Terpstra and Lambers (1983) indicated that highly purified chlorophyll was not suitable as chlorophyllase substrates when compared to the partially purified chlorophyll. It has been indicated that chlorophyllase-catalyzed chlorophyll hydrolysis is remarkably influenced by the presence of lipids; both enzyme and substrate being affected. The modification of chlorophyllase associated lipid conformation is due to the interaction of magnesium ion with these lipids, which are essential for the enzyme activity (Terpstra and Lambers, 1983). The breakdown products of chlorophyll, comprises chlorophyll *a* and *b*, and depending on the degree of decomposition, larger or smaller amounts of pheophytin *a* and *b* (Niewiadomski *et al.*, 1965). The chlorophyllase from *P. tricornutum* hydrolyzed chlorophyll *a* much faster than chlorophyll *b*; the affinity of chlorophyll *a* to the active site may be much higher than that of chlorophyll *b* (Schoch and Brown, 1987). In acid media, the enzyme hydrolyzed predominately chlorophyll *b*, whereas chlorophyll *a* is hydrolyzed at a pH of 7.0 (Tarasenko *et al.*, 1986).

Chlorophyll breakdown reactions have been classified as Type I reaction and Type II reaction (Brown *et al.*, 1991). Type I reaction includes the primary loss of the magnesium moiety to form pheophytin, followed by the cleavage of the phytol chain to form pheophorbide. Type II reaction involves the primary cleavage of the phytol chain or the macrocyclic ring system to form chlorophyllide and subsequently pheophorbide (Fig. 2).

The phytol group may be replaced in chlorophyll or pheophytin, chemically or enzymatically, to yield the corresponding chlorophyllide or pheophorbide (Vernon and Seely, 1966; Hendry *et al.*, 1987). The loss of magnesium ion in chlorophylls might be due to an enzymatic (Owens and Falkowski, 1982) or a chemical reaction (Hendry *et al.*, 1987).

2.6.5. Immobilization of Chlorophyllase

2.6.5.1. Definition

Immobilized enzymes have been a topic of ongoing research since the early 1960's. This was due to the need to improve the biocatalysis and stability of enzymes in organic systems (Clark, 1994).

Immobilization is the entrapment of a biocatalyst in a distinct phase that allows exchange with, but is separated from the bulk phase in which the substrate, or the inhibitor are dispersed and monitored (Trevan, 1980). Immobilization achieves the separation of the enzyme and the product during the reaction using a two-phase system (Mailcata *et al.*, 1990), one phase contains the enzyme and the other contains the product. With an increase in the support to enzyme ratio to a certain limit, a larger surface area for more enzyme molecules to bind is provided; consequently it would enhance the rate of enzymatic activity (Klein and Ziehr, 1990).

2.6.5.2. Advantages

An immobilized enzyme shows a higher catalytic activity, or withstands a higher concentration (up to 90-95 volume percent) of organic solvent without complete loss of catalytic activity, or shows a higher resistance to irreversible denaturation

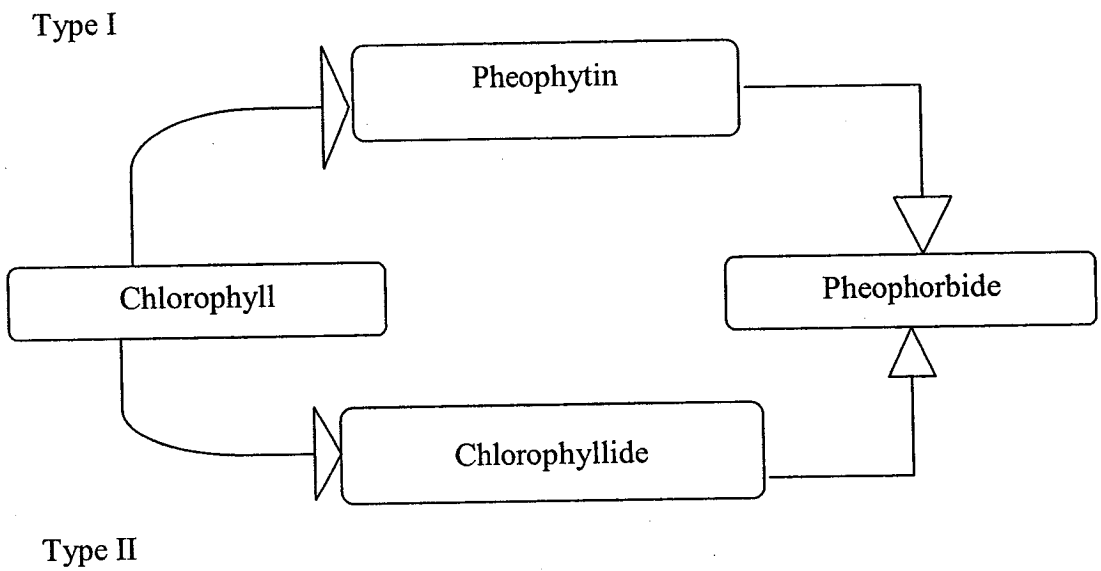

Figure 2. Degradation pathways of chlorophyll and its derivatives.

(Horvath, 1974) in comparison with a free enzyme. A higher percentage bioconversion was obtained with the immobilized enzyme compared to that with the free one (Gaffar and Kermasha, 1999). These findings suggest that a higher percentage bioconversion may occur for the immobilized chlorophyllase extract due to the occurrence of a lower diffusional resistance involved in the transportation of the substrate from the bulk solution to the enzyme as well as the diffusion of end-product from the enzyme back to the bulk solution. Hence the enzyme support system can be easily withdrawn from the system without contaminating the reaction mixture (Tramper, 1985). The enzyme is entrapped within its phase, allowing its reuse or continuous use but preventing it from contaminating the product; other molecules including the reactants, are able to move freely between the products (Parrado and Bautista, 1994).

Tramper (1985) reported that immobilization restricts the movement of the backbone and side chains of the enzyme molecule thereby preventing intermolecular interaction and unfolding of the polypeptide chain. These advantages increase the stability of the enzyme and retain its activity after repeated uses.

2.6.5.3. Chlorophyllase Immobilization

Immobilization of chlorophyllase was optimized by physical adsorption on various inorganic supports, the adsorption of chlorophyllase onto silica gel had the highest immobilization efficiency of 85 % as well as a specific activity of 0.44 μmole hydrolyzed chlorophyll per mg protein per min (Gaffar and Kermasha, 2000). The enzyme was also immobilized in different media, including water, Tris-HCl buffer solution and a ternary micellar system containing Tris-HCl buffer solution, hexane and Span 85 as a surfactant. The highest immobilization efficiency and specific activity were obtained when chlorophyllase was suspended in Tris-HCl buffer solution. The experimental results showed that the optimum pH and temperature for the immobilized chlorophyllase were 8.0 and 35°C, respectively. The use of optimized amounts of selected membrane lipids increased the specific activity of the immobilized chlorophyllase by approximately 50 % (Gaffar and Kermasha, 1999). Immobilized chlorophyllase was more reactive with chlorophyll than with pheophytin.

2.7. Biocatalysis in Organic Solvents

2.7.1. Introduction

Biocatalyst is the name used for enzymes, microbial cells, animal cells and cellular organelles, which are traditionally applied to aqueous reaction. High specificity characteristic of enzymes made them more superior in catalytic reactions than any other catalysts. In the past, all enzymatic reactions were performed in aqueous solutions, mainly to the understanding that organic media destroy and denature enzymes since they are proteins in nature (Mozhaev *et al.*, 1989). However, recent studies showed that enzymes could work in nearly anhydrous organic solvents, since water needed for enzymatic activity is tightly bound to the enzyme molecules and remains bound even when the bulk water is replaced with organic solvents.

It is well known that enzymes catalyze biochemical reactions in aqueous media. The poor solubility of the enzyme and its substrate chlorophyll in aqueous solutions, precluding hydrolytic activity, is overcome by including an organic solvent in the reaction medium (Bacon and Holden, 1970). The presence of organic solvents makes it possible to shift the thermodynamic equilibrium of many enzymatic reactions in favor of the desired products. Suspending enzymes in organic solvents can dramatically alter a number of their functional properties, such as substrate specificity, incubation temperature and pH dependence.

2.7.2. Water Content or Water Activity

Water plays an important role in the catalytic activity of an enzyme this is due to its direct and indirect role in non conventional interactions such as electrostatic, hydrogen bonding, Van der Waals forces and hydrophobic bonding that help maintain the catalytically active conformation of the enzymes (Carrea, 1984). As long as the enzyme is fully surrounded by water molecules, replacement of the rest of the water with an inorganic solvent is possible without adversely affecting the enzyme. The evidence suggests that an enzyme molecule in aqueous solutions becomes fully hydrated when surrounded by a few layers of water (Zaks and Klibanov, 1988). One common hypothesis is that the enzyme molecule requires small hydration layer that acts as the primary

component of the enzymatic microenvironment (Zaks and Klibanov, 1985; Fukui *et al.*, 1987).

2.7.3. Nature and Choice of Solvent

Certain factors can be taken into account in determining which solvent is most appropriate for a given reaction (Dordick, 1989). The first and most important one is the compatibility of the solvent with the reaction of interest. A second factor is that the solvent selected must be inert to the reaction. Additional factors, which may influence the choice of the solvent, are the following: solvent density and viscosity, surface tension, denaturing or inhibitory effects, reactant/product partition coefficient, toxicity, flammability, waste disposal and solvent cost.

The nature of the organic solvent affects the enzymatic catalysis in three distinct ways. First, the solvent can cause inhibition or inactivation by directly interacting with enzymes (Cremonesi *et al.*, 1975). Second, solvents may interact with diffusible substrates or products of the reaction. Finally, solvents may interact directly with the essential water in the vicinity of the enzyme (Dordick, 1989). The nature of the solvent is crucial for maintaining the layer of essential water around the enzyme. The best solvents to use are the most hydrophobic ones (Reslow *et al.*, 1987; Zaks and Klibanov, 1988) because there is no incentive for the essential water to partition into them and thus it remains on the enzyme. Most enzymes are inactive in hydrophilic, water-miscible organic solvents due to the partitioning of water from the enzyme into them (Kazandjian *et al.*, 1986).

2.7.4. Classification of Organic Solvent System

2.7.4.1. Water/miscible-Organic Solvent System

Water mixed in a miscible/organic solvent system creates a homogeneous reaction mixture (Lilly *et al.*, 1987). Common solvents used in this case are methanol, ethanol, *N*, *N*-dimethylformamide, and dimethyl sulfoxide. This system has been used to dissolve water-miscible, lipophilic compounds to prepare homogeneous reaction systems and to shift reaction equilibrium in a desired reaction (Lilly, 1982). Unfortunately the main drawbacks of these solvents are their high viscosity, inability to dissolve substrates of low

polarity, and the rather high nucleophilic properties of their molecules. Therefore, water/organic co-solvent systems cannot be regarded as a universal medium for enzymatic reactions.

2.7.4.2. Macroheterogeneous (biphasic) Reaction Media

A biphasic organic system consists of water and a water-immiscible solvent (Martinek *et al.*, 1981; Carrea, 1984). The enzyme and hydrophilic cofactors are dissolved in the water phase and the hydrophobic substrates in the organic solvent phase (Carrea, 1984). Shaking the reaction medium would result in enzyme-substrate interaction, which would be followed by a mass transfer of substrate product. This mass transfer can occur at the interface where the substrate, in our case chlorophyll, is soluble on the organic solvent phase and the end product, chlorophyllide, is soluble in the aqueous phase (Khamessan, 1994). Advantages of this system include high ratio of reactants to products concentration in the reactor (Lilly, 1982), reduction of product inhibition (Klibanov *et al.*, 1977; Schwartz and McCoy, 1977) and ease of product recovery (Carrea, 1984). On the other hand, these systems have several drawbacks; one of them is related to the fact that the non-polar solvents used as the organic phase are, to a certain extent, still soluble in water. The presence of these mixtures in the aqueous phase, even at low concentrations, can in principle result in the deterioration of the catalytic properties of the enzyme dissolved in this phase. The direct interaction of organic solvent and the enzyme is a major problem with soluble enzymes in biphasic organic systems (Marini and Martin, 1971).

2.7.4.3. Micellar System

Enzymes act mostly on or near the water/organic medium interface in living cells (Martinek *et al.*, 1981). Amphiphilic molecules of surfactants in water/organic solvent media spontaneously form micelles, whose dimensions are comparable with the molecular dimensions of proteins (Martinek *et al.*, 1986). A micellar system provides similar model for cell membrane and organelles (De Kruijiff *et al.*, 1980).

There are two main types of micelles, namely normal micelles designated as oil in water (O/W) which exist in water at low concentrations of organic solvents, and reversed

micelles (W/O) that form in an organic solvent with moderate water content. Reversed micelles are an example of the ternary micellar system consisting of surfactant/water/non-polar solvent, which forms aggregates called microemulsions (Overbeek *et al.*, 1984). A microemulsion is a clear, translucent, and thermodynamically stable homogeneous dispersion of two immiscible liquid containing appropriate amounts of surfactant and cosurfactants (Reed and Healy, 1977; El-Nokaly *et al.*, 1991).

In surfactant/water/organic solvent ternary systems much larger associates and liquid-crystalline structure, such as cylindrical, lamellar, etc., can spontaneously form depending on the composition of the mixture (Rob *et al.*, 1997).

The basic components, hydrocarbon, surfactant and water, are sufficient to form the micellar solutions. A lyophilized enzyme or its aqueous solution is introduced with stirring into a solution of surfactant (such as detergents and phospholipids) in an organic solvent (Menger and Jerkunica, 1977; Martinek *et al.*, 1986).

The enzyme molecules are spontaneously entrapped in reversed micelles. Reversed micelles are closed, almost spherical aggregates of surfactant molecules, the outer shell of which is formed by hydrophobic tails of the surfactant molecules whereas the inner core is composed of the polar heads of these molecules. The most important property of hydrated reversed micelles is their ability to entrap protein molecules into their inner cavities (Khmelnitsky *et al.*, 1988).

2.7.5. Surfactants

Surfactants or surface-active agents are amphiphilic compounds with hydrophilic and hydrophobic parts well separated in their molecular structure. The hydrophobic groups of surfactants are generally long-chain hydrocarbon residues, such as straight or branched chain alkyl groups (C_8-C_{20}) (Myers, 1988). Surfactants function in two ways; they reduce the interfacial energy and thus the energy required to create more surfaces, and they prevent coalescence of the droplets once these are formed.

Among surfactants, the non-ionic ones are of great importance because chemically, most food surfactants are non-ionic in nature and they are esters of fatty acids

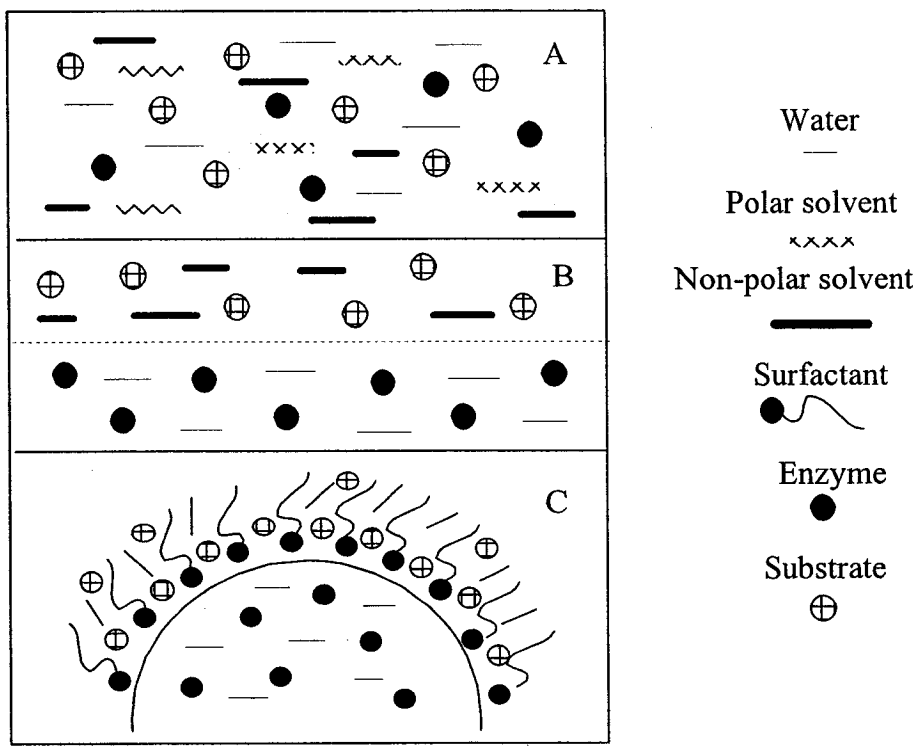

Figure 3. Schematic presentation of different organic solvent systems:

(A) Water/miscible system

(B) Biphasic system

(C) Micellar system

with naturally occurring alcohols (Myers, 1988). Non-ionic surfactants are effective in much lower concentrations than the ionic surfactants, because of their low critical micelle concentration values (c.m.c). Moreover, they show a relatively higher solubilizing power which may be related to looser packing of the surfactant molecules in the micelles of the non-ionic materials, making more space available for the incorporation of additive molecules (Attwood and Florence, 1983).

3. MATERIALS AND METHODS

3.1. Characterization of Green Pigments in Crude Canola Oil by High Performance Liquid Chromatography

3.1.1. Reagents and Standards

All chemicals used throughout this study were of HPLC grade such as acetone and methanol (Fisher Scientific, Pittsburgh, PA). Standards of chlorophylls *a* and *b* were purchased from Sigma Chemical (Sigma Chemical Co., St-Louis, MO). Standards of pheophytins *a* and *b* were prepared from chlorophylls *a* and *b* according to the method of Fraser and Frankl (1985), using a 9.2 % (v/v) solution of HCl. Calibration curves were prepared with standard solutions of chlorophyll *a*, chlorophyll *b*, pheophytin *a* and pheophytin *b* as described in Endo *et al.* (1992).

3.1.2. Methodology

The separation and identification of chlorophyll and its derivatives obtained from different varieties of crude commercial canola oil, was performed using an HPLC system (Beckman Instruments) equipped with a fluorescent detector (Waters Model 474 Scanning model) with an excitation wavelengths (λ_{ex}) at 420 nm and an emission wavelengths (λ_{em}) at 660 nm. Automatic injection (Beckman, autosampler 507) was carried out with a 20 µL volume loop onto a Restek Ultra C-18 reverse phase column (250 x 4.6 mm i.d., 5 µm). The analysis was performed with a flow rate of 1 mL/min, as elution solvent composed of a mixture of deionized water, methanol, and acetone at a ratio of 4:36:60 (v/v/v).

3.1.3. Oil Sample Preparation

Eight different varieties of crude commercial canola oil obtained from Canamera Foods (Canamera Foods, Oakville, Ontario) were analyzed. The sample was composed of a dissolved mixture of cold acetone (–20°C) : crude canola oil at a ratio of 75:25 (v/v). Before injection the sample was filtered (Acetate Plus filters, 0.22 µm).

Calculation of the limit of detection (LOD) for each of the chlorophyll and pheophytin standards was based on the external standard method. Dilutions of stock solutions of 1 mg of substrate/mL of a solution of acetone/oil mixture at a ratio of 75:25 (v/v) were injected in triplicate, and used to create a standard curve (peak area versus concentration in micrograms per milliliter). Furthermore, r-square and precision were calculated for each compound.

3.2. Optimization of *P. tricornutum* Growth Conditions

The marine alga *P. tricornutum* Bohlin (Bacillariphyceae), used as a source of chlorophyllase, was grown according to the method described by Kermasha *et al.* (1992). The strain CCMP 1327 was obtained from the culture collection of Provasoli-Guillard National Center for Culture of Marine Phytoplankton (West Boothbay Harbor, ME). The strain was maintained on culture medium, consisting of a mixture of *f*/2 synthetic medium (Guillard and Ryther, 1962) prepared in our laboratory and artificial seawater of 3.2 % salinity (diluted with distilled water to 2.8 % salinity) prepared by LARSA (Laboratory of Aquatic Sciences of Laval University, Quebec, Canada), to make a total volume of 30 L. Table 1 illustrates the composition of the *f*/2 synthetic medium. The pre-culture was used to inoculate 200 L of medium in a 250 L aquarium (when the biomass attained an optical density of 0.7). The final biomass optical density was 0.05 (proper dilution was made from the initial pre-culture). The culture was incubated in a 250 L container at a temperature of 18°C for 18 h during the day and 10°C for 6 h during the night. Illumination was provided by cool-white fluorescent lamps projecting an incident intensity of 330 $\mu mol/m^2.s$ at the surface of the culture chamber for 18 h of light and 6 h of dark photocycle. An inflow rate of sterile-filtered air was used to ensure adequate aeration and agitation. The pH of the culture was maintained at 8.4, adjusted by a stream flow of CO_2 (10 KPSI) which was controlled by a connected nanometer. After incubation, the algal cell mass was recovered by centrifugation (7,000×g, 5 min).

Table 1. The composition of Guillard and Ryther *f*/2 synthetic medium for the culture of *P. tricornutum*.

Quantity (mL)	Compound	Stock Solution
1.0	$NaNO_3$	75.0 mg/L H_2O
1.0	$NaH_2PO_4.H_2O$	5.0 mg/L H_2O
1.0	$Na_2SiO_3.\ 9H_2O$	30.0 mg/L H_2O
1.0	*f*/2 Trace Metal Solution	-
0.5	*f*/2 Vitamin Solution	-

3.3. Recovery and Partial Purification of Chlorophyllase

Biomasses of alga *P. tricornutum* were harvested after 5, 6, 7, 8, 9, 10 and 14 days of culture incubation. Chlorophyllase extractions were carried out using fresh and lyophilized cells of *P. tricornutum.* Figure 4 shows the partial purification of chlorophyllase. The chlorophyllase enzyme was recovered and partially purified from the photosynthetic membrane fraction according to the procedure described previously by Kermasha *et al.* (1992) with some slight modifications. The algal biomass was first washed with Tris-HCl buffer solution (20 mM, pH 8.0) containing 2 % NaCl (w/v). The washed cells were subsequently re-suspended in Tris-HCl buffer solution (20 mM, pH 8.0) containing 0.75 mM EDTA for cell homogenization, followed by centrifugation (10,000×g, 15 min). The supernatant, containing the chlorophyllase extract, was subjected to further ultracentrifugation (96,768×g, 60 min) to obtain the photosynthetic membrane fraction possessing the enzyme. Suspension of the cells in $Na_4P_2O_7$ buffer solution (10 mM, pH 7.4) was then performed to remove ribulose diphosphate carboxylase attached to the membranes, followed by an incubation step at 4°C for 4 h in a low ionic strength NH_4HCO_3 buffer solution (1 mM, pH 7.8). Chlorophyllase was partially purified using a 90 % (v/v) acetone concentration, which separated chlorophyllase from associated chlorophyllide. The precipitate was lyophilized, and the enzymatic powder was stored at -80°C.

3.4. Biocatalysis of Chlorophyllase in Canola Oil

3.4.1. Preparation of the Enzyme Suspension

The enzymatic suspension was prepared using the procedure of Khamessan *et al.* (1994). The enzymatic extract consisting of 1 mg lyophilized enzyme extract suspended in 1 mL of Tris-HCl buffer solution (20 mM, pH 8.0), was homogenized at 4°C for 5 min with a tissue grinder (Wheaton, Millville, NJ) so that a homogeneous enzyme suspension was obtained. The enzyme suspension, considered to be as the partially purified chlorophyllase, was freshly prepared prior to the assays.

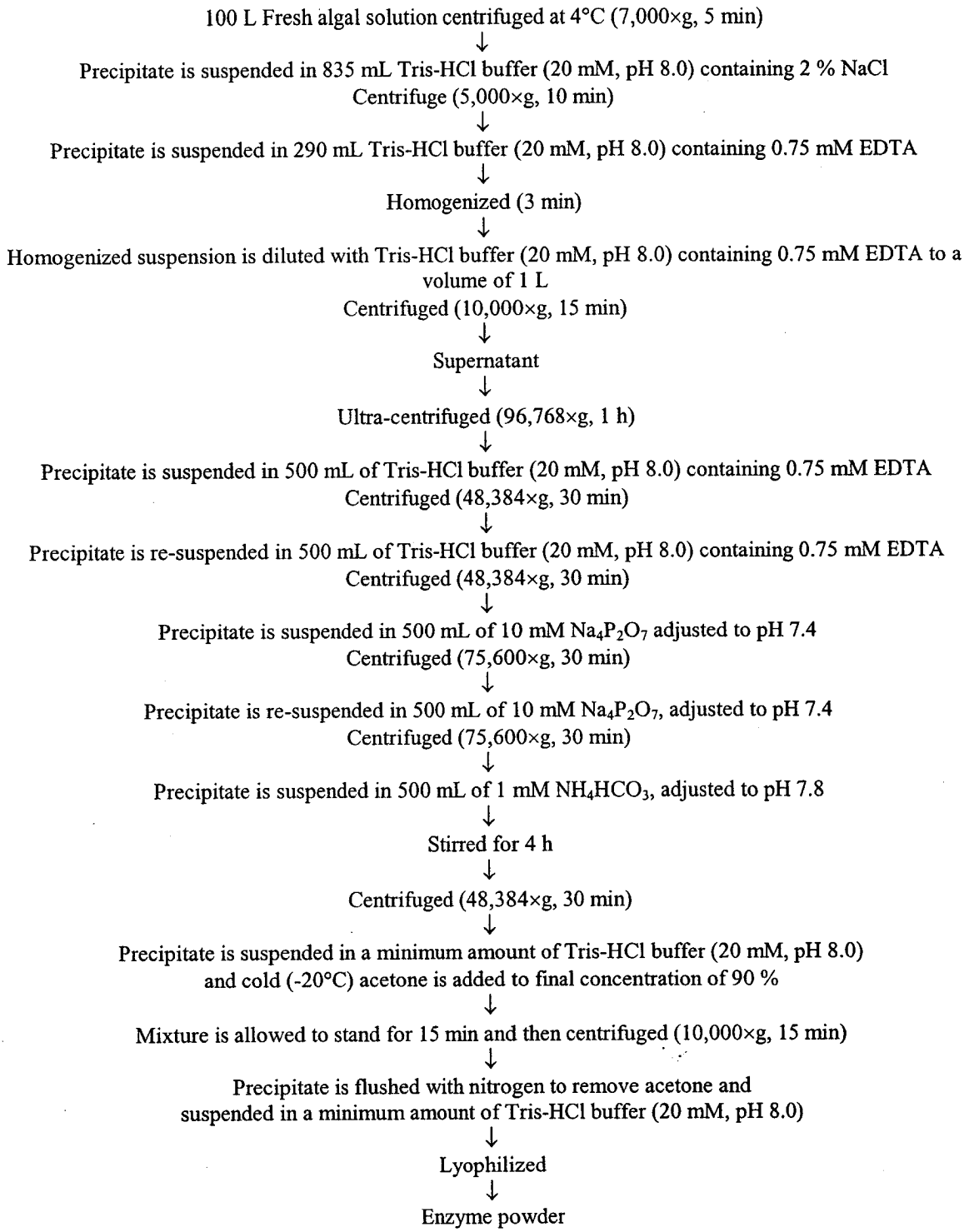

Figure 4. Schematic representation of the procedure for the partial purification of the chlorophyllase extract from the alga *P. tricornutum*.

Partially purified chlorophyllase was assayed for its protein content, using a modification of the Lowry method (Hartree, 1972). Bovine serum albumin (Sigma Chemical Co.) was used as the protein standard for calibration. A suspension of 1 mg of partially purified enzyme powder per mL containing 200 μg of proteins was used in the chlorophyllase reaction assays.

3.4.2. Preparation of Substrates

3.4.2.1. Partially Purified Chlorophyll

Partially purified chlorophyll, prepared from fresh spinach leaves according to the procedure described previously by Khalyfa *et al.* (1992), was used as a substrate for the determination of chlorophyllase activity. Lyophilized partially purified chlorophyll was dissolved in cold (-20°C) acetone (Fisher Scientific) at a concentration of 1 mg/mL (1.12 μmol/mL) and subjected to subsequent dilutions to give a wide range of chlorophyll concentrations ranging from 0.10 μmol/mL to 0.64 μmol/mL (Kermasha *et al.*, 1992). The substrate stock solution and subsequent dilutions were freshly prepared prior to the enzyme assay.

3.4.2.2. Partially Purified Pheophytin

Chlorophyll was converted to pheophytin according to the method of Fraser and Frankl (1985), using a 9.2 % HCl (v/v) solution. The partially purified pheophytin substrate obtained from the partially purified chlorophyll was dissolved in cold (-20°C) acetone (Fisher Scientific) at a concentration of 1 mg/mL (1.12 μmol/mL) and diluted to give a wide range of pheophytin concentrations ranging from 0.10 μmol/mL to 0.64 μmol/mL. The substrate solution and subsequent dilutions were freshly prepared prior to the enzyme assay.

3.4.3. Chlorophyllase Assay Using Refined Bleached Deodorized Canola Oil

The chlorophyllase assays were carried out in a water-miscible organic solvent system containing refined bleached deodorized (RBD) canola oil, and partially purified chlorophyll and pheophytin as substrates.

The assay was performed according to a modification of Khamessan *et al.* (1994) procedure. The reaction mixture was prepared in a 50 mL Erlenmeyer flask and consisted of 70 µL of the substrate solution (440 µM or a final concentration of 10.26 µM), 1030 µL Tris-HCl buffer solution (20 mM, pH 8.0), 300 µL of acetone and 600 µL of refined bleached deodorized (RBD) canola oil. The reaction was initiated by the addition of 1 mL of the enzymatic solution containing 200 µg proteins. A mixture, containing all the components except the enzyme suspension was prepared as a substrate blank and a mixture, containing all the components except the substrate suspension was prepared as an enzyme blank. The enzymatic reaction was carried out at 35°C and 30°C for chlorophyll and pheophytin, respectively, for 0.5, 1, 2, 3, 4, 5, 6, and 7 h with continuous agitation (200 rpm), using an incubator shaker (New Brunswick Scientific, Edison, NJ).

The enzymatic reaction was halted by adding a mixture composed of 4 mL cold acetone (-20°C), 2 mL petroleum ether (petroleum ether has a boiling point range of 35-60°C) and 1.66 mL 2 % (w/v) of NaCl solution. The reaction mixture was then vortexed for 15 sec, and transferred to a 15 mL test tube for separation into two separated layers. The residual substrate was extracted in the upper petroleum ether layer, whereas the product chlorophyllide or pheophorbide, remained in the lower acetone layer. The unhydrolyzed substrate located in the upper petroleum ether layer was measured spectrophotometrically at 663 and 666 nm, for chlorophyll and pheophytin, respectively (Beckman DU-65 spectrophotometer). The amount of residual unhydrolyzed substrate was determined from a calibration curve ranging from 0.0 to 11.7 µmoles/L of chlorophyll or pheophytin. The specific activity of chlorophyllase was defined as µmoles of hydrolyzed chlorophyll or pheophytin per mg protein per min. Table 2 represents the method performed for the calculation of the specific activity of chlorophyllase.

The specific activity of chlorophyllase was determined from the following calculations:

1. Calculate the hydrolyzed substrate absorbance.

Absorbance of hydrolyzed substrate = (absorbance of substrate blank + absorbance of enzyme blank) – absorbance of enzyme and substrate assay.

Table 2. Representation for the calculation of the specific activity of chlorophyllase.

Time (h)	Enzyme blank	Substrate blank	Enzyme substrate sample	Bioconversion (%)	Hydrolyzed substrate absorbance	Hydrolyzed substrate concentration (μmol/L)
0	0.03	0.5952	0.5952	0.00	0.0000	0.000
0.5	0.03	0.5952	0.5620	2.00	0.0632	0.316
1	0.03	0.5952	0.5306	15.13	0.0946	0.478
2	0.03	0.5952	0.4906	21.53	0.1346	0.685
3	0.03	0.5952	0.4562	27.03	0.1690	0.863
4	0.03	0.5952	0.4241	32.17	0.2011	1.029
5	0.03	0.5952	0.3959	36.68	0.2293	1.175
6	0.03	0.5952	0.3709	40.67	0.2543	1.305

2. Convert the absorbance of hydrolyzed substrate to a unit of μmol/L.

Chlorophyll and pheophytin standard curves were prepared by measuring the absorbance of several concentrations of the substrates. The concentrations varied from 0.65 μM to 10.45 μM. The extinction coefficient of each of chlorophyll and pheophytin were 0.050 and 0.056 $cm^2/\mu mol$ respectively.

3. Plot the graph of incubation time versus hydrolyzed substrate in μmol/L (Fig. 5).

4. Determine the slope of the polynomial second order equation.

$$y = ax^2 + bx + c \qquad \text{(Equation 1; with reference to Fig. 5)}$$

5. From the slope of the equation, the specific activity is obtained:

$$SA = \frac{b \times V_{reaction} \times Prot}{V_{enzyme}} \qquad \text{(Equation 2)}$$

where, *SA stands for chlorophyllase specific activity defined as μmol/mg.min; b stands for the slope of Equation 1 defined as μmol/L.min;* $V_{reaction}$ *is the total volume of the reaction defined in L;* V_{enzyme} *is the volume of the enzyme added defined in L; and Prot is the protein content defined in mg/L.*

3.4.4. Chlorophyllase Assay in Crude Canola Oil

The chlorophyllase assays were carried out as described previously with some slight modifications; an enzymatic suspension of 6 mg crude enzyme/mL and a 600 μL volume of crude canola oil were used in the reaction medium and the substrates, chlorophyll or pheophytin, were not added, as the crude canola oil already contained endogenous green pigments. The enzymatic reaction was carried out at 30°C for 6, 18 and 24 h with continuous agitation (200 rpm). The enzymatic reaction was halted and the residual substrate, located in the upper petroleum ether layer, was measured at 666 nm (Beckman DU-65 spectrophotometer).

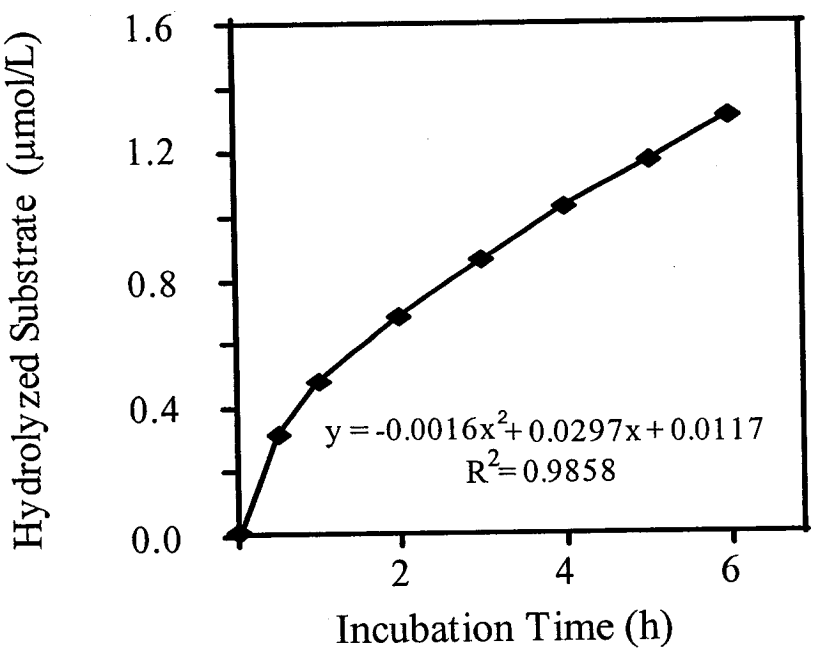

Figure 5. Representation of the slope of the curve, which is considered a factor while determining the specific activity of chlorophyllase.

3.5. Kinetic Studies of Chlorophyllase Activity

3.5.1. Effect of Oil Content on Chlorophyllase Activity

The effect of oil content on the specific activity of chlorophyllase was determined by using different oil percentages ranging from 0 to 40 % (v/v) of oil in the enzymatic reaction media.

3.5.2. Effect of Acetone Content on Chlorophyllase Activity

The effect of acetone content on the specific activity of chlorophyllase was determined by using different acetone percentages ranging from 0 to 20 % (v/v) in the enzymatic reaction media.

3.5.3. Effect of Agitation Speed on Chlorophyllase Activity

The effect of agitation speed on the specific activity of chlorophyllase was determined by incubating the enzymatic reactions at different agitation speeds ranging from 100 to 200 rpm.

3.5.4. Effect of Incubation Temperature on Chlorophyllase Activity

The effect of incubation temperature on the specific activity of chlorophyllase was determined by incubating the enzymatic reactions at different temperatures ranging from 20 to 40°C.

3.5.5. Effect of Enzyme Concentration on Chlorophyllase Activity

The effect of enzyme concentration on the specific activity of chlorophyllase was determined by using different chlorophyllase concentrations ranging from 0.16 to 0.66 mg of crude powder enzyme/mL in the enzymatic reaction media.

3.5.6. Effect of Substrate Concentration on Chlorophyllase Activity

The effect of chlorophyll and pheophytin substrate concentrations on the specific activity of chlorophyllase was determined by using different substrate concentrations ranging from 3.26 μM to 14.93 μM in the enzymatic reaction media.

4. RESULTS AND DISCUSSION

4.1. Characterization of Green Pigments in Crude Canola Oil

Eight different varieties of crude commercial canola oil were investigated with respect to their green pigment content. Preliminary identification of chlorophyll and its derivatives was based on comparing retention time data obtained for standard compounds.

Figure 6 is a representative chromatogram of a crude oil extract with 7 eluted peaks. Similar chromatograms were obtained for the extracts of the eight different varieties of crude canola oil, possessing different green pigment concentrations. The elution order was based on the hydrophobicity of the molecules. The polar molecules eluted first since the column used was a reverse phase column. The pheophytins eluted first, followed by the pyropheophytins and carotenes, respectively. The *b* derivatives eluted before the *a* derivatives since the latter were more hydrophobic due to the presence of the methyl group on the seventh carbon instead of the aldehyde group.

The results (Fig 6, Table 3) indicate the different chlorophyll derivatives present in the crude canola oil extracts with their relative percent composition. The highest percentage of pigment present was pyropheophytin *a*, followed by pheophytin *a* and carotenes, respectively while the presence of chlorophylls *a* and *b* was not detected. The presence of high and low concentrations of pyropheophytins and chlorophylls, respectively, indicated that most of the chlorophylls in the crude canola oil were oxidized to pheophytins, which were later subsequently oxidized to pyropheophytins. This phenomenon could be explained by the fact that crude canola oil was subjected to thermal treatments earlier in the oil processing procedure, leading to the oxidation of chlorophyll pigments (Levadoux *et al.*, 1987).

Table 4 shows the concentrations of the pheophytin *a* and *b* pigments and their derivatives found in the eight different varieties of crude commercial canola oil. The lowest and highest pheophytin *a* concentration were, 4.17 μg/mL and 25.21 μg/mL,

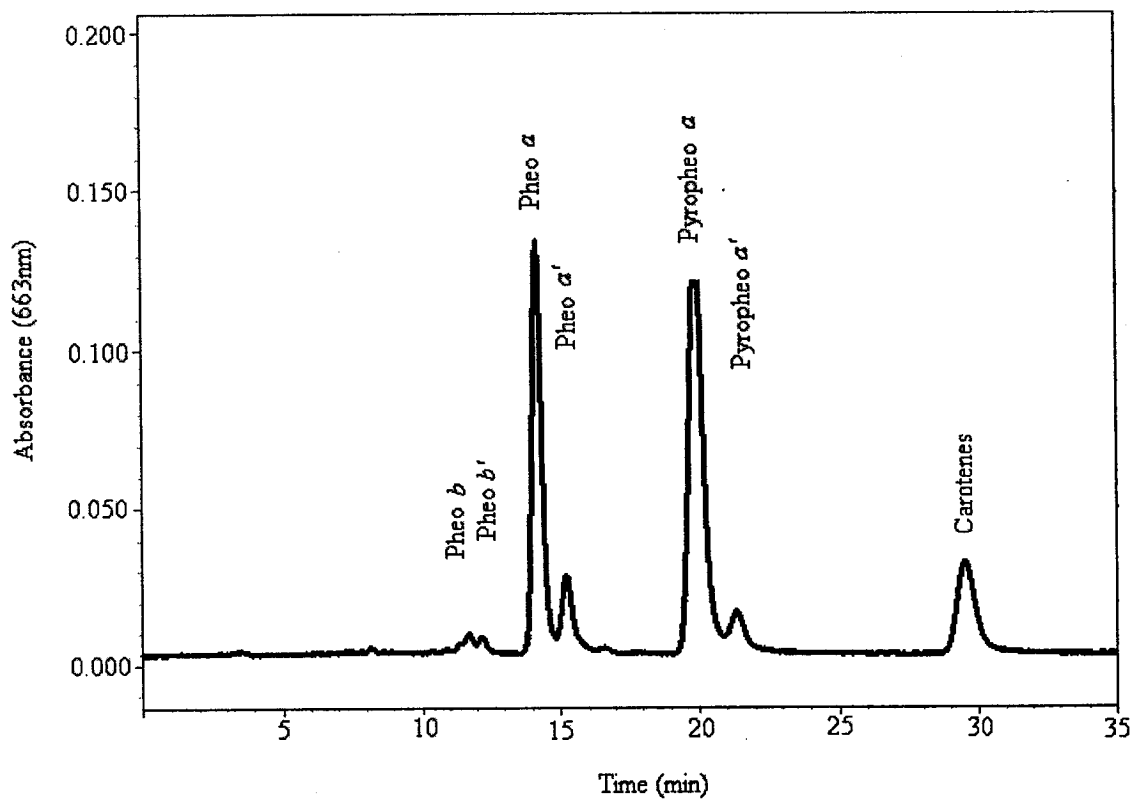

Figure 6. HPLC chromatogram of a crude commercial canola oil extract.

Table 3. Relative percentage of green pigments present in selected varieties of crude commercial canola oil.

	Relative percentage (%)[a]						
Variety[b]	Pheo *a*	Pheo *a'*	Pheo *b*	Pheo *b'*	Pyropheo *a*	Pyropheo *a'*	Carotenes
1	30.2 (0.68)[c]	6.6 (1.33)[c]	1.4 (4.00)[c]	0.9 (0.59)[c]	44.6 (0.23)[c]	3.9 (9.76)[c]	12.4 (4.25)[c]
2	26.8 (1.31)[c]	5.5 (4.28)[c]	0.9 (0.21)[c]	0.8 (3.33)[c]	48.2 (1.68)[c]	3.8 (0.97)[c]	13.9 (1.51)[c]
3	36.6 (0.18)[c]	9.6 (2.5)[c]	1.5 (4.26)[c]	1.0 (3.72)[c]	36.4 (0.32)[c]	4.5 (0.56)[c]	9.9 (1.33)[c]
4	38.9 (2.76)[c]	8.6 (2.04)[c]	3.5 (3.38)[c]	0.9 (1.54)[c]	34 (2.36)[c]	2.9 (1.07)[c]	1.1 (2.85)[c]
5	10.9 (0.9)[c]	2.4 (1.21)[c]	0.3 (4.31)[c]	*ND*[d]	58.5 (1.69)[c]	2.6 (0.43)[c]	25.1 (3.83)[c]
6	10.9 (0.06)[c]	2.1 (3.16)[c]	0.3 (0.42)[c]	*ND*[d]	58.9 (0.15)[c]	2.3 (1.81)[c]	25.6 (0.49)[c]
7	14.3 (4.63)[c]	2.2 (3.3)[c]	1.9 (2.65)[c]	0.7 (6.9)[c]	53.4 (1.79)[c]	3.5 (0.8)[c]	24 (0.35)[c]
8	21.7 (3.03)[c]	4.3 (4.17)[c]	2.4 (6.26)[c]	0.8 (1.59)[c]	48.4 (1.92)[c]	4.5 (0.49)[c]	17.8 (0.55)[c]

[a]Mean of the percentage of crude canola oil varieties (duplicates).
[b]Crude commercial canola oil variety.
[c]Relative percentage standard deviation (% RSTD) calculated as (standard deviation/mean) x 10^2.
[d]Not detected.

Table 4. Quantification of pheophytin *a* and *b* and their derivatives in different varieties of crude commercial canola oil by HPLC analysis.

Variety[a]	Pheophytin *a* (μg pheophytin *a*/300 μL crude canola oil)	Pheophytin *b* (μg pheophytin *b*/300 μL crude canola oil)
1	4.89	0.18
2	4.17	0.17
3	25.21	0.73
4	23.01	1.20
5	10.90	0.14
6	9.73	0.12
7	9.43	0.35
8	6.41	0.37

[a]Variety of crude commercial canola oil.

respectively, whereas; the lowest and highest pheophytin b concentrations were, 0.12 μg/mL and 1.2 μg/mL, respectively. Variations in the green pigment content of the crude oil were expected, this could be explained either by a difference in the genetic composition within varieties or by the application of different cultural practices for the growth of the canola plant.

The green pigment content (pheophytin *a* and *b* concentrations) of the eight varieties of crude canola oil varied from 4.24 μg/mL to 25.94 μg/mL compared to vegetable oils where the green pigment content was 5 μg/mL (Suzuki and Nishioka, 1993). These high concentrations of green pigments in canola oil were expected since crude canola oil has the highest green pigment content compared to other vegetable oils (Kalmokoff *et al.*, 1988).

4.2. Alga Culture

4.2.1. Optimization of the Culture Growth Conditions for P. tricornutum

Optimization of culture conditions for the biomass production of the alga *P. tricornutum* with respect to chlorophyllase activity was investigated. The alga *P. tricornutum* was grown using different culture conditions, including incubation temperature, light intensity, and photoperiodic cycles. Table 5 shows the culture growth conditions used for nine different cultures.

The findings indicate that chlorophyllase activity was not detected in cultures 1, 2 and 3 which may be due to the cultures' high pH value of 10. In the fourth culture, the pH was adjusted to 8.4 by adding hydrochloric acid but there was still no chlorophyllase activity, this indicates an inhibitory effect of the acid. While in the fifth culture when the pH was adjusted by introducing a stream flow of CO_2, the presence of chlorophyllase activity first was detected. The mixture of CO_2 and water produced carbonic acid, H_2CO_3, a weak acid, which lead to an excess of hydrogen ions to the solution thereby reducing the pH of the culture medium. Cultures 6 to 9 show the positive effect of changes in light intensity, incubation temperatures and harvest period, on the chlorophyllase activity. The major factors that regulate algal growth are sunlight and temperature which enhance photosynthesis leading to an increase in algal growth and a gain in algal biomass

Table 5. Optimization of culture growth conditions for the biomass production of the alga *P. tricornutum* with respect to chlorophyllase activity.

Culture	Culture day[a]	Temperature (°C)[b]	Photoperiod[c]	Light intensity (μmol/m^2.s)[d]	pH	pH control	Chlorophyllase activity[e]
1	10	18	18L/6D	340	10	X	-
2	7/10	18	14L/10D	150	10	X	-
3	7/10/14	18D/14N	14L/10D	150	10	X	-
4	7/10/14	14	14L/10D	150	8.4	HCl	-
5	7/10/14	18D/10N	18L/6D	185	8.4	CO_2	+
6	7/8/9/10	18D/10N	18L/6N	185	8.4	CO_2	++
7	5/6/7	18D/10N	18L/6D	330	8.4	CO_2	+++
8	5/6/7/8	18D/10N	18L/6D	330	8.4	CO_2	++++
9	7	18D/10N	18L/6D	330	8.4	CO_2	+++++

[a]Recovery day of the culture biomass.
[b]Culture incubation temperature (°C) used as day (D) and night (N) periods.
[c]Photoperiod divided in light (L) and darkness (D) periods.
[d]Illumination provided by fluorescent lamps projecting an incident light intensity.
[e]Chlorophyllase activity scaled from - (absence of activity) to + (presence of activity). The increase in + sign signifies a higher activity.

(Hoek, 1995). The gain in algal growth and biomass increased the green pigment content which in turn affected positively on the activity of chlorophyllase.

In order to obtain the highest total chlorophyllase activity, the biomass of *P. tricornutum* was recovered after a period of culture growth of 7 days. In addition, the incubation temperature was maintained at 18°C for 18 h and 10°C for 6 h, while illumination was at an incident intensity of 330 μmol/m^2.s on an 18 h of light and 6 h of dark photocycle. The pH value, maintained by a stream flow of CO_2, of 8.4 was determined to be optimal for the culture medium.

4.2.2. Effect of Algal Growth Period on Chlorophyllase Activity

The effect of culture incubation time on the biomass production of *P. tricornutum* and chlorophyllase activity was investigated.

The spectrophotometric scans in Figure 7 show that there was an increase in absorbance at the wavelength of 678 nm that occurred during the ten days of culture incubation. The culture biomass at day 0 (Fig. 7A) showed low absorbance values corresponding to low growth of the alga, whereas at day 6 (Fig. 7B) there was a gradual increase in the absorbance values. The maximum absorbance values of 2.5 were attained at day 10, where the biomass was at its highest (Fig. 7D). From the scans, it was observed that an increase in the optical density of the biomass resulted in a prominent peak at 678 nm where green pigments are known to absorb. This increase in optical density at 678 nm may be due to an increase in the green pigment content of the alga which could in turn positively affect the activity of chlorophyllase. Kermasha *et al.* (1992) showed that there was a net increase in the chlorophyll *a* content of the alga during the log to the exponential phase of growth thereby suggesting the occurrence of a significant change in the photosynthetic activity of the alga during the cell cycle and the existence of a relationship between chlorophyll content and chlorophyllase activity.

Figure 8 shows that the biomass production of the alga *P. tricornutum* culture followed a typical microbial growth curve consisting of four distinct phases. The growth curve started with a lag phase of 2 days, followed by an exponential phase from day 3 to

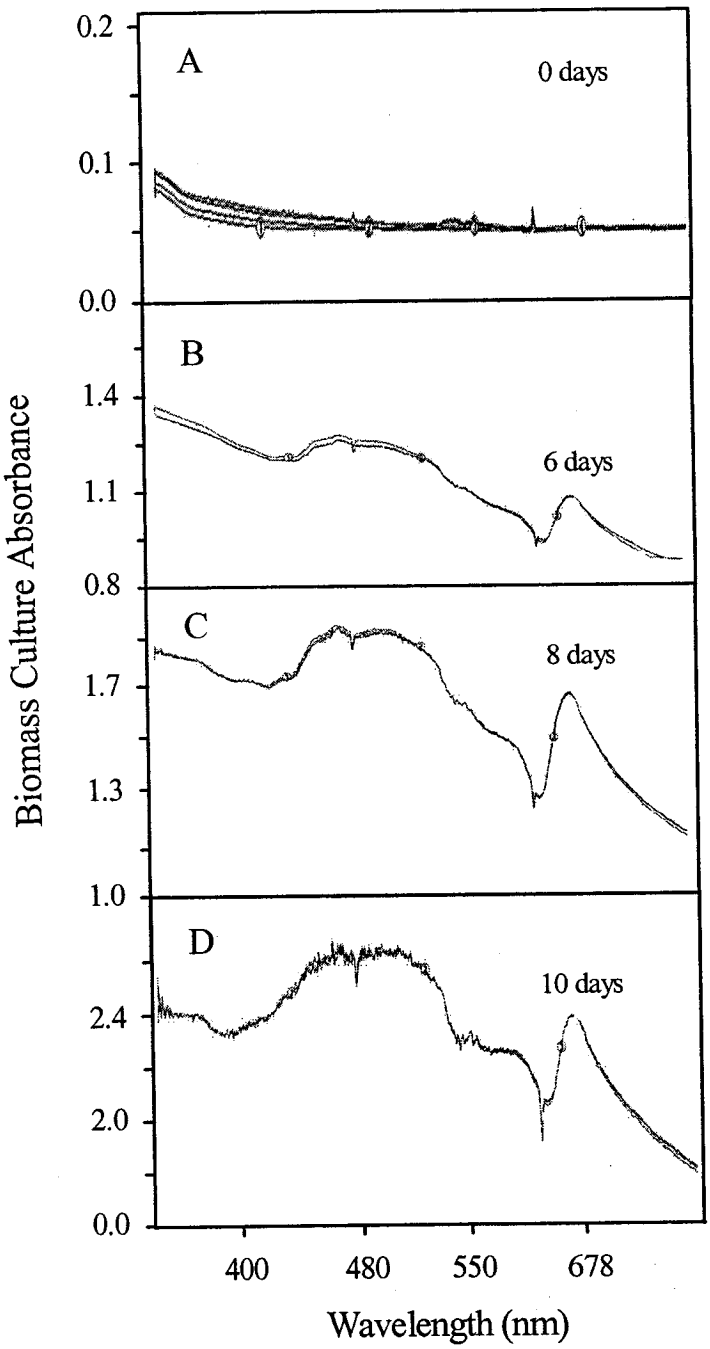

Figure 7. Spectrophotometric scans of the biomass of the alga *P. tricornutum* after 0, 6, 8 and 10 days of culture incubation.

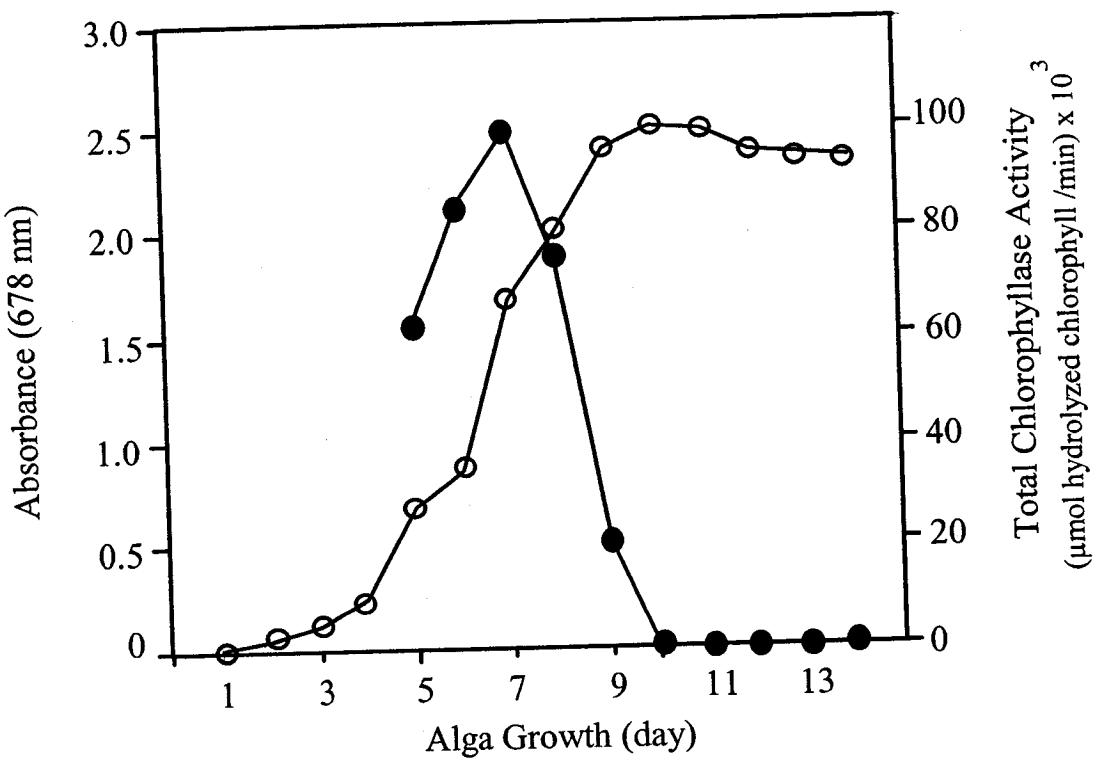

Figure 8. Effect of the alga *P. tricornutum* growth period on total chlorophyllase activity. Optical density of the culture biomass (o) and total activity of chlorophyllase (•).

9 with a specific growth rate of 0.4996 day^{-1}. A stationary phase followed the exponential phase at day 10 where the maximum absorbance reached 2.5 and formed a plateau until day 14.

Figure 8 shows that the growth curves of the alga growth and enzymatic activity followed the same pattern appreciably up to day 7. The total chlorophyllase activity increased from 60 µmol/min to 100 µmol/min, during the growth period of 5 to 7 days, respectively. Beyond the seventh day of growth, the total chlorophyllase activity decreased gradually. The decrease in the hydrolytic activity of chlorophyllase could be explained by a change in the physical conditions of the culture medium which could have resulted in the exhaustion of some critical nutrients or an accumulation of waste products thereby affecting the stability of the enzyme (Kazandjian *et al.*, 1986). Another possible explanation could be that increases in lipid concentrations occur as the cells age, which could inhibit the chlorophyllase enzyme (Khamessan *et al.*, 1992) as nutrient limitations in the culture have been positively associated with an increase in lipid abundance in *P. tricornutum* cells. Lambers and Terpstra (1985) also found that negatively charged lipids had a pronounced inhibiting effect on enzyme activity even at relatively low lipid concentrations.

In order to measure the level of enzymatic chlorophyllase activity, the specific activity and the protein content were measured. Figure 9 demonstrates the relationship between the specific activity, total activity and the total protein content of the enzymatic extract of the algal cells. The results show that as the growth rate increased (Fig. 9), the total protein content increased but the specific activity decreased. The specific activity decreased by 1.4 times during the growth period of day 5 to day 7.

The overall findings (Fig. 9) indicate that after a period of 7 days, optimal total chlorophyllase activity was obtained from the biomass of *P. tricornutum* and used for subsequent studies.

4.2.3. Effect of Lyophilization on the Hydrolytic Activity of Chlorophyllase

The effect of lyophilization on chlorophyllase activity (Fig. 10) was investigated. Chlorophyllase activity was measured using chlorophyll as a substrate, in a

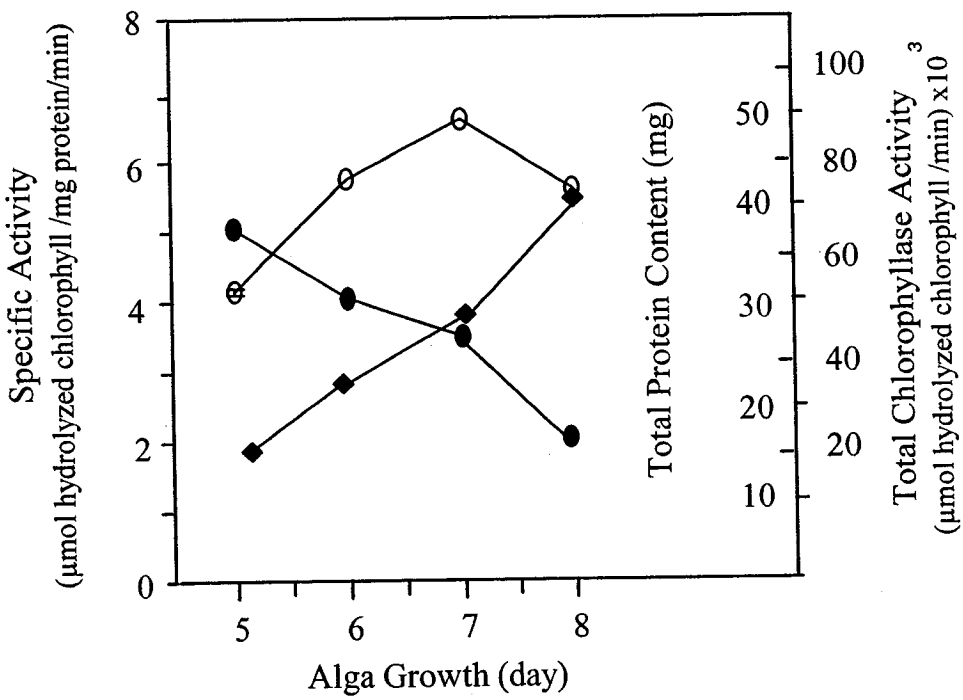

Figure 9. Effect of alga growth period on the total activity (o), specific activity (•), and total protein content (♦) of the partially purified chlorophyllase extracts.

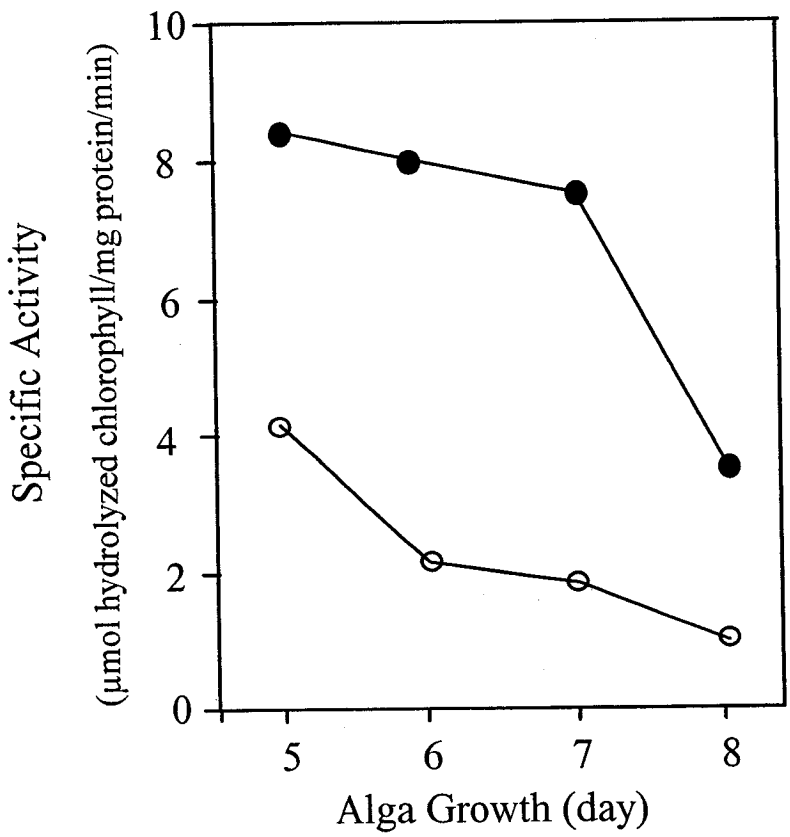

Figure 10. Effect of lyophilized (o) and fresh (•) algal *P. tricornutum* extracts on the specific activity of chlorophyllase.

water-miscible system of 7 % (v/v) acetone. Figure 10 indicates that lyophilization of the algal biomasses obtained at five, six, seven and eight days of culture growth decreased the hydrolytic activity by 2.03, 3.68, 4.05, and 3.55 times, respectively, compared to the use of fresh algal biomasses.

Many possible explanations for chlorophyllase inactivation have been suggested. First, a disruption in protein configuration (unfolding of the helical structure) could have been caused by an increase in salt concentration within the cells as water was removed as ice (Golblith, 1975). Meryman (1966) suggested that a sudden fall in temperature has a damaging or lethal effect on many types of cells in the liquid state since freezing of plant and animal tissues leads to immobilization of water as ice and results in an increased concentration of solutes. While activity of some enzymes is not appreciably affected by the presence or absence of salts, the activity of others can greatly be influenced by the nature and concentration of ions present depending on the enzyme and the nature and concentration of the salts. The combined effect of solute concentrations and lowering of temperature can therefore greatly reduce the specific activity of enzymes (Oetjen, 1999). Meryman (1966) demonstrated that the impairment of certain metabolic functions after freezing could result probable alterations of enzyme systems involved.

The second reason for freezing damage is through the formation of intra cellular ice crystals that may alter the permeability control mechanisms of the cell thereby leading to cell wall breakage and rendering the enzyme more accessible and decreasing its activity (Golblith *et al.*, 1975). During enzyme purification, the activity of the enzyme decreased throughout the homogenization step. These findings suggest that mechanical homogenization may have altered protein activity as the mechanical forces involved are a factor to consider in keeping the structure and stability of the protein intact while purifying. Although shear force is needed to disrupt the cells, it may have damaged the enzymes making the proteins inactive (Hower, 1979).

Based on these overall results, the chlorophyllase enzyme was extracted from the fresh biomass of *P. tricornutum* and used in subsequent kinetic studies.

4.3. Optimization of Chlorophyllase Bioconversion in the Presence of Canola Oil

4.3.1. Biocatalysis of Chlorophyllase in RBD Canola Oil

After optimization of the growth conditions for the alga *P. tricornutum* as related to the development of chlorophyllase activity, a study on optimizing the biocatalysis of chlorophyllase in the presence of refined bleached deodorized (RBD) canola oil, using chlorophyll and pheophytin as substrates was investigated.

4.3.1.1. Effect of Oil Concentration on Chlorophyllase Activity

The effect of refined bleached deodorized (RBD) canola oil on chlorophyllase activity, using chlorophyll and pheophytin as substrates, in an aqueous/miscible organic solvent system containing 10 % (v/v) acetone, was investigated at 0, 10, 20, 30 and 40 % (v/v) canola oil concentrations. Figures 11A and 12A indicate that the presence of RBD canola oil to the system decreased the hydrolytic activity of chlorophyllase. The presence of 10 % RBD canola oil decreased the hydrolytic activity by 1.4 and 1.2 times, for chlorophyll and pheophytin as substrates, respectively, while the presence of 30 % RBD canola oil decreased the hydrolytic activity by 3.8 and 4.1 times, respectively. In contrast, Kalmokoff *et al.* (1988) indicated that chlorophyllase hydrolyzed only chlorophyll and not pheophytin, in a buffer/acetone system in the presence of RBD canola oil. The variation in these results may be due to differences in the conditions of the enzymatic assay including the experimental approach, the buffer/acetone ratios and the oil concentration.

A possible estimation of chlorophyllase inactivation could be determined by calculating the inactivation constant k and the inhibitor factor I_{50} (the concentration of RBD canola required to decrease the chlorophyllase activity by 50 %). The inactivation of chlorophyllase by canola oil was found to follow first order kinetics, since the plot of the logarithm of the specific activity against the oil concentration showed a straight line with a good correlation coefficient of 85.9 % and 89.9 %, for chlorophyll and pheophytin, respectively (Fig. 11B and 12B). Table 6 shows the values of the pseudo first order constant k and I_{50} values estimated from the slope of the straight lines of Figures 11B and 12B.

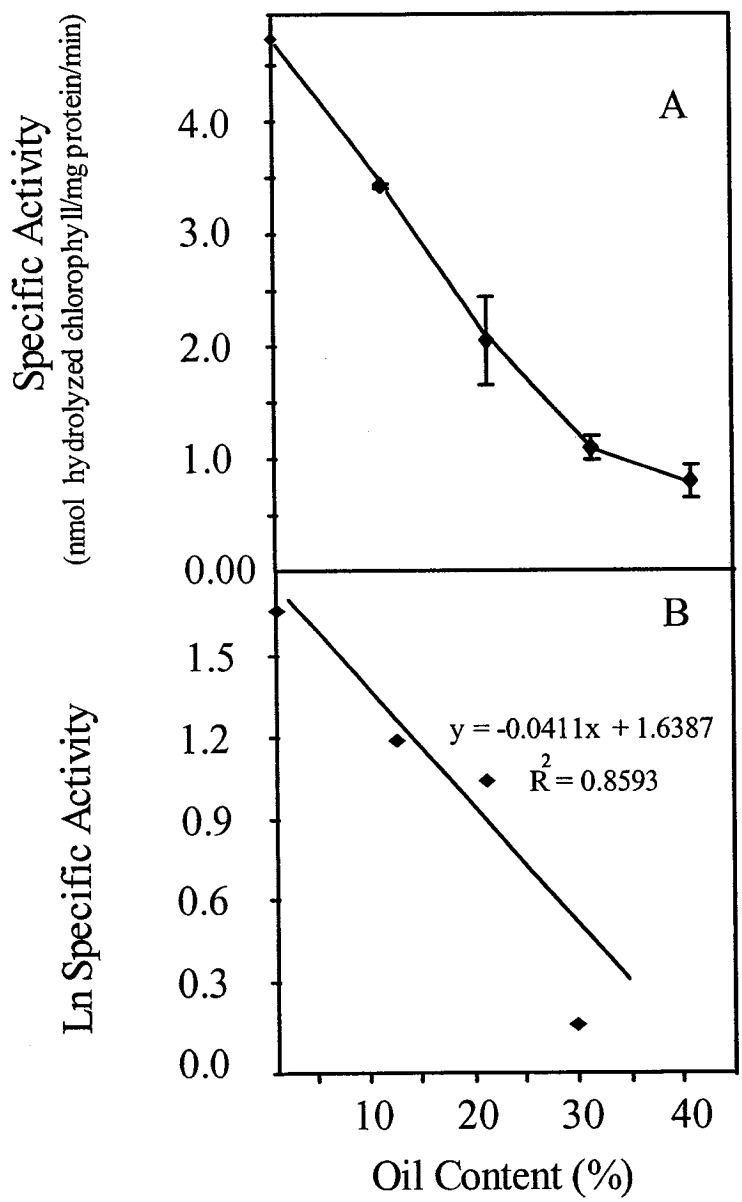

Figure 11. Effect of oil content on (A) specific chlorophyllase activity and (B) Ln specific activity in a system containing chlorophyll as a substrate.

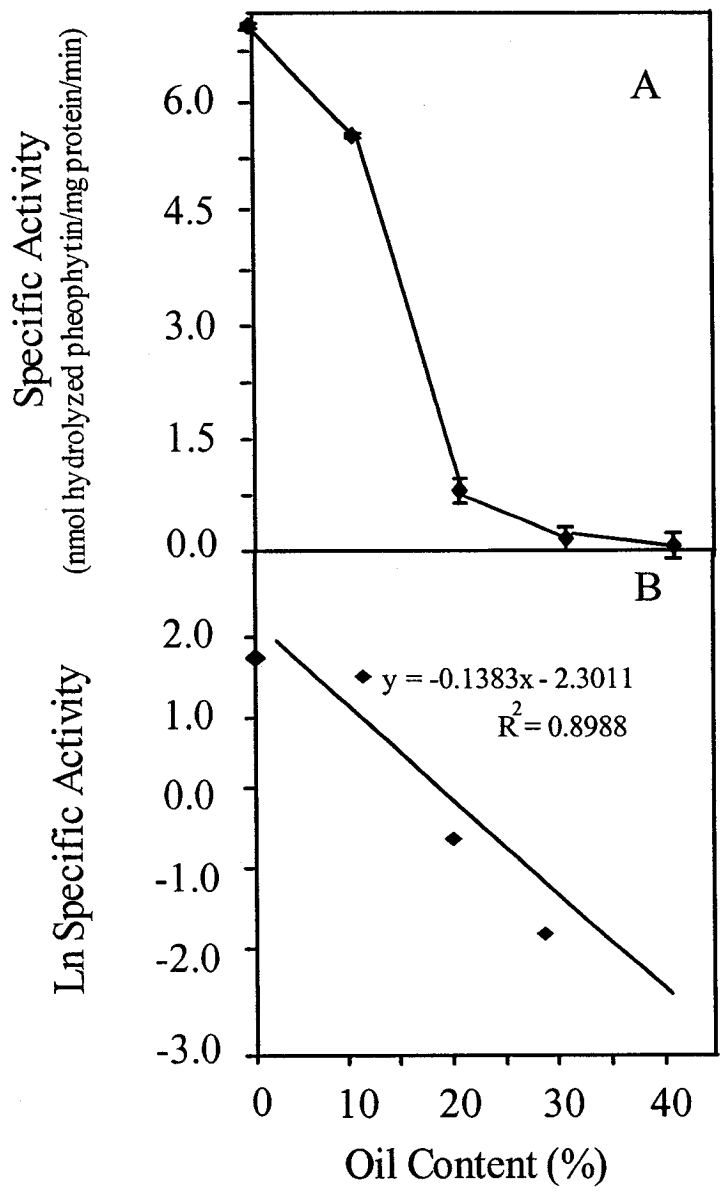

Figure 12. Effect of oil content on (A) specific chlorophyllase activity and (B) Ln specific activity in a system containing pheophytin as a substrate.

Table 6. Inhibitory effect of RBD canola oil on the activity of chlorophyllase using chlorophyll and pheophytin as substrates.

Substrate	k[a]	I_{50}[b]
Chlorophyll	0.0411	18.9
Pheophytin	0.1383	7.8

[a]k is the inactivation constant defined as 1/% (determined from the slope of the equations of Figures 11B and 12B).

[b]I_{50} is the inhibitor factor defined as the percentage of oil (% v/v) required to decrease the chlorophyllase activity by 50 %.

The inactivation of the enzyme by canola oil can be described by a first order kinetic equation.

$$A_o = A_i e^{-kI_{50}} \qquad \text{(Equation 3)}$$

where, *A_o is the specific activity at an oil percentage, defined as mmoles of hydrolyzed substrate/mg protein/min; A_i is the initial specific activity, defined as µmoles of hydrolyzed substrate/mg protein/min; k is the inactivation constant defined as 1/%; I_o is an oil percentage, defined as % (v/v); and I_{50} is the inhibitor factor defined as the oil percentage (v/v) where the chlorophyllase activity decreased by 50 %.*

Table 6 shows that the addition of 18.9 % and 7.8 % RBD canola oil (v/v) to chlorophyllase reaction media containing chlorophyll and pheophytin, respectively, decreased the specific activity of chlorophyllase by 50 %. Khamessan and Kermasha (1996) reported that the presence of 5 % canola oil decreased chlorophyllase activity by 50 % in a ternary micellar system containing Span 85. Based on our calculation, their I_{50} value was 2.3 % (with an activation constant of 0.068); which was much lower than the one obtained in our work (Table 5).

Table 6 demonstrates that the I_{50} values were lower in the presence of pheophytin than those obtained with chlorophyll. This difference may be attributed to the effect of oil on the conformation of the enzyme which may result in a change in the affinity of chlorophyllase for each of its substrates. These findings also suggest that the availability of the substrates to the enzyme decreased in the presence of oil due to the degree of solubility of both substrates thereby resulting in a partitioning of the substrates between the water-miscible system and the oil. Buskov (1985) reported that pheophytin was less available to the enzyme in the presence of oil than chlorophyll due to the difference in their polarities.

The refined bleached deodorized canola oil was found to act as a chlorophyllase inhibitor since it may have disrupted the tertiary structure of the enzyme thereby denaturing the protein and causing it to lose its activity (Buskov *et al.*, 1985).

Based on these findings, an RBD oil concentration of 20 % (v/v) was used in the following experimental work. This oil percentage was considered since it was an intermediate percentage of enzymatic inhibition.

4.3.1.2. Effect of Acetone Concentration on Chlorophyllase Activity

The effect of acetone, ranging from 0 to 20 % (v/v), on chlorophyllase activity in a water/miscible organic solvent system containing 20 % (v/v) RBD canola oil was investigated. Figure 13 demonstrates that the presence of 5 % acetone increased the hydrolytic activity by 1.1 and 1.2 times for chlorophyll and pheophytin, respectively, while the addition of 10 % acetone increased the hydrolytic activity by 1.5 and 1.8 times, respectively. However at higher acetone concentrations, the hydrolytic activity decreased. The optimum acetone content for the chlorophyllase-catalyzed hydrolytic reaction was 10 % for chlorophyll and pheophytin, respectively. McFeeter *et al.* (1971) reported that the highest hydrolytic activity was obtained at 17.5 % acetone in a water/miscible-organic-solvent system in the absence of canola oil while higher concentrations of acetone caused enzymatic inhibition.

The acetone acted as an activator and as an inhibitor at low and high concentrations, respectively.

The increase in the specific activity of chlorophyllase in the presence of low concentrations of acetone may be explained by the role of acetone as a co-solvent that shifts the partition coefficient of chlorophyll and pheophytin more in favor of the buffer solution thereby increasing the affinity of chlorophyll and pheophytin at the oil/buffer interface and consequently improving the proximity of the enzyme to the substrate. Garcia and Galindo (1991) reported that the hydrolytic activity of chlorophyllase increased at acetone concentrations. The overall findings suggest that acetone may disaggregate chlorophyll dimers and oligomers to form monomers that could be more appropriate for substrate-enzyme interactions (Khamessan *et al.*, 1994).

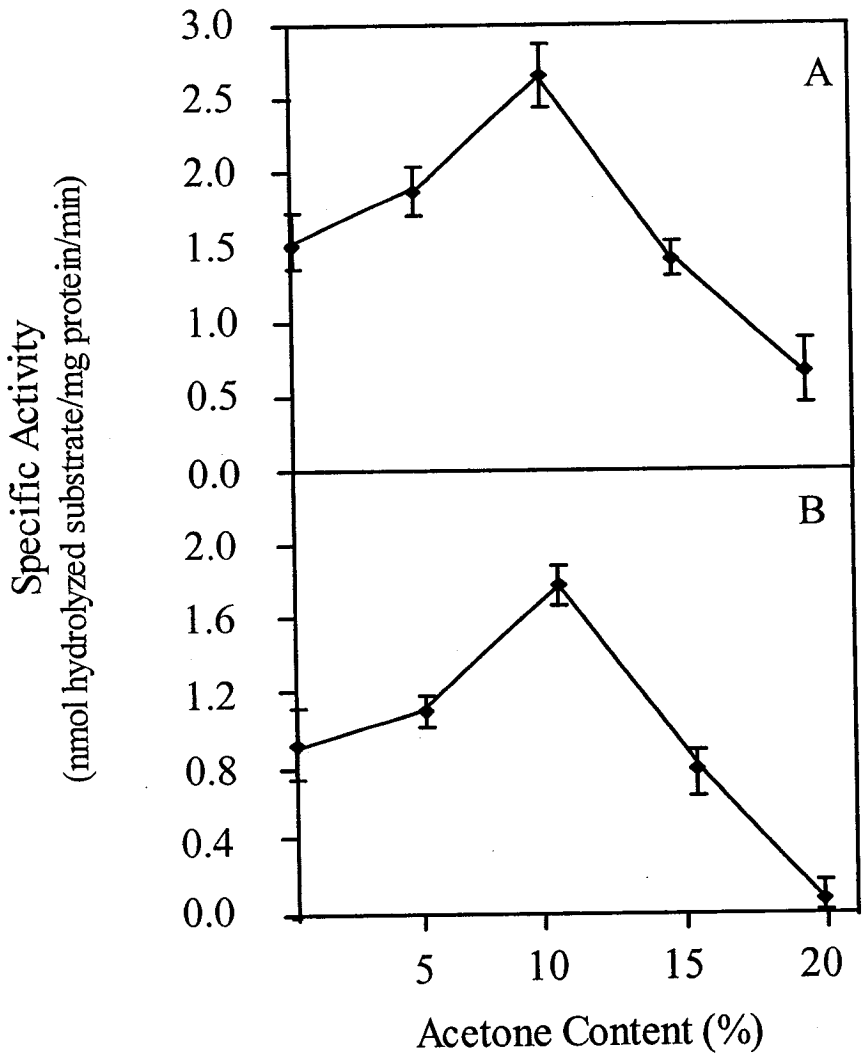

Figure 13. Effect of acetone content on chlorophyllase activity in a system using (A) chlorophyll and (B) pheophytin as substrate.

The decrease in the hydrolytic activity of chlorophyllase in the presence of acetone concentrations above 10 % could be explained by the following hypotheses: (a) the hydrophilic nature of acetone which could have stripped the essential water surrounding the enzyme molecule; (b) inactivation due to protein precipitation (McFeeter *et al.* 1971); (c) inactivation due to alteration of the native confirmation of the protein due to disruption of the hydrogen bonds and hydrophobic interactions (Dordick, 1989). The phenomenon of acetone inhibition beyond a concentration of 30 % was reported by Khamessan *et al.* (1994). In addition, Fernandez-Lopez *et al.* (1992) indicated that chlorophyllase activity decreased in the presence of acetone concentrations higher than 35 %.

Based on these findings, an acetone concentration of 10 % (v/v) was used in the following experimental work.

4.3.1.3. Effect of Enzyme Concentration on Chlorophyllase Activity

The effect of enzyme concentration on chlorophyllase activity, using chlorophyll and pheophytin as substrates in a water-miscible system containing 10 % (v/v) acetone and 20 % (v/v) RBD canola oil, was investigated using 0.16, 0.33, 0.50, and 0.66 mg of crude enzyme extract/mL. Figures 14A and 15A show that increases in the bioconversion rates occurred with concomitant increases in enzymatic concentration. The highest enzyme concentration of 0.66 mg/mL produced 4.0 μmole/L and 2.9 μmole/L of hydrolyzed chlorophyll and hydrolyzed pheophytin, respectively, whereas the lowest enzymatic concentration of 0.16 mg/mL produced 2.7 μmole/L and 2.6 μmole/L of hydrolyzed chlorophyll and hydrolyzed pheophytin, respectively.

In general, the overall results show that chlorophyll and pheophytin were rapidly hydrolyzed within the first 3 h by chlorophyllase to produce chlorophyllide and pheophorbide, respectively. In the first 3 h, the hydrolyzed substrate concentrations increased at a high rate, it is followed by a decrease in the rate until around 7 h where it stabilized.

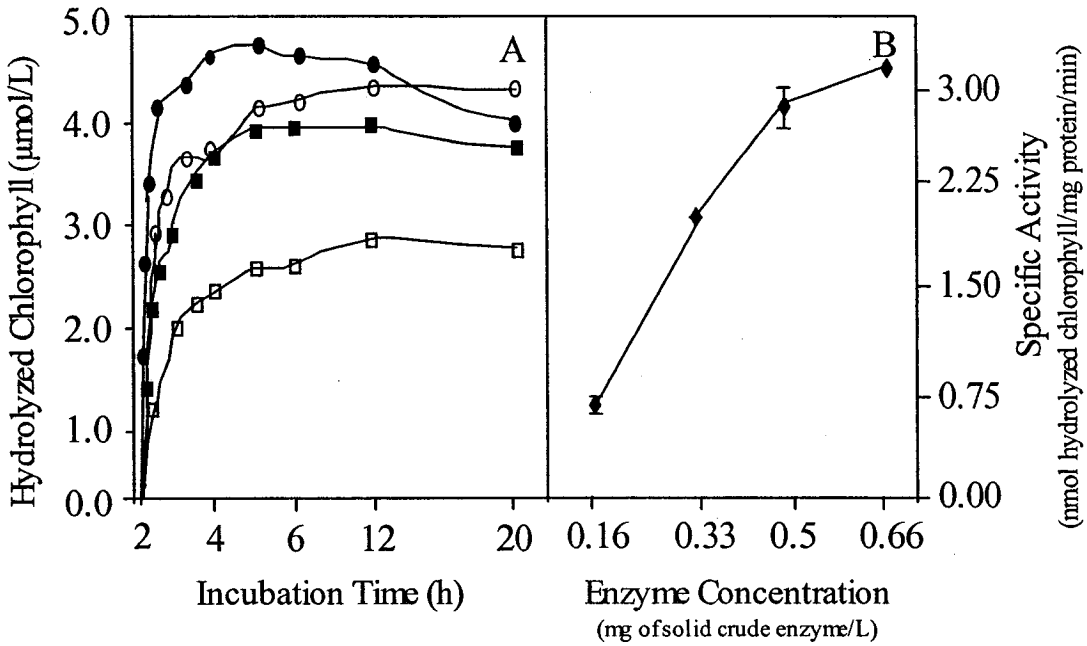

Figure 14. Effect of enzyme concentration on chlorophyllase activity in a system containing chlorophyll as a substrate: (A) enzyme activity at different enzyme concentrations of 0.66 mg/mL (•), 0.5 mg/mL (○), 0.33 mg/mL (■), and 0.16 mg/mL (□) versus incubation time and (B) specific activity at different enzyme concentrations.

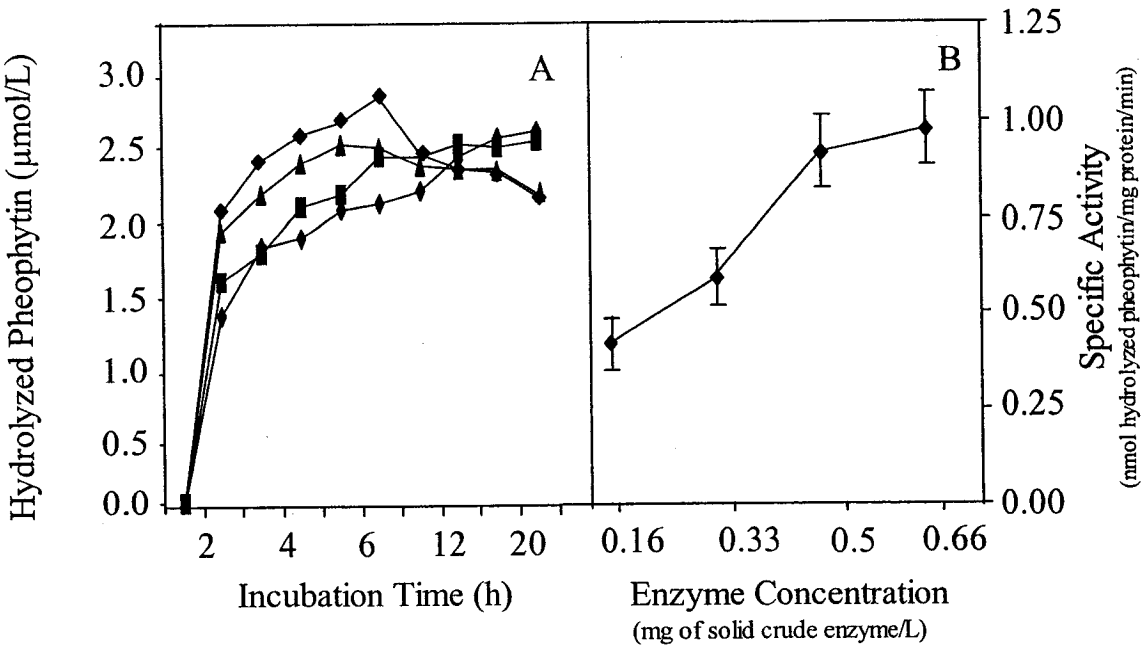

Figure 15. Effect of enzyme concentration on chlorophyllase activity in a system containing pheophytin as a substrate: (A) enzyme activity at different enzyme concentrations of 0.66 mg/mL (♦), 0.5 mg/mL (■), 0.33 mg/mL (▲), and 0.16 mg/mL (•) versus incubation time and (B) specific activity at different enzyme concentrations.

No further increases in the hydrolytic activity suggest that the enzyme was inactivated due to either an increase in incubation temperature with time (Guraya *et al.*, 2001) or due to the presence of a phytol group that was a by-product of the reaction. Levadoux *et al.* (1987) reported that phytol showed a strong non-competitive inhibitory effect on chlorophyllase activity. Non-competitive inhibitors can combine with an enzyme molecule to produce a dead-end complex, regardless of whether the substrate molecule is bound to the enzyme molecule, and decrease the hydrolytic activity of the enzyme while not affecting substrate binding (Khamessan *et al.*, 1994).

The results (Figs. 14B and 15B) show that an increase in chlorophyllase concentration from 0.16 to 0.33 mg/mL increased the hydrolytic activity of the chlorophyllase by 3.34 times for chlorophyll and 1.40 times for pheophytin, while an increase in chlorophyllase concentration from 0.16 to 0.66 mg/mL, increased the hydrolytic activity by 4.9 and 2.4 times for chlorophyll and pheophytin, respectively.

Figure 14B indicates that the rate of reaction was directly proportional to the amount of chlorophyllase with chlorophyll as the substrate. However, Figure 15B demonstrates the formation of a plateau after an enzyme concentration of 0.66 mg/mL thereby suggesting that a substrate saturation point was attained, with pheophytin as the substrate. The differences in these findings may be due to differences in the affinity of the enzyme for chlorophyll and pheophytin as substrates.

Based on these findings, an enzymatic concentration of 0.33 mg/mL was used in the following experimental work.

4.3.1.4. Effect of Agitation Speed on Chlorophyllase Activity

Since the rate of reaction in an aqueous/miscible organic solvent system containing refined bleached deodorized (RBD) canola oil can be affected by the mass diffusion of the substrate from the oil phase into the aqueous phase, the effect of diffusional limitations on the reaction rate was investigated.

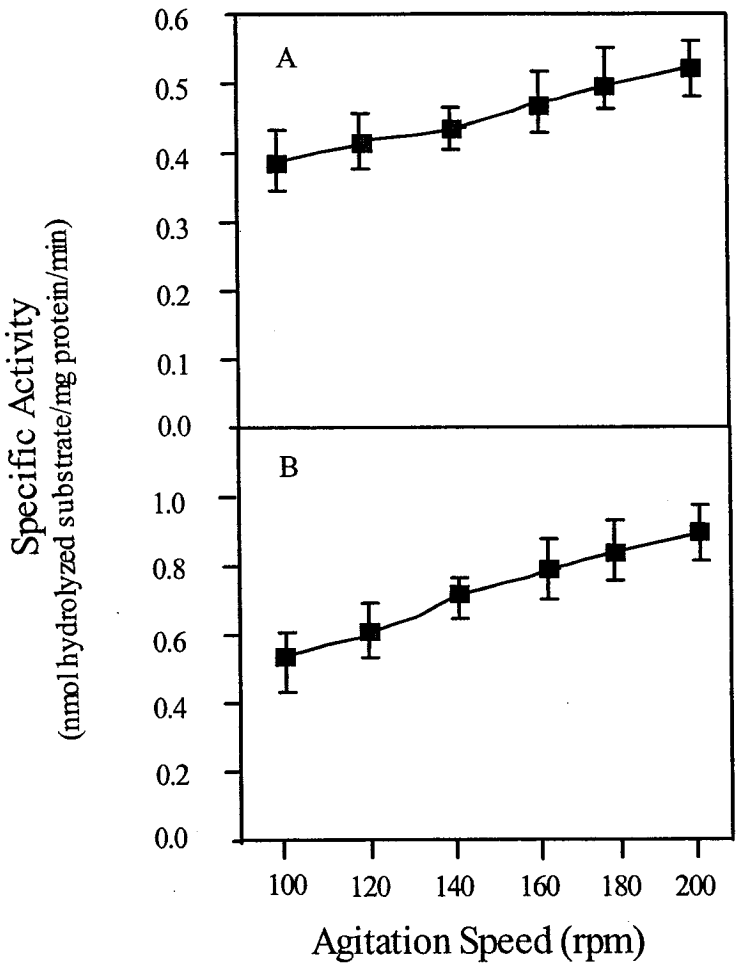

Figure 16. Effect of shaker speed on chlorophyllase activity using (A) chlorophyll and (B) pheophytin as substrates.

The effect of diffusional limitations on reaction rate was studied by examining the effect of the agitation speed on enzyme activity. The effect of agitation speed on the activity of chlorophyllase, using chlorophyll or pheophytin as substrates, in a water-miscible organic solvent system containing 10 % acetone and 20 % oil was investigated at 100, 120, 140, 160, 180 and 200 rpm. Figure 16 indicates that an increase in speed from 100 to 200 rpm, increased chlorophyllase activity by 1.4 and 1.7 times with chlorophyll and pheophytin, respectively. The difference in these values indicated that agitation speed had a higher effect on the hydrolytic activity of chlorophyllase in the presence of pheophytin as substrate compared to that obtained with chlorophyll. These results may be attributed to differences in polarity between the two substrates. The slope of the chlorophyll curve was 1.6×10^{-3} with a correlation coefficient of 0.9755, whereas the slope of the pheophytin curve was 3.9×10^{-3} with a correlation coefficient of 0.9617.

These findings suggest that mass transfer in the reaction medium increased at higher agitation speeds thereby resulting in an improvement in the physical contact between the chlorophyllase enzyme and the substrates. A decrease in substrate particle size may have occurred in the oil phase, contributing to an increase in the number of substrate particles in the aqueous phase, thus, making the substrate more available to the enzyme's active sites at higher agitation speeds. Levadoux *et al.* (1987) demonstrated that maximum chlorophyllase activity in a buffer/canola oil medium required intensive mixing of the substrate, enzyme, and liquid phases. Intensive mechanical mixing could increase the interfacial area, and hence enhance enzyme activity by physically improving the contact between the enzyme and the substrate (Levadoux *et al.*, 1987).

Based on these results, the agitation speed of 200 rpm was used in the following experimental work.

4.3.1.5. Effect of Incubation Temperature on Chlorophyllase Activity

The effect of incubation temperature, ranging from 20°C to 40°C, on the chlorophyllase activity in water/miscible organic solvent system containing 10 % (v/v) acetone and 20 % (v/v) RBD canola oil was investigated. Figures 17A and 18A show that the specific activity increased by a factor of 1.9 and 2.4, for chlorophyll and pheophytin,

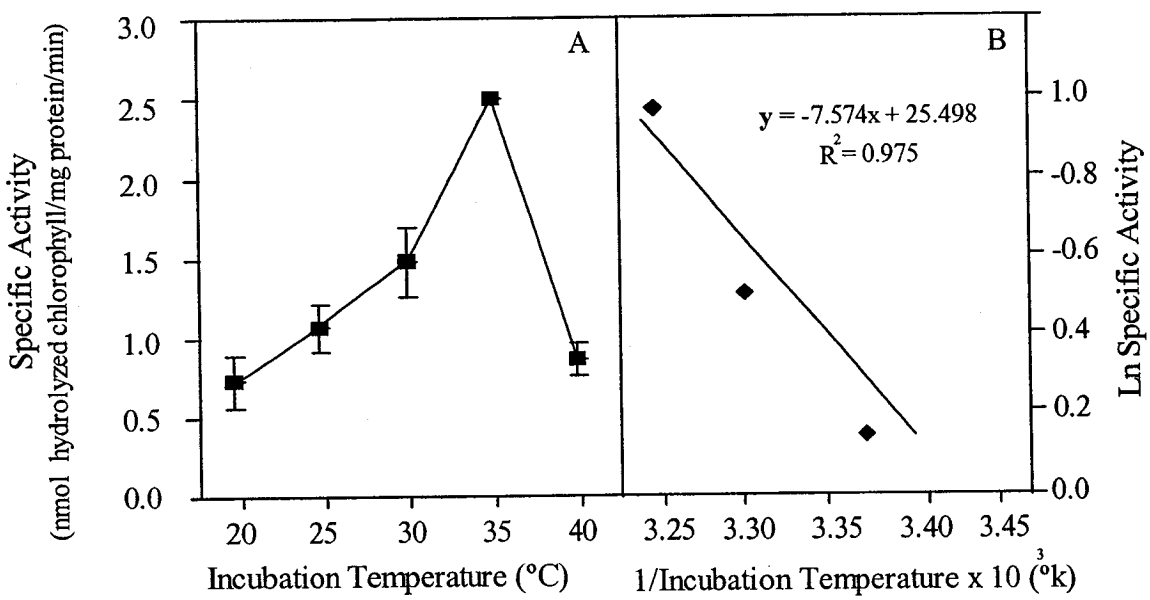

Figure 17. Effect of incubation temperature on chlorophyllase activity with chlorophyll as a substrate: (A) incubation temperature versus specific activity and (B) Arrhenius plot.

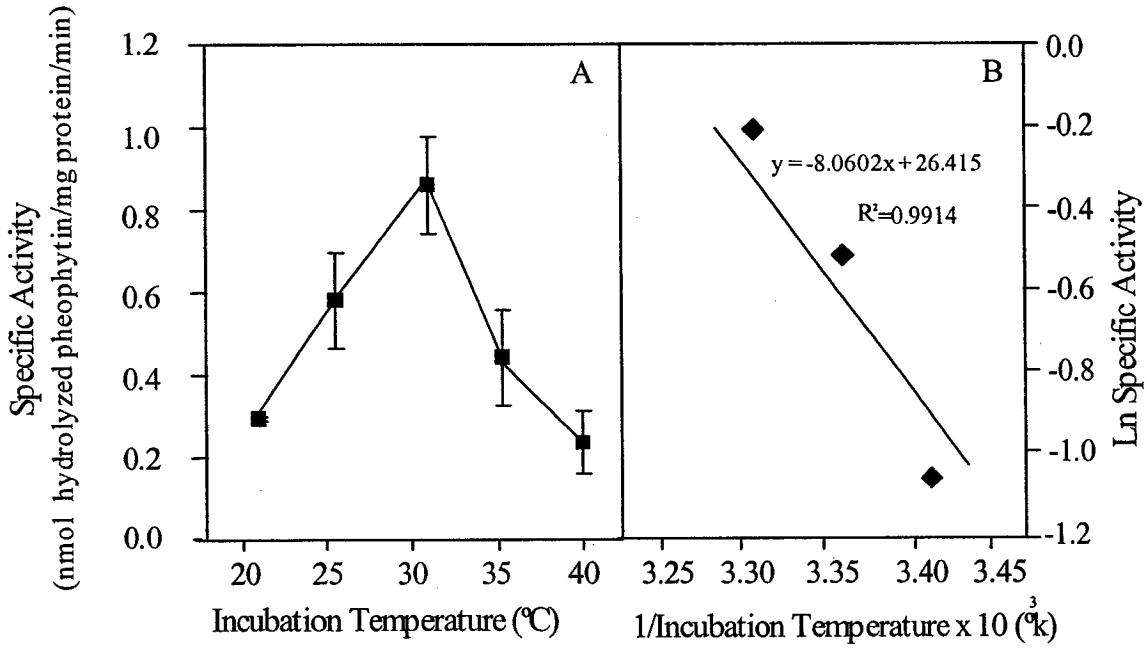

Figure 18. Effect of incubation temperature on chlorophyllase activity with pheophytin as a substrate: (A) incubation temperature versus specific activity and (B) Arrhenius plot.

respectively, when the incubation temperature increased from 20°C to 30°C. The results also demonstrate that increasing the incubation temperature to 35°C increased the hydrolytic activity of chlorophyllase by 3.2 times with chlorophyll as substrate, but increased the hydrolytic activity by 1.3 times with pheophytin. At higher temperatures, the decrease in hydrolytic activity may be due to thermal inactivation of the enzyme.

The ascending part of the curves in Figures 17A and 18A followed the Arrhenius law, as shown in Figures 17B and 18B (the logarithmic plot of specific activity versus 1/Temperature was linear). The slopes of these plots were used to determine the activation energies of chlorophyllase in the presence of chlorophyll and pheophytin.

The increase in the activity of a reaction as a function of temperature can be described by Arrhenius law.

$$V = A_i e^{-(Ea/RT)} \qquad \text{(Equation 4)}$$

where; *V is the rate constant at a specific temperature defined as mmoles of hydrolyzed substrate/mg protein/min; A_i is a pre-exponential constant; E_a is the activation energy defined as kJ/mole; R is the gas constant (8,31 mole.°k/J); and T is the temperature in Kelvin (k).*

Table 7 shows that the optimal incubation temperatures for chlorophyllase with chlorophyll and pheophytin were 35°C and 30°C, respectively. These findings might suggest that the effect of incubation temperature on chlorophyllase stability was higher in the presence of pheophytin as substrate rather than with chlorophyll.

It was reported in the literature, that depending on the plant source, chlorophyllase shows maximal activity in the temperature range of 30°C to 40°C (Drazkiewicz, 1994). In agreement, Khamessan *et al.* (1996) reported the optimum temperature of 35°C for chlorophyllase activity in micellar ternary systems with chlorophyll as a substrate.

The activation energy values (Table 7) were in the range of those reported in the literature for catalytic reactions of other enzymes. In general, the activation energies varied from 40 to 200 kJ/mole (Rob *et al.*, 1997). The activation energy for the hydrolysis of azocasein was determined to be 65 kJ/mole (Secades *et al.*, 1999), for polyphenol

Table 7. Optimal temperature and activation energy of chlorophyllase using chlorophyll and pheophytin as substrates.

Substrate	Optimal Temperature	E_a [a]
Chlorophyll	35°C	66.9
Pheophytin	30°C	64.4

[a] E_a is the activation energy defined as kJ/mole.

oxidase (PPO) 90 kJ/mole (Arogba *et al.*, 1998), and for peroxidase 80 kJ/mole (Rob *et al.*, 1997). The activation energies were reported to be 88, 42, 12, and 34 kJ/mole for the tyrosinase biocatalysis in the reaction medias of heptane, toluene, dichloromethane and dichloroethane, respectively (Kermasha *et al.*, 2001).

The activation energy values in the presence of chlorophyll and pheophytin as substrates were slightly different thereby indicating that the sensitivity of the reaction rate to temperature was slightly higher in the presence of pheophytin compared to chlorophyll. These findings suggest that chlorophyllase stability was greater in the presence of chlorophyll than pheophytin.

The overall findings suggest that the hydrolytic activity of chlorophyllase decreased with an increase in incubation temperature, which could be attributed to the occurrence of chemical reactions that denature the enzyme (Ahern and Klibanov, 1999). A fundamental step in the thermo inactivation of enzymes is the unfolding of the protein which results due to a decrease in the different covalent forces that maintain the native catalytically active structure (Guadalupe *et al.*, 1986). Khamessan *et al.* (1995) also found that 60 % of the hydrolytic activity of chlorophyllase in a biphasic system was lost when the incubation temperature was raised to 40°C.

Based on these findings, the optimal incubation temperatures of 35°C and 30°C for the chlorophyllase reactions involving chlorophyll and pheophytin, respectively, were used in the subsequent experimental work.

4.3.1.6. Effect of Substrate Concentration on Chlorophyllase Activity

The effect of chlorophyll and pheophytin concentration on chlorophyllase activity was determined in an aqueous/miscible organic solvent system, containing 10 % acetone and 20 % RBD canola oil. The chlorophyll and pheophytin concentrations investigated varied from 0 to 14.9 μM. Maximum velocity (specific activity) was calculated from the results obtained (Figs. 19A and 20A). The objective of this study was to determine the capacity the system could attain in relation to its substrate concentration.

Figures 19A and 20A show that the reaction velocity increased with an increase in substrate concentration. A maximum bioconversion rate was attained after which it stabilized, since the enzyme was inhibited either due to thermal inactivation or inhibition by the phytol group. The products of the reaction were chlorophyllide and phytol from chlorophyll and pheophorbide and phytol from pheophytin. Phytol showed a competitive inhibitory effect due to the influence on the equilibrium state of the bioconversion of chlorophylls and pheophytins into chlorophyllides and pheophorbides, respectively (Khamessan *et al.*, 1993). The molecule phytol decreased the hydrolytic activity of the enzyme by binding to the esterification active site (Levadoux *et al.*, 1987).

Figure 19B indicates that the specific activity increased by a factor of 1.9 when the chlorophyll concentration increased from 3.3 to 7.9 μM, whereas Figure 20B shows that the specific activity increased by a factor of 3.44 when the pheophytin concentration increased from 3.3 to 9.3 μM. As the substrate concentration increased, the specific activity also increased at a directly proportional rate up to concentrations of 12.6 μM and 9.3 μM for chlorophyll and pheophytin, respectively.

At higher concentrations, the hydrolytic activity decreased which could be due to chlorophyllase inactivation by the inhibitory phytol product. The optimal substrate concentration for chlorophyll and pheophytin were 12.6 and 9.3 μM, respectively since the reactions did not follow Michaelis-Menten law, the K_m and V_{max} values estimated from the Lineweaver-Burk plot were not calculated. Comparison of both substrates showed that a lower concentration of pheophytin was required to decrease the specific activity of chlorophyllase in comparison to chlorophyll. These findings suggest that the presence of high concentrations of pheophytin would inhibit the activity of chlorophyllase.

4.3.2. Biocatalysis of Chlorophyllase in Crude Canola Oil

The enzyme assays were performed using the optimized conditions obtained in section 4.3.1. using 20 % (v/v) crude canola oil, 10 % (v/v) acetone at 30°C with an agitation speed of 200 rpm.

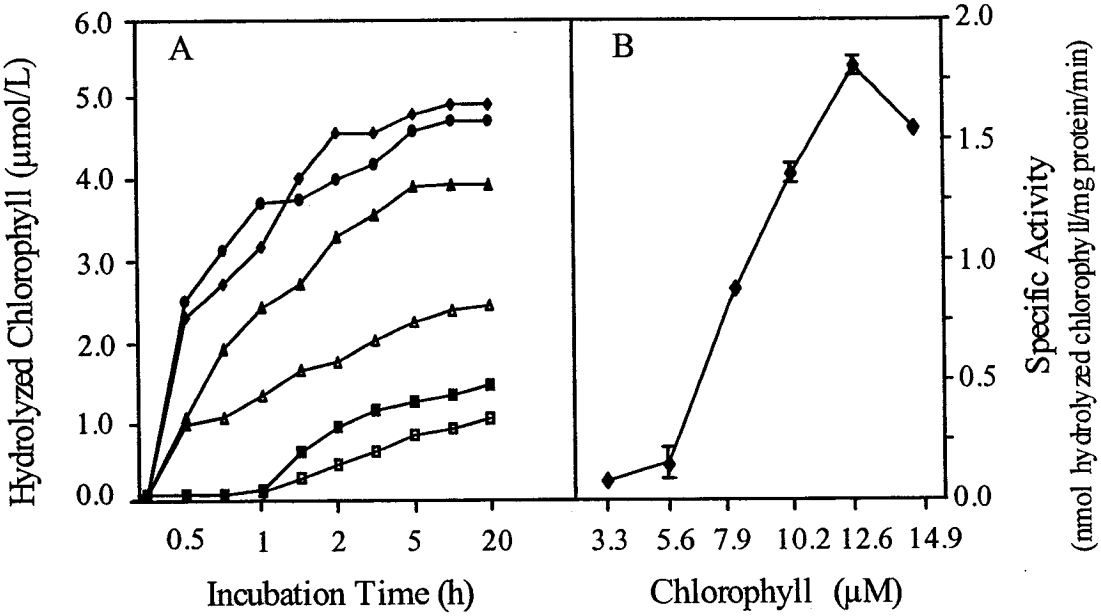

Figure 19. Effect of substrate concentration on chlorophyllase activity with chlorophyll as a substrate: (A) enzyme activity at different substrate concentrations 3.3 µM (□), 5.6 µM ('), 7.9 µM (!), 10.2 µM ("), 12.6 µM (o), and 14.9 µM (•) and (B) specific activity at different substrate concentrations.

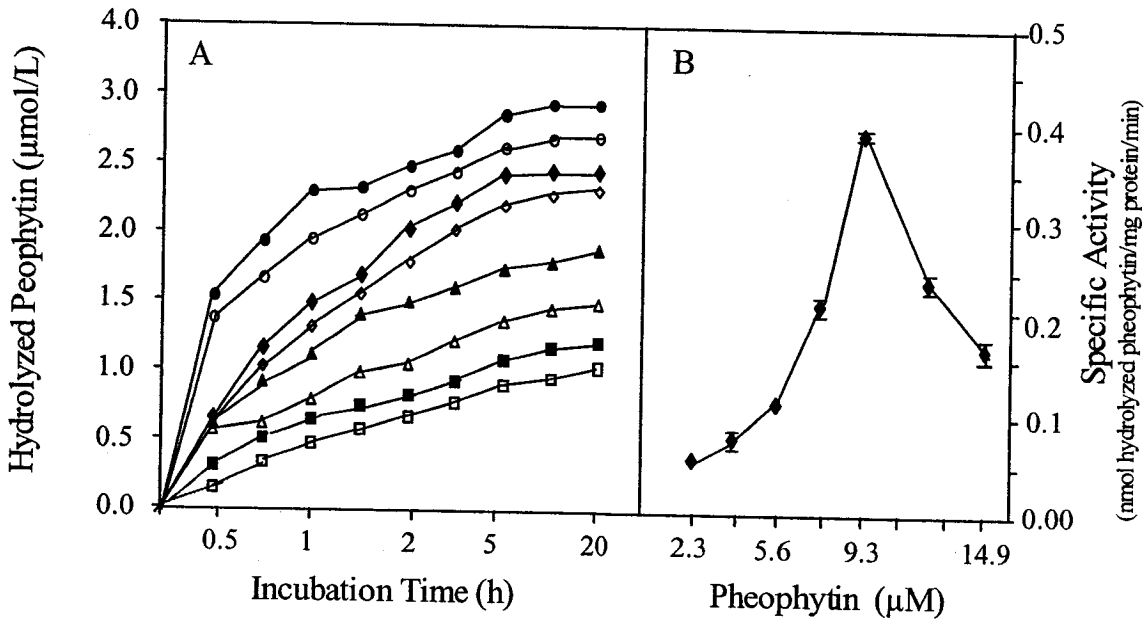

Figure 20. Effect of substrate concentration on chlorophyllase activity with pheophytin as a substrate: (A) enzyme activity at different substrate concentrations 2.3 μM (□), 3.3 μM (■), 5.6 μM (Δ), 7.9 μM (▲), 9.2 μM (◊), and 10.2 μM (♦), 12.6 μM (o), and 14.9 μM (•) and (B) specific activity at different substrate concentrations.

Table 8 shows the specific activity of chlorophyllase in reaction media containing crude canola oil were quite different from those obtained with media containing RBD canola oil with chlorophyll and pheophytin as substrates.

The specific activity of chlorophyllase in media containing RBD canola oil and chlorophyll was 0.12 nmol/mg.min whereas with pheophytin it was 0.04 nmol/mg.min. The specific activity decreased by a factor of 500 and 100 times in media containing crude canola oil compared to that obtained in RBD canola oil assays with chlorophyll and pheophytin as substrates, respectively. These lower specific activity values were expected since crude canola oil contains impurities composed of phosphatides, gums and some carotene pigments which could act as chlorophyllase inhibitors thereby decreasing its specific activity.

Contrary to our successful hydrolysis of endogenous green pigments in crude canola oil, Kalmokoff *et al.* (1988) reported the absence of chlorophyllase activity in the hydrolysis of pigments in natural green canola oil. Levadoux *et al.* (1987) also reported no chlorophyllase activity in crude canola oil assays. However, the same authors stated that only after removal of phospholipids and proteinaceous materials (degumming), the presence of chlorophyllase was detected.

Table 8. Effect of crude commercial canola oil on the specific activity of chlorophyllase.

Variety[a]	Total Pheophytin Concentration (μg pheophytin/mL crude canola oil)	Specific Activity (nmol pheophytin/mg proteins/min)
1	5.07	7.50
2	4.23	7.50
3	25.94	17.50
4	24.21	20.00
5	11.04	2.50
6	9.85	2.50
7	9.78	1.00
8	6.78	0.02

[a]Varieties of crude commercial canola oil.

5. CONCLUSION

The results gathered in this study indicated that the biomass culture of the alga *P. tricornutum*, harvested after 7 days of growth, possessed the highest chlorophyllase activity. Furthermore, lyophilization of the fresh alga resulted in a decrease in the specific activity of chlorophyllase.

Optimization of the biocatalysis of chlorophyllase was carried out in an aqueous/miscible organic solvent system containing refined bleached deodorized (RBD) canola oil and after chlorophyll or pheophytin as substrates. The results showed that the chlorophyllase activity was inhibited by the addition of RBD canola oil. However, the presence of low concentrations of acetone showed an activatory effect on chlorophyllase activity followed by an inhibitory effect at higher concentrations. In addition, an increase in incubation temperature inhibited the enzyme which may have been due to the unfolding of the protein's tertiary structure. Moreover, chlorophyllase showed a limited affinity towards pheophytin as substrate compared to that obtained for chlorophyll.

The development and optimization of the reaction conditions in the presence of RBD canola oil with chlorophyll and pheophytin as substrates and crude commercial canola oil should lay the ground work for further investigations aimed at the potential application of chlorophyllase for the removal of green pigments in edible oils.

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