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PRODUCTION OF FATTY ACID ALCOHOL ESTERS BY ESTERASE ACTIVITY FROM *PSEUDOMONAS FRAGI*

by

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

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SHORT TITLE

ESTERASE ACTIVITY FROM *PSEUDOMONAS FRAGI*

ABSTRACT

M. Sc. Safwan Ismail

Production of Fatty Acid Alcohol Esters by Esterase Activity from *Pseudomonas fragi*

Pseudomonas fragi CRDA 037 was used as source of intracellular esterase, which remained attached to the cell membrane, and characterized. Several mechanical methods of disruption were used including glass beads (MSK), sonication, French press and combinations of these methods. The cellular debris were also treated with detergents such as CHAPS and Triton X-100 in the presence of chelating agent ethylenediaminetetra acetic acid (EDTA). However, these methods were not effective in liberating the enzymes. The esterase activity remained in the cellular debris which was therefore use as a source of enzyme for kinetic studies. In the case of glass beads homogenization, the activity was found to decrease as a function of time of disruption; the optimum time of ethyl valerate production by *P. fragi* esterase was 2 min. On the other hand, the use of sonication and the French press resulted in an enrichment of esterase activity from cellular debris. In the case of cellular debris has an esterase activity that increases after 1 to 3 passages but which reduces after 4 to 6 passes. The optimum passes of extraction was 3 passes for French press and 2 or 4 min for sonication. The results of chemical treatment showed that the esterase was characterized in terms of detergent and EDTA action as well as substrate specificity. On the other hand, Triton X-100 and EDTA had no effect on the esterase activity and did not denature the enzyme, since the esterase activity was found in the debris after the treatment. The optimum esterase activity was obtained at 2% (v/v) of Triton X-100 and 2.5 mM of EDTA. The optimum time for the production of ethyl valerate from valeric acid and ethanol by the esterase activity of *P. fragi* (whole cells) in culture medium was found to be 78 h. However, the biogeneration of ethyl valerate was optimal at 24 h, 11°C and 150 rpm with the best storage time of 1 days. The substrate specificity with cells and cellular debris were carried out. The ratio of fatty acid to alcohol used 1:2, 1:1 and 2:1. The valeric acid was the best in term of esterase activity among fatty acids used in the study such as butyric, caproic, heptanoic and octanoic acids. The

ratio of fatty acid to alcohol substrate was found to be 1:1 of most favorable in bioconversion with reproducibility 10% or less. At low concentration from 0 to 250 ppm, ethyl (methyl) caproate was the highest percent obtained among other esters. It was not significantly different between the cell and cellular debris in their specificity.

RÉSUMÉ

M.Sc. Safwan Ismael

Production d'alcool ester d'acide gras par l'activité estérase de *Pseudomonas fragi*

Pseudomonas fragi CRDA 037 a été utilisé comme source d'estérase intracellulaire, qui est restée attachée à la membrane cellulaire, puis caractérisée. Plusieurs méthodes de broyage mécanique ont été utilisées incluant les billes de verre (MSK), la sonication, la presse de French, et une combinaison de toutes ces méthodes. Les débris cellulaires ont été traités avec des détergents tels que CHAPS et Triton X-100 en présence d'un agent chélatant, l'acide éthylènediaminetétraacétique (EDTA). Cependant, ces méthodes se sont avérées inefficaces pour libérer les enzymes. L'activité estérase est demeurée dans les débris cellulaires, qui par la suite sont utilisés pour les études cinétiques. Dans le cas de l'homogénéisation avec les billes de verre, une activité décroissante en fonction du temps de broyage a été observée, le temps optimal de production de valérate d'éthyle par l'estérase de *P. fragi* était de 2 minutes. D'un autre coté, l'utilisation des ultra-sons et de la presse de French ont resulté en un enrichissement de l'activité estérase des débris cellulaires. Dans ce cas, des débris cellulaires ont une activité estérase augmentant après 1 à 3 passages, mais diminuant après 4 à 6 passages. Le nombre optimal d'extractions fut de 3 passages pour la presse de French et de 2 à 4 minutes aux ultra-sons. Les résultats du traitement chimique ont montré que l'estérase a été caractérisée en terme de détergent et d'action (EDTA), aussi bien qu'en spécificité de substrat. Paralèllement. Le Triton X-100 et l'EDTA n'eurent aucun effet sur l'activité estérasique et n'ont pas dénaturé l'enzyme, étant donné qu'une activité estérase fut trouvée dans les débris cellulaires après le traitement. Les optimales activité estérase obtenues furent 2% (v/v) et 2.5 mM pour le Triton X-100 et l'EDTA respectivement. Le temps optimal de production d'ethyl valérate et d'ethanol par l'activité estérasique de *P. fragi* (cellules entières) fut 78 h. Toutefois, la biogénération de valérate d'éthyle fut optimale à 24 h. 11°C et 150 rpm avec un temps d'accumulation optimal de 2 jours. La spécificité du substrat avec des cellules et des débris cellulaires a été effectuée. Les rapports acides gras: alcool utilisés furent 1:2, 1:1 et

2:1. L'activite estérasique de l'acide valérique fut la meilleure parmi tous les acides gras étudiés tels que les acides butyrïque, caproïque, heptanoïque et octanoïque. Le ratio des substrats acide gras: substrat alcool, le plus favorable en terme de bioconversion trouvé, fut 1:1 avec une RSD 10% ou moins. À basses concentrations allant de 0 à 250 ppm, le meilleur pourcentage obtenu parmi plusieurs esters fut celui du caproate (de méthyle) d'éthyle. Il n'y eut aucune différence significative de spécificité entre les cellules et les débris cellulaires.

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1. INTRODUCTION

Due to consumers increasing preference for food products containing natural ingredients, rather than artificial ingredients, has led to growing demand of natural aroma components (Buchel, 1989; Welsh *et al.*, 1989). This demand has led to more interest in the biotechnology. The flavor production by enzymatic action and fermentation are considered to be natural (Schreier, 1992). Enzymatic reactions are commonly mediated by living cells and can be accomplished through a direct esterification or through a pathway involving the activation of the fatty acid moiety by coenzyme (Nordstrom, 1964).

Natural ingredients are important components in food production. People can judge the food by its natural substance, and it is playing an important role in the success or failure of new products in the market. Biotechnology has become a very attractive tool for the production of these natural ingredients. To be classified as natural, all ingredients must be of natural origin, and among these the flavorings ones are most important to provide the desirable taste. Currently, most of the flavor and fragrance components are provided by traditional methods which included chemical synthesis or extraction from natural sources (Armstrong, 1986). Accordingly, with the great current interest in "natural" products more pressure has been placed on expensive and labor intensive extraction processes since the FDA specifies that only products derived from living sources can be termed "natural" (Armstrong, 1986). Hence, the flavor industry is more and more interested in the use of biotechnology to produce natural flavors.

Whey is a non-environmental product, pollutant and one of the dairy waste products; it contains sugars, proteins, and minerals and trace amounts of vitamins. Whey is a by-product of the cheese manufacturing industry and it is a good medium for the growth of microorganisms. Moreover, whey medium has been used for growing *P. fragi*, as it is easily available and an inexpensive source of nutrients needed for the production of the bacterial cells. However, preliminary research of production of strawberry aroma by bacteria (*P. fragi*) when grown in skim milk, whey and whey permeate, has been carried

out to determine the optimum conditions for ester production. The production of odour-active compounds was most intense during the stationary growth phase. On the other hand, the enzyme responsible for ester production needs to be investigated. If the enzymes can be isolated and purified, they will be available for industry in biotechnological production of naturally- produced fruity aromas.

Esterases are carboxylic ester hydrolases that are specific for the soluble esters, whereas lipases are carboxylic ester hydrolases that are more active with insoluble fatty acid esters and hydrolyze the ester bonds present only at the water-oil interface. *Pseudomonas fragi* is responsible for development of "fruity" flavors in processed dairy products by virtue of the organisms ability to hydrolyze milk fat and esterify certain of the lower fatty acids with ethanol. A similar esterase is present in certain lactic cultures used in the manufacture of cheddar cheese. Microbial enzymes play an important role in the development of natural ingredients in a wide variety of foods and food products. The developments in biotechnology have result in a wide use of microorganisms in the food industry. Fatty acid ethyl esters are an important group of flavor compounds displaying a pleasant fruity aroma. Fermentation and bioconversion catalysis by whole microorganisms or enzymes are attractive means of producing these flavor compounds. The spoilage of milk products by *Pseudomonas fragi* is characterized by the production of a strawberry-like aroma mainly due to ethyl esters of butyric, hexanoic (Reddy *et al.*, 1968; Morgan, 1970) and 3-methylbutanoic acids (Pereira and Morgan, 1958). Fatty acid ethyl ester production through fermentation has been reported for *Geotrichum penicillatum* (Janssens *et al.*, 1988) and *Geotrichum candidum* (Latrasse *et al.*, 1988). The characteristics of resembling strawberry aroma could make an excellent natural source of strawberry ingredient from *P. fragi*. In nature, enzymes are functioning in aqueous media. Therefore, it is not surprising that enzymology thus has used water as the reaction medium virtually in all studies.

Bacteria can be classified as either gram-positive or gram-negative, based on stainability with gentian violet (crystal violet) (Doyle, 1992). *P. fragi* is a non-pathogenic,

aerobic, gram-negative, psychrophilic, non-fermentative, short and rod-shaped bacterium (Bassette *et al.* 1986). *P. fragi* has been reported as major spoilage microorganism in food including milk. Reddy *et al.* (1970) revealed that the esterase from *P. fragi* were intracellular. "Esterase activity from *P. fragi* produces a fruity aroma by producing of esters when grown in whey" and by using a purge and trap method demonstrated that 26 were odor-active compounds. Ethyl esters were the major contributors in milk cultures" (Cormier *et al.*, 1991), and the esters are flavor components for certain other dairy products (Bills *et al.*, 1965). Ethyl valerate was obtained using gas stripping with whole cells of *P. fragi* CRDA 037 at a concentration of more than 800 ppm in a bioconversion medium containing valeric acid and ethanol as substrate (Morin *et al.* 1994). It is important to investigate the enzyme responsible for the aroma production. It may be possible to create natural ingredients by either using the extracted enzyme or enzymes or the produce of natural aroma by exposing them to cheap substrates of natural origin, or by using genetic engineering to create clones of the enzyme(s).

The objectives of this research were:

1) To investigate the production of the biomass of *P. fragi* with reference to optimal esterase activity, using ethyl valerate as a model system.

2) To investigate the methods used for disruption of *P. fragi* cells using glass beads, French press, ultrasonic and combined treatments with chemical.

3) To study the effect of storage on esterase activity.

4) To determine the esterase specificity using different ratio of fatty acids to alcohol, *in vivo* and *in vitro*.

2. LITERATURE REVIEW

2.1. Definition of Esterase

Esterase is an enzyme that catalyzes the hydrolysis of various esters bond. They are subdivided into many groups depending on the ester bond that they hydrolyze. They are acting on carboxylic esters (3.1.1), thiolesterase (3.1.2), phosphoric monoester hydrolases, the phosphatases (3.1.3), phosphodiester hydrolases (3.1.6), diphosphoric monoesterase (3.1.7) and phosphoric triester hydrolases (3.1.8). Lipases are enzymes that catalyze the hydrolysis of triacylglycerols of animal fats and vegetable oils or esterases, since they hydrolyze the ester bonds of triacylglycerol molecules (Brockerhoff and Jensen, 1974). Although most of the lipases in microorganisms are extracellular (Desnuelle, 1972), esterase of gram-negative bacteria are often described as intracellular enzyme among bacteria including the *P. fragi* (Lawrence *et al.*, 1967).

2.2. Sources of Esterase

Esterases of gram negative bacteria are often described as intracellular enzymes among different *Enterobacteriaceae* (Goullet, 1978, 1980, 1981; Goullet and Picard, 1984; 1985), phytopathogenic bacteria (El-Sharkawy and Huisingh, 1971), *Pseudomonas fragi* (Lawrence, Fryer and Reiter, 1967) and an *Acinetobacter* species (Breuil and Kushner, 1975). In *Acinetobacter calcoaceticus*, Shabtai and Gutnick (1985) studied an extracellular esterase in relation to emulsan release, whereas Claus *et al.* (1985) described an outer-membrane esterase. An esterase has also been localized on the outer membrane of *Pseudomonas aeruginosa* (Ohkawa *et al.* 1979) and *Escherichia coli* (Pacaud, 1982).

The fruity aroma in many dairy products has been attributed to *P. fragi*. Eichholz (1902) first isolated this organism from milk possessing a strawberry-like odor mainly due to ethyl esters of butyric, hexanoic (Reddy *et al.*, 1968; Morgan, 1970) and 3-methylbutanoic acids (Pereira and Morgan, 1958). Hussong *et al.* (1937) proposed the name *P. fragi* as a psychrophilic microorganism, responsible for fruity flavor defects in dairy products. A similar esterase is present in certain lactic cultures used in manufacturing cheddar cheese. When grown in skim milk, *P. fragi* strain CRDA-037

produced a mixture of 26 different odor-active compounds of which 13 were identified as fatty acid ethyl esters (Cormier *et al.*, 1991).

2.3. Enzyme Mechanism

Enzymes are proteins that catalyze biochemical reactions. Each enzyme is highly specific for the type of reaction it catalyzes. The catalytic site on the enzyme contains the groupings that are directly involved in the catalysis. The catalytic site is usually only a small localized region on the enzyme surface. The substrate binding site and the catalytic site are usually near one another and frequently overlapping. Together they are referred to as the active site.

2.3.1. Aroma defects from enzymatic reaction by *P. fragi*

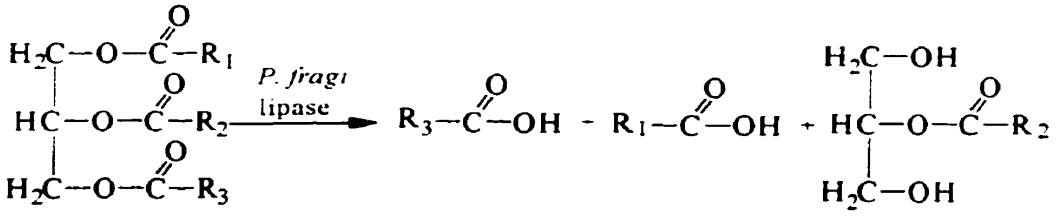
Development of flavors in a number of food products using microbial enzymes has an important value in food industry. The addition of esterase and lipases to cheeses, butterfat or condensed milk yield a variety of flavor-modified dairy ingredients. The advantage of enzyme modification is that it shortens reaction times as compared to traditional processes which results in substantial reduction in process costs.

Lipases and esterases are used interchangeably since these enzymes can catalyze the hydrolysis of carboxylic acid ester bonds and the esterification of fatty acids and alcohols. They are classified among group of enzymes which are belonging to the class of 3.1.1 under the classification of the International Union of Biochemistry (IUB).

The esterases are group of enzymes widely distributed in nature. They are highly capable of catalyzing the hydrolysis of carboxylic acid esters. Depending on their specificity toward either the alcohol moiety or the acid of the ester substrates, esterases can be subclassified into: (a) carboxylic ester hydrolases, (b) thiolester hydrolases, (c) phosphoric monoester hydrolases, (d) phosphoric diester hydrolases and (e) sulfuric ester hydrolases (Whitaker, 1994). However, in the presence of water, esterases can hydrolyze the carboxylic esters of fatty acids to simple alcohols and carboxylic acid.

2.3.2. Mechanism of esterase/lipase reaction

Pseudomonas fragi hydrolyzed the milk fat at 7°C, producing short - chain fatty acids apparently responsible for the development of fruit flavor in cottage cheese (Reddy *et al.*, 1971). The organism is strongly lipolytic (Hussong *et al.*, 1937; Nashif and Nelson, 1953) and a 1, 3 - position specificity for triglycerides has been observed with both crude and purified lipases obtained from the organism (Alford *et al.*, 1964; Mencher and Alford, 1967). Since most of the butyric and caproic acids of milk fat are esterified in the 3-position of constituent triglycerides (Pitas *et al.*, 1967), these are likely major products of the lipolysis of the fat in milk medium by this organism.



where R = $-(CH_2)_2CH_3$
$-(CH_2)_4CH_3$

Scheme 1. Mechanism of ethyl ester formation by *P. fragi* (Morgan, 1976).

However, marked increases have been noted in production of esters of these acids by this organism in milk medium when the milk fat was increased (Pereira and Morgan, 1958). Reddy *et al.* (1969) have also noted that the addition of milk fat or butyric acid to milk media containing ethanol were conductive to the production of a fruity aroma. Moreover, Reddy *et al.* (1968) acknowledged that *P. fragi* does produce some ethanol, thus only 0.2% ethanol was added to increase the level of fruity compounds. They easily isolated six esterases from milk cultures of *P. fragi* which contained an enzyme system responsible for the esterification of butyric and caproic acids with ethanol (Morgan, 1970). It also seemed likely that production of the fruity aroma esters by the organism

would be enhanced in both milk and cottage cheese containing increased amount of ethanol due to silage feeding (Bassette *et al.*, 1966) or by the growth of other organisms which produce either acetaldehyde or ethanol (Keenan *et al.*, 1967; Morgan, 1976).

Primarily a lipase produced by *P. fragi* will hydrolyze triglyceride into free fatty acid and glycerol, which are then esterified in the presence of ethanol and *P. fragi* esterase to give the fruity ethyl esters. Elucidation of the mechanism involved in the production of the fruity aroma has been completed by Hosono *et al.* (1974) and Hosono and Elliott (1974) who demonstrated the presence of an esterase in crude cell extracts from *Pseudomonas* cells capable of esterifying butyric and caproic acids with ethanol. Hosono *et al.* (1974) showed that some lactic acid bacteria commonly present in cheddar cheese also possess an esterase enzyme similar to that found in *Pseudomonas* cells. A summary of the mechanisms involved in the formation of ethyl esters by *P. fragi* is presented in Scheme 1. This means, it is not known whether only one enzyme is used or if the enzymes involved are different, or there could be a different esterase performing the ester synthesis. Studies on whole cells of *P. fragi* have been reviewed but the characterization of enzymes extracted from the bacterium for the purpose of flavor production will be mentioned with more details in this study.

2.4. Food Biotechnology

Biotechnology is of special interest to the flavor industry because it may enable the mass production of large quantities of any desired flavoring materials at relatively low cost. Conceptually, the production of flavor compounds and other natural products using biotechnological techniques seem straightforward (Wasserman and Montville, 1989). Food biotechnology includes (1) microbial fermentation processes for flavorful food, preservation of foods and biomass production, (2) purified enzyme processing for improving quality attributes of food (3) bioprocessing such as membrane processing (Committee Report, 1988). The term fermentation shall be used to describe several processes such as any process involving biomass production of microorganisms, food spoilage, the production of alcoholic beverages, a mode of energy-yielding metabolism

that involves a sequence of oxidation-reduction reactions in which both the substrate (primary electron donor) and the terminal electron acceptor(s) are organic compounds and energy is obtained during fermentation largely by substrate-level-phosphorylation (Singleton and Sainsbury, 1978; Prescott *et al.*, 1990a). The Canadian Committee on Food Biotechnology presented a working definition of the Food Biotechnology as a process for manipulations of the enzyme in the free and immobilized forms, and in the cells for the purpose of (1) producing compounds to increase the attractiveness of food quality, (2) to increase the shelf life for fresh food by reducing metabolic process and (3) to analyze food ingredient and food toxicants. Biotechnology has recently been applied to many different areas including the food and flavor industry.

The sources of microorganisms present different ranges of problems whereas the collection of bacteria and other unicellular organisms during the log phase of growth is usually desirable to the end of the exponential phase before growth rate slows at the end of the stationary phase (Scopes, 1994 and Stanier *et al.* 1990). In microbial transformation the microorganisms used are grown, or kept as permanent or treated cells, where as in fermentation grown cells are used. Product concentration is high and reaction time is short in microbial bioconversion, whereas product concentration is low and the reaction time is long in fermentation. On the other hand, fermentation uses cheap carbon and nitrogen sources, whereas bioconversion uses specific and sometimes expensive substrates. One of the most important differences is that the fermentation involves complex reactions, but the bioconversion uses simple catalytic reactions (Schreier, 1992).

The effects of controlled availability of substrate on the production of methyl ketones in milk-fat-coated microcapsules were studied. The substrate specificity of one enzyme (thiohydralase) in the pathway of methyl ketone synthesis by *Penicillium roqueforti* is thought to control the production of the pathway (Pannell and Olson, 1991). The complex mixture of methylketone produced by *Penicillium roqueforti* is an example of a complex flavour system. Single ester compounds of vanilla are produced by *Corynebacterium*, fruity ester by *Lactococcus*, *Pseudomonas* and *Hansenula anomala*, pyrazines (roasted, nutty ester) by *Lactococcus lactis* and lactones (coconut, peachy

ester) produced by *Candida spp.* and *Sporobolomyces odorus* (Gatfield, 1988). Utilization of microorganisms to produce natural ingredients was carried out by a culture medium to grow a cellular microorganism in a fermentation process, or in bioconversion reactions. However, fatty acid ethyl ester production through fermentation has been reported for *Geotrichum penicillatum* (Janssens *et al.* 1988) and *Geotrichum candidum* (Latrasse *et al.* 1988).

Enzyme technology essentially deals with enzymes and the techniques by which they can be used in manufacturing food products (Tombs, 1991). The enzyme industry succeeded in overcoming a shortage of animal rennet by developing several microbial rennets, chiefly produced by thermophilic fungi (Norris and Richmond, 1981). The ester-synthesizing capabilities of the esterase-lipase enzymes preparation derived from *Mucor miehei* have been investigated. The enzyme has found use in cheese manufacture (King and Cheetham, 1988). The lipase enzyme was obtained and partially purified from *P. fragi* (Schuepp *et al.* 1997); this enzyme produced fruity aromas (Bassette *et al.* 1986). There is thus a commercial potential in producing food additives through biotechnology process. However, potential esters are chemicals that when added to food, enhance the aroma attributes of the food. The biotechnological processes of ester production are more expensive than chemically produced ones, because the former is considered a natural product and the latter is artificial. Moreover, the consumers demand natural products more than chemical products. Although biochemically produced natural ingredient compounds are some times more expensive than chemically produced ones, they are still generally cheaper than identical compounds extracted from plants (Knights, 1992). Furthermore, almost all-new enzyme preparations developed are of microbial origin. There are several reasons for this: (i) microorganisms grow fast and are well suited for cultivation under industrial conditions, (ii) the growth substrates are relatively inexpensive and generally consist of common agricultural products available worldwide in almost unlimited quantities, and (iii) furthermore, the choice of suitable microorganisms is very wide and can easily be increased.

2.5. Esterase Activity from *Pseudomonas Fragi*

2.5.1. Bacteria And Bacterium *P. fragi*

The term bacteria is a plural form of the Latin bacterium, meaning staff or rod. The bacteria are organisms in which the chromosomes are not separated from the cytoplasm by a membrane. The vast majority of bacteria play a positive role in nature. They digest sewage into simple chemicals are extract the nitrogen from the environment to make it available to plants as a fertilizer after being converted into amino acids and proteins, production of natural substance by using bacteria as a biocatalyst such as *P. fragi*. They break down the remains of all that die and recycle the carbon and produce foods for human consumption and products for industrial technology. On the other hand, some bacteria are harmful. Certain bacteria digest the tissues of human body or produce poisonous toxins that lead to disease. Other bacteria infect plant crops or animal herds. However, bacteria have adapted to living conditions more different than any other group of organisms. They inhabit the environmental air, water and soil and exist in plants and animals. The shapes of bacteria walls are governed by the presence of a rigid boundary wall. *Pseudomonas. fragi* is catalase positive and does not produce prosthecae. In nature, *P. fragi* can be separated from soil and water and is commonly found in the environment of milk (Hussong et al. 1937; Singleton and Sainsbury, 1978). In another study by Shelley *et al.* (1987) indicated that while the lipolytic psychrotrophic microorganism most commonly isolated from spoiled raw milk was *P. fluorescens*, *P. fragi* may have been responsible for more significant spoilage of the dairy products.

Fruity aroma in dairy products is a common defect caused by *Pseudomonas fragi* which may spoil refrigerated foods such as butter, eggs and meat (Singleton and Sainsbury, 1978). In addition, fruity aroma production is detected in a culture where the role of leucine as a probable substrate in the production of the isovalerate ester by *P. fragi* is explained (Pereira and Morgan, 1958) and this microorganism is able to produce a fruity flavour in a medium which contained leucine as the only carbon source (Pereira and Morgan, 1957). *Pseudomonas fragi* is an aerobic, gram negative, nonfermentative, psychrophilic, short and rod-shaped bacterium (Bassette *et al.*, 1986, Fairbairn and Low,

1986). Hussong *et al.* (1937) linked the odor of their cultures to that of the flower of the mayapple and proposed the name of *Pseudomonas fragi* as an organism spoiling dairy products.

2.5.2. Whey

Whey protein was considered as a by-product of the dairy industry. It was considered a waste product either dumped in sewer or used as fertilizer (Considine and Considine, 1982). From a point of view of environmental pollution, whey presents a high pollution capacity as it has a very high biochemical oxygen demand, which is an indication of the amount of organic matter in the water (Prescott *et al.*, 1990b). However, it was estimated that about 6 to 11 liter of whey was produced for each kilogram of cheese manufactured that means a serious environment problem (Macrae *et al.*, 1993). Using whey protein as an ingredient in food industry is reducing the environment pollutant. However, it is an important source of functional ingredients now (Kinsella, 1989). It contains 93% water, 4.5 % lactose, 0.7 % protein, 0.7 % ash and 0.1 % milk fat. The main protein in whey is β-lactoglobulin (50 %). The other proteins are α-lactalbumin (12 %), bovine serum albumin (5 %), immunoglobulins (10 %), proteose peptones (23 %); other minor constituents are lactoperoxidase, lysozyme, lactoferrin and lactollin (Bottomley *et al.*, 1990). Whey protein functionality is affected by several physicochemical properties including amino acid composition and sequence, molecular size net surface charge, conformation and hydrophobicity.

2.5.3. Enzyme extraction

Cell disruption by grinding with glass beads is an important process for the recovery of intracellular enzyme. This method has been adapted to small- (Schütte and Kula, 1988) and large-scale (Kula and Schütte, 1987) downstream processes as well as involving sold shear such as bead milling (Harrison, 1991) and to strategies for screening microorganisms producing intracellular enzymes (Hummel and Kula, 1989). Laboratory methods of cell disruption using glass beads at the milliliter- and microliter scale with a commercial mixer mill have been successful (Schütte and Kula, 1988; Hummel and Kula,

1989; Morin *et al.*, 1992; Morin *et al.*, 1993). There are a widely used analytical cell disruption methods, such as ultrasonication (Doulah, 1977) and French press (French and Milner, 1955; Schuepp *et al.*, 1997). The method of ultrasonication is extensively used to disintegrate biological cells for the release of intracellular compounds (Neppiras and Hughes, 1964). This method of cell disruption is viewed as sonic energy used for cell disintegration on the basis of a physical model of cavitating ultrasonic fields in which elastic waves are generated by imploding bubbles (Doulah, 1977). On the other hand, "it may be necessary to solubilize the enzymes using detergents, in which case the detergent, bound to the protein, takes the place of the lipid-containing membrane" (Scopes, 1994). The addition of Trition X-100 (a nonionic synthetic detergent) resulted in enzymatic activity apart from that fraction; after the treated fraction was centrifuged, the enzymatic yield increased in the supernatant while it decreased in the pellet and the most efficient Triton X-100 concentration was 1.25 to 2% (vol/vol) to obtain the best resolution (Debette and Prensier, 1989). Esterase activity from *Xanthomonas maltophilia* was much higher in EDTA-Triton extract (10,800 U) than in Mg^{2+}-Triton extract (2,200 U) (Debette and Prensier, 1989). The detergent can be removed as follows:

Preparative Procedures

During the course of purification of a membrane protein, it may be necessary to remove excess detergent at three distinct points in the protocol. The first arises after initial solubilization of membranes. As this step usually requires detergent excess in order to give maximal dispersion of membrane proteins and lipids, removal of excess detergent at this stage usually improves the quality of subsequent chromatographic steps. It is often carried out so that the detergent is removed in the beginning of a purification strategy. This second point at which detergent may need to be removed occurs during the process of detergent exchange. The detergent initially used for solubilization may not be appropriate for subsequent chromatography or analytical procedures. The detergent exchange is a common feature of many preparative protocols. The replacement of one detergent with another is usually achieved by a chromatography procedure. The most efficient processes are those which involve adsorption of the detergent-membrane protein complex to a

chromatographic support, followed by extensive washing with buffer containing a new detergent. Alternatives involve gel filtration chromatography and equilibrium methods such as dialysis. Finally, the detergents are removed at the end of the preparative procedure to facilitate analytical procedures such as amino acid analysis, amino acid sequencing, and analytical high-performance liquid chromatography.

Since membrane proteins are not soluble in aqueous media, the goal of these procedures is to replace the detergent-containing aqueous medium with a solvent system which is compatible with the analytical procedure.

Methods frequently recommended for final removal of detergents include adsorption of detergent to hydrophobic media, precipitation with polyethylene glycol, and chromatography on various hydrophobic or reversed phase media. Amongst choices, the latter is preferred due to unavoidable losses involved with simple adsorption or precipitation procedures.

Analytical Procedures

Many analytical procedures have specific requirements related to the physical properties of the detergents to be used and thus preparation of samples may necessitate detergent exchange or removal. Firstly the preparation of proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis for subsequent use in sequencing. Secondly the interference that ionic detergents cause in the use of isoelectric focusing or other charge-based electrophoretic methods, and a third example would be the interference of an ionic detergent such as cholic acid with analytical high-performance ion-exchange chromatography.

2.5.4. Enzyme assay

The growing demand for natural products has led the research workers to use various lipolytic enzymes for rapid flavor development in cow milk cheese (Kanawjia and Singh, 1991). There are many important enzymes in food systems which can catalyze the hydrolysis of esters. The most important esters involved in the hydrolysis, come from

carboxylic acid and phosphate esters. In each of these categories there are two groups of interest. The carboxyl ester hydrolases are specific for soluble esters (esterases) or insoluble fatty acid esters (lipases). The phosphate ester hydrolases may be involved with various kinds of water-insoluble phospholipids (phospholipases) or with soluble phosphate esters (phosphatases) (Stauffer, 1989). However, there is a clear-cut difference between lipase and esterase based upon the water-solubility of the substrate. Some researchers demonstrated that pancreatic lipase reacted with fatty acid esters having moderate solubility only when the solubility concentration was exceeded and a lipid/water interface was formed. Therefore, the nature of the substrate depended upon the specificity of the enzyme being studied. A carboxyl esterase may be most active on ethyl propionate, pectin esterase requires methoxylated pectin, and cholesterol esterase acts on O-acetyl cholesterol. Esterases are often used as markers in studying plant or insect genetics. Extracts of a tissue were run on an electrophoresis slab, the developing of electrophoresis slab gel was incubated with a solution of α-naphthyl acetate for a while, and then sprayed with a solution of some diazotized salt. It reacted with the α-naphthol which was freed by esterase action, depositing a colored diazo dye in the zone of active enzyme (Stauffer, 1989). These techniques revealed a large number of esterase isozymes and as much as 17 have been found in wheat.

Lactonizing lipase is an enzyme that catalyses the synthesis of macrocyclic lactones (intermolecular ester). Ihara *et al.* (1991) determined the esterase activity by measuring the amount of *p*-nitrophenol formed from *p*-nitrophenyl acetate. Lactonizing activity was assayed by measuring the amount of cyclo hexadecanolide formed from methyl 16- hydroxyhexadecanoate. To characterize a lactonizing lipase, the lipase was purified from the crude powder of lipase P. Fraction of a lactonizing lipase was determined both by esterase activity to produce p-nitrophenyl acetate and formation of cyclohexadecanolide from methyl 16- hydroxyhexadecanoate. Two esterases were observed at isoelectric focusing (pI) values of 5.3 and 7.0 respectively. One unit of esterase activity was defined as the amount of enzyme, which liberates 1 μmol of *p*-nitrophenol of fatty acid/min.

Nishio *et al.* (1987) assayed the ester synthesis-activity of PEG_2-lipase in benzene according to the method described previously, using lauryl alcohol and lauric acid as substrates (Inada *et al.*, 1984), and the activity was found to be 13.6 μmoles/min/mg protein. Nishio *et al.* (1988) determined the ester synthesis using the method outlined below. For ester synthesis from oleyl alcohol and various carboxylic acids, reaction mixtures consisted of 10 mmol of oleyl alcohol, 10 mmol of acid and enzyme solution (1,500 units). When the reaction was stopped, free fatty acid was titrated with NaOH. The amount of synthesis (%) was calculated from the amount of acid consumed in the reaction mixture.

Nishio and Kamimura (1988) determined the ester activity by reacting the same concentration of alcohol and carboxylic acid in n-hexane in the presence of lipase. When the reaction was stopped, the carboxylic acid was immediately titrated with NaOH. The degree of ester formation was calculated from the amount of acid consumed in the reaction mixture. Thin-layer chromatography conducted on a silica gel plate and infrared spectroscopy were done to identify the synthesized esters.

2.5.5. Substrates And Substrates specificity

Short-chain fatty acids are characterized by sharp unpleasant, strongly pungent and irritating odors in high concentration. As the molecular weight of the acid increases, the character is replaced by rancid, buttery, cheesy notes and then becomes more fatty or aldehydic. However, fatty acids above C_{14} are waxy solids with only slight, tallow-like odors. The low flavor impact of acids has lessened their perceived value as flavorings, however, they contribute to complex aromas and accentuate aroma characteristics, such as C_3 to C_6 acids accentuate fruity notes while C_4 and C_6 to C_{10} acids heighten cheese flavor. In addition, fatty acids are basic building blocks of the lipids in dairy product. In their free form they make significant contributions to the flavor of different types of cheese and they act as precursors for the formation of other aroma components, such as esters, aldehydes, alcohol and ketones (Kinsella and Hwang, 1976; Scott, 1981).

A number of biotechnological processes have been developed for production of natural propionic acid and Swiss and Emmental cheese flavors (Paulsen *et al.*, 1980). Naturally produced propionic acid can be used as a preservative in natural bakery goods and for the preparation of propionate esters (Boyaval and Corre, 1987).

Substrate specificity measurements of esterases of *P. fragi* was carried out by using phenyl acetate, ethyl butyrate and triacetin. The volume of gas liberated during 40 min incubation by 1 ml of the sonically treated cell preparation was 290 µliters when phenyl acetate was the substrate, 35 µliters when ethyl butyrate was the substrate and 47 µliters when triacetin was the substrate. Therefore, the enzymes were more active with aromatic esters than with aliphatic esters (Reddy *et al.* 1970).

2.5 6. End-product specificity

The fruity flavor defect in milk and cottage cheese has been primarily attributed to *Pseudomonas fragi*, and it appears that the short chain fatty acids resulting from the hydrolysis of milk triglycerides could play an important role in production of the fruity ester defect in milk and cottage cheese (Reddy *et al.*, 1968). Cormier *et al.* (1991) has shown that all 26 active compounds were identified in the strawberry aroma produced by *P. fragi* incubated at 15°C in skim milk supplemented with ethanol. Pereira and Morgan (1958) reported that formate, acetate, propionate and isovalerate esters were detected in steam distillates of milk cultures of *P. fragi*. The spoilage of milk products by *P. fragi* characterized by the production of a strawberry-like aroma due mainly to ethyl esters of butyric, hexanoic (Reddy *et al.*, 1968; Morgan, 1970). The utilization of artificial strawberry flavoring in beverages, ethyl valerate, aldehyde C16 and ethyl lactate are used in the highest quantity of flavoring compounds (Matthews, 1991).

Microbial enzymes play an important role in the development of flavor in a wide variety of food and food products. The development of flavor in cheese depends on the action of enzymes present in the cheese in the course of its manufacture. These include the enzymes that are present in the milk. All enzymes produced by the starter culture

organisms reside in the cheese in varying proportions at various stages during the ripening process. Certainly, the esterase or lipase produced by these organisms is of major importance in developing the delicate aroma and natural substance associated with each type of cheese. This means that they are the factors responsible for the characteristic lipolytic activity present in rennet paste. Harper (1957) compared preparation of pregastric esterase with rennet paste in their behavior towards the butterfat. When the enzymes were incubated with cream containing 20% butterfat, a characteristic distribution of free fatty acids was obtained with each enzyme (Huang and Dooley, 1976).

The production of the pregastic type of fatty acid profile parallels the production of the characteristic goaty ester in Italian types of cheese (Long and Harper, 1956). From the increase in the content of butyric acid in the cheese during the ripening period due to the addition of several enzyme preparations to the milk, it was indicated that production of butyric acid closely parallels development of natural ingredient (Huang and Dooley, 1976). A similar study (Arnold *et al.*, 1975) indicated a direct correlation between natural ingredient intensity and butyric acid concentration in romano and provolone cheese samples. On the other hand, Arnold *et al.* 1975 reported the possibility of production of aroma compounds from degradation of the polyunsaturated fatty acids under the action of the enzyme intrinsic in apple pomace.

The production of the major odour-active metabolites of *P. fragi* in whey was monitored. The aroma produced were ethyl hexanoate, ethyl butyrate, 2-hexanoic acid ethyl ester, ethyl crotonate, ethyl 2-methyl hexanoate and ethyl isovalerate (Raymond *et al.* 1991).

2.5.7. Enhancement of fruity aroma

The influence of temperature, agitation speed, pH and biomass on the synthesis of 19 metabolites contributing to a strawberry aroma was followed over a 72 h fermentation of skim milk by *Pseudomonas fragi* (Raymond *et. al.*, 1990). These parameters have been examined to determine if they have any effect on the production of fruity aroma by *P. fragi*.

The influence of culture media on the production of fruity aroma and as growth of *P. fragi* was studied by (Raymond *et al.*, 1991). The production of 19 odor-active metabolites contributing to the aroma produced by *P. fragi* on three different culture media (skim milk, whey permeate and whey) incubated at 150 rpm and at 11°C was monitored. The optimal production of most odor-active metabolites were higher in skim milk medium than in other media, and fruity aroma production occurred after an incubation time of 60 h in both skim milk- and whey- based media, and after 48 h in whey permeate containing medium.

The pH of the cultures grown in whey- and whey permeate-based media increased from 6.5 to 7.0 within the first 24 h, and from 6.5 to 6.7 in the cultures grown in skim milk. Later, the pH of all cultures dropped slowly to 6.2 between 24 h and 60 h of incubation (Raymond *et al.*, 1991). In another study the pH of 6.5 of all cultures grown at 130 rpm and at different temperature was stable up to 36 h of incubation of *P. fragi* (Raymond *et al.*, 1990). A slight acidification of all cultures occurred from 36 h to 72 h of fermentation. The higher the incubation temperature, the lower was the drop in the pH. For instance, the pH of the culture grown at 11, 20 and 25°C decreased to 6.3, 5.9 and 5.7, respectively.

Raymond *et al.* (1990) examined the effects of temperature on the production of fruity aroma by *P. fragi*. The highest odor-active metabolites were obtained at 60 h of fermentation with strongest aroma at 11°C for most compounds, although the level of ethyl 2- hexanoate was highest after 60 h of incubation at 15°C, the overall aroma was weaker and was characterized by off- aromas of rancidity. Growth of *P. fragi* was similar at 15, 20 and 25°C and the stationary growth phase was reached after 12 h of incubation where 5×10^8 CFU ml^{-1} were detected (Bassette *et al.*, 1986). At 5 and 11°C, the stationary phase was attained at 36 and 24 h respectively with the same number of cells. At all growth temperatures investigated, the production of aroma was maximum ca 36 h after the beginning of the stationary phase.

Myhara and Skura (1990) found out that the optimal conditions of the production of the extracellular proteinase from *P. fragi* ATCC 4973 was 12.5°C, incubation time 38 h, initial pH 6.8, organic nitrogen concentration 314 mmol nitrogen/l and a gas mixture containing 16.4% oxygen flowing over the medium (7.42 ppm dissolved oxygen). Oxygen was the major factor influencing proteinase production by *P. fragi*. It was important to observe that the optimal temperature of extracellular proteinase by *P. fragi* ATCC 4973 was close to that of *P. fragi* CRDA 037 cells.

The agitation speed in previous studies was proved to have effect on the production of aroma by *P. fragi*. The highest productions of aromas were obtained at 60 h of incubation, 150 rpm for 4 out of 6 of the major metabolites and for 8 of the 13 other compounds. Although the highest levels of 2- methyl hexanoate and isovalerate ethyl esters were attained at 200 rpm, the odor was characterized by off-aromas of rancidity.

The supplementation of the whey- based culture medium with ethanol and C_3 - C_7 fatty acids had a direct effect on the production of the fruity aroma by the presence of *P. fragi*. The addition of five fatty acids (C_3 - C_7) at 0 h increased the production of ethyl valerate, ethyl heptanoate, ethyl propionate, ethyl butyrate and ethyl hexanoate up to ca 1570, 1450, 180, 55 and 12 times, respectively. The addition of ethanol and / or C_3 - C_7 fatty acids after 48 h incubation had less effect on the production of the different odor - active metabolites. The production of esters was monitored after the supplementation of the whey-based culture medium with ethanol. The concentrations of ethyl butyrate and ethyl valerate were increased 1.4 and 1.7 times respectively. The concentration of ethyl valerate and ethyl heptanoate were increased 2.1 and 155 times after the addition of the five C_3 - C_7 fatty acids. The addition of both ethanol and fatty acids resulted in a 3.4-, 1.5- and 932- fold increase in ethyl valerate, ethyl hexanoate and ethyl heptanoate, respectively (Raymond *et al.*, 1991). The cells were grown on whey and whey permeate with and without supplementation with butyric acid or valeric acid for 70-72 h at 12°C. Cells grown on whey supplemented with 0.1% butyric acid had the highest ethylating activity (Morin *et al.*, 1994).

2.6. Characterization of end-products using gas-liquid chromatography

2.6.1. Principles

Gas chromatography is a physical method of separation, in which the components to be separated are distributed between two phases, one of these phases constituting a stationary bed of large surface area. The other one of the phases is a gas which acts to transport the various solutes through the fixed sorbent bed (Nogare and Juvet, 1963).

This characteristic of gas chromatography is primarily due to low density of the carrier gas used in the system. The mobile phase or carrier gas is an inert gas which is made to flow at a constant rate and permits rapid diffusion of the vaporized solute through a capillary column (which is a small diameter tube containing the sorbent or stationary phase). The low viscosity of the carrier gas also permits large flow rates resulting in a fast elution of the solutes from the gas-liquid chromatography capillary columns. The carrier gas of He, N_2 or H_2 are gases of choice with thermal conductivity (TC) detectors because the signal from a TC cell depends on the difference between the thermal conductivity of the solute vapor, and He or H_2 is preferred as a carrier gas (Nogare and Juvet, 1963). These gases provide a factor of 4 to 5 over the sensitivity with N_2. The H_2 cannot be used with thermistors (metal oxides) above about 100°C because of its reducing properties, but it is satisfactory with metal filament TC cells.

The basic apparatus requires units of a carrier gas supply as well as a detector gases of H_2 and air compress and flow control, introducing the sample by injection into a sample port, a capillary column and a flame ionization detector (FID) for determining the composition of the column effluent. Programming the injector, column and detector to obtain excellent separations for volatile compounds could control the sample running through the gas chromatography. The carrier gas enters the sample port in which the sample is placed. The sample port must be heated to promote rapid vaporization of all components. The sample vapor is then swept into the column where the separation process has begun according to their vapor pressure and interaction with the column packing. The separated components in the carrier gas enter the sensing side of the

detector and pass out to the atmosphere. When solute enters the sensing side, a change in signal produces a differential output from the bridge proportional to the concentration or rate at which solute enters the sensing side. The recorded output of the differential bridges are peaks reflecting the distribution process which the solutes were subjected in their passage through the column.

The gas chromatography has the simplicity of the apparatus requirement for using the technique in a wide range of separation problems. The potential advantages of using gas chromatography describe the character of efficiency, sensitivity and convenience. As mentioned above, the determination of the composition of the effluent gas stream can be made conveniently and with high sensitivity by any of a number of relatively simple detector. It is the high sensitivity with which the gas stream composition can be determined that is responsible for extensive analytical applications of gas chromatography. It is possible to determine parts per million concentrations of impurities. It is capable of both quantitative and qualitative analyses of mixtures which in many instances are too complex for standard analytical techniques. The sample injected in the system should be small. The detector cell volume should also be small. Large cell volume results in peak broadening and mixing with other peaks by closing the space between peaks and loss of resolution. The detector temperature should be higher or at least that of the column if condensation of both solute and liquid phase in the detector cell is to be avoided.

2.6.2. Extraction and /or Enrichment of volatile

Cormier *et al.* (1991) used the purge and trap sampling method. *Pseudomonas fragi* produced a pleasant strawberry-like odor when grown in skim milk at 15°C. Volatile from the culture broth were extracted, enriched, and analyzed by using sniffing flame ionization detector-gas chromatography (FID-GC).

Douillard and Guichard (1990) used the extract of strawberry volatile from strawberries. The strawberry slurry was extracted and centrifuged to recover the organic extract. Because of the liability of furaneol in extracts, this compound together with the acids was determined in the total organic extract immediately after extraction and

concentration. Acids were then removed from the total organic extract by neutralization with sodium hydrogen carbonate solution. Then, the neutral extract was concentrated.

Lesschaeve *et al.* (1991) used dimethyl-3,3-butyric acid and water to extract the strawberry volatile from jam with the Likens-Nickerson apparatus. The slurry was in one side and distilled trichloro-1, 1, 1- ethane in the other side which was concentrated. To separate volatile from condenses, the water was condensed in two traps and then the volatile components were extracted and concentrated.

Jollivet *et al.* (1992) extracted the flavor compounds from culture medium and concentrated by distillation. Gas chromatography and gas chromatography–mass spectrophotometer (GC-MS) were used to analyze the samples.

Almosnino and Belin (1991) extracted volatile from apple pomace using steam stripping. The suspension was homogenized and then extracted (Berger and Drawert, 1984). The volatile compounds were extracted by steam stripping, and the aroma was recovered by liquid-liquid extraction in hexane. The concentration of the aroma was carried out by hexane evaporation with a stream flow of nitrogen. The sample was analyzed by gas chromatography.

2.6.3. Internal standard

The use of an internal standard in gas-liquid chromatography is a well-known technique for decreasing differences between runs. The study of internal standard in GC analyses overcomes most of the experimental difficulties related to quantitative aspects of the procedure and compensates for variation between runs and confers reliability of results. The internal standard should be a substance which is not one of the sample components. Neither it should interfere with the peaks nor prolong the assay. Because of the difficulties in finding such a compound, a number of chromatographic procedures lack an internal standard. Despite this difficulty, chromatograms of the methyl esters derivatives have shown that both pentanoic and hexanoic acids can be used as internal standards for gas-chromatographic analyses of acidic fermentation end-products, since

they are well separated and resolved (da Silva, 1995). In addition, some compounds have been used as internal standard such as heptanoic acid methyl ester (Cormier *et al.*, 1991). The quantity of compounds was determined from peak area relative to that of the internal standard. Internal standards were used in several studies (Lesschaeve *et al.*, 1991). Adding 3-pentanol as internal standard (Jollivet *et al.*, 1992) made the quantitative evaluation of the aroma compounds. However, some compounds such as 2-methyl pentanoic acid and the methyl ester of benzoic acid have been suggested for use as internal standards, both for identification and quantitative purposes (Mayhew and Gorbach 1977), as well as the methyl ester of hexanoic acid (Drucker 1970).

2.6.4. Ester analysis

Cormier *et al.* (1991) used gas chromatography to separate the volatile produced by *Pseudomonas fragi*. Gas chromatography analysis of the volatile fraction of strawberry smelling cultures of *P. fragi* revealed a complex profile that consisted of approximately 90 peaks, several of the 26 odor-active components were ethyl esters of short-chain (C_3-C_7) fatty acids. Influences of freezing on strawberry aroma and characterization of their cultivars were studied by Douillard and Guichard (1990), and performing gas chromatographic analysis of the total organic extracts of the strawberry cultivators.

Separation and quantification of volatile from strawberry jam were carried out by Lesschaeve *et al.* (1991) by using gas chromatography. Five trained persons noted odors from the GC effluent (Etiévant *et al.*, 1983). Jollivet *et al.* (1992) analyzed the volatile produced by coryneform bacteria using GC analyses. In addition, Hosono *et al.* (1974) observed that the quantitative estimation of ethyl butyrate and ethyl hexanoate produced by the enzyme reaction was determined from GLC standard curves prepared by plotting GC peak height against concentration for the respective esters.

Almosnino and Belin (1991) using a Packard chromatography unit examined volatiles from the apple pomace enzyme system. Douillard and Guichard (1990) identified and quantified sixty compounds in strawberry cultivars by GC-MS. Lesschaeve *et al.* (1991) identified the volatile constituents of strawberry jam using a Nermag MS couple

with GC. In addition, Jollivet *et al.* (1992) identified flavor compounds from strains of coryneform bacteria by GC coupled with MS. Thirty-two flavor compounds were identified consisting of fatty acids, alcohols, methylketones, sulfur compounds, aromatic compounds and pyrazine.

3. Materials and Methods

3.1. Bacterial Strains

The gram negative bacteria of *Pseudomonas fragi* CRDA 037 was obtained from the Food Research and Development Center of Agriculture and Agri-Food Canada (CRDA, St Hyacinthe, Québec, Canada).

The microbial cells were kept on BHI agar plates (Brain Heart Infusion, Difco Laboratories Detroit, USA) at temperature between 4 to 20°C and transferred every 2 to 4 weeks, and on agar slants at 4°C.

3.2. Chemicals

Whey powder, used for fermentation medium was obtained from Saputo (St Hyacinthe, Québec). Brain Heart Infusion (BHI) and Bacto-Agar were obtained from Difco Laboratories (Detroit, MI). Butyric, pentanoic (valeric), hexanoic (caproic) acids, phosphate and EDTA were purchased from ACP Chemical Inc. (St-Léonard, Québec). Heptanoic and octanoic (caprylic) acids with their ethyl esters, CHAPS and polyoxyethylene-sorbitan monolaurate (Tween 20) were obtained from ICN Biomedicals Inc. (Aurora, OH). Aldrich Chemical Co. (Milwaukee, WI) supplied ethyl butyrate, ethyl caproate, methyl butyrate, methyl valerate and Triton X-100. Ethyl pentanoate (valerate), bovine serum albumin (BSA) and potassium phosphate dibasic, anhydrous were got from Sigma Chemical Co. (St-Louis, MO). Methanol and sodium hydroxide provided by Fisher Scientific (Fair Lawn, N.J.) and ethanol (94%) purchased from Société des Alcools du Québec (Montréal, Québec). Methyl hexanoate (caproate), methyl heptanoate and methyl octanoate (caprylate) were received from Poly Science Co. (Niles, IL). Potassium dihydrogen orthophosphate and Antifoam "B" were bought from BDH Inc. (Toronto, ON).

3.3. Biomass Production

The inoculation of *P. fragi* CRDA 037 was carried out according to the procedure described previously (Raymond *et al.*, 1990). Three successive subcultures of the bacteria were incubated in sterilized BHI broth at 30°C and shaking at 200 rpm in an Environ-Shaker (Lab-line Instruments, Inc., Chicago, IL). The first subculture was initiated by inoculation, using one colony of *P. fragi* from agar slants. The first and second subcultures were incubated for 17 to 24 h whereas the third one was incubated only for 17 h. A 0.01% (v/v) inoculum was used to initiate the second subculture from the first one and the third subculture from second one. Twenty five milliliter of sterilized BHI broth were dispensed into 125 ml Erlenmeyer flasks which were plugged with sterilized cotton. BHI was only used for inoculum.

The whey medium of 6% (w/w) was adjusted to pH 8.0 with 4 N NaOH prior to sterilization (121°C, 10 min). The medium was cooled to room temperature and butyric acid (0.1%, v/v) and 0.2% (v/v) of food grade ethanol (94%) were added. A 0.3% antifoam B was supplemented and the medium was adjusted to pH 6.5 with NaOH. The third subculture was used as an inoculum for the whey protein medium. A 1% of inoculum standardized of BHI with an optical density of 0.3 measured at 600 nm, using Beckman DU-650 spectrophotometer (Beckman, San Ramon, CA). Whey was only used for the fermentation.

Four hundred milliliter quantities of culture medium should occupy 1/5 of the volume of Erlenmeyer flask of 2 L which were then plugged with cotton and covered with foil and autoclaved at 121°C for 10 minutes. The cultures were incubated at 11°C for 78 h in orbital shaking incubator at 150 rpm.

3.3.1. Measurement of *P. fragi* esterase activity and optical density

Measurement of activity and optical density were done from 0 to 108 h during the time of biomass production. The technique available in our laboratory was based on spectrophotometry. The light intensity will be decreased when it is passed through the

bacterial suspension because part of the light intensity is absorbed by the bacteria and other part is scattered by them. However, since the bacteria are uncolored, the effect of the absorption of the light by the bacteria is considered negligible. When the culture has low densities, the absorbance of the culture is proportional to the dry weight of the culture, but at higher densities the correlation is no longer linear. If the concentration of the culture is too high, the stream of light will be "plugged". This means, the medium will have to be diluted to a value that lies in the linear portion of the absorbency range. This value will then be multiplied by the dilution factor (Ingraham *et al.*, 1983). The optical density was measured by taking 0.5 ml of culture medium, diluted with 1.0 ml of deionized water and measuring the optical density at 600 nm against a blank of deionized water by spectrophotometer. The values were later multiplied by a factor of three to correct for the effect of the dilution. By increasing the optical density was indicated an increase in the cellular bacterial growth.

3.3.2. Cell harvesting

The *P. fragi* cells were harvested and separated by centrifugation (12,000 x g, 2 h) at 4°C and assayed for esterase activity. Cultivated cells were maintained in ice-bath (0°C), the cells were washed twice with samples of chilled potassium phosphate buffer (0.05 M, pH 7) and centrifuged (39,000 x g, 20 min) at 4°C

3.4. Cell Disruption

Microorganism preparation (25 %, w/v) of *P. fragi* strain CRDA 037 was stored at 4°C. The disruption of the cells were carried out by using a wide range of disruption methods, including glass beads (MSK Braun), French press, ultrasonic and a combination of two of the disruption methods glass beads, ultrasonic and French press techniques.

3.4.1. French press

A 25% cell suspension (w/v) in potassium phosphate buffer (0.1 M, pH 7) was prepared. The bacterial suspension was placed under high pressure range from 5,000 PSI to 8,000 PSI at a working volume of 40 to 50 ml and then was extruded through a narrow

hole by slightly opening a ball or needle valve. The combination of the release of pressure and the shear at the hole will cause cell disruption. The pressure cell used was an Aminco French press cell (SLM Instruments, Inc., Urbana, IL) with a Power Laboratory Press (American Instrument Co., Inc., Silver spring, MD). Cell suspension was passed through the French press several times (1 to 6). The disrupted cell suspension was centrifuged (39,000 xg, 20 min) at 4°C and the supernatant was collected. A 25% debris suspension was prepared by using chilled potassium phosphate buffer (0.1 M, pH 7) and *P. fragi* esterase activity assay was carried out for each individual pass.

3.4.2. Glass beads (MSK)

Small glass beads of 0.10 to 0.11 mm in diameter were poured into a 70 ml disruption bottle and the solution contained a 25% cell suspension (w/v) in chilled potassium phosphate buffer (0.1 M, pH 7) by (1/1, v/v) at a working volume of 40 ml. The disruption of cells was carried out at low temperatures using a stream flow of CO_2. The cells were vibrated for various periods of time with interruptions about every 1 to 2 min. to check the cooling. The suspension was decanted, and the glass beads rinsed with buffer to remove the debris. The cell suspension was centrifuged (39,000 x g, 20 min) and the supernatant was collected. The *P. fragi* esterase activity was determined for the fresh debris and supernatant. The supernatant obtained was "freeze-dried".

3.4.3. Ultrasonic

Cells suspensions of 25% (w/v) were prepared in chilled phosphate buffer (0.1 M, pH 7). A 20 ml of cell suspension (50 ml flask) was introduced and placed in an ice-bath. The disruption was carried out by programming the ultrasonic at 3.5 speed for 15 sec disruption, following by 15 sec interruption. The disrupted cells were centrifuged (39,000 x g, 20 min) at 4°C. The esterase activity assay was carried out on the debris and the supernatant. Protein determination was carried out on the debris and the supernatant.

3.4.4. Combined treatment

Two combined methods of homogenization using glass beads and ultrasonic

techniques as well as French press and ultrasonic techniques were used for the disruption of cells, and homogenization of debris. The debris obtained, after centrifugation of a cell-disrupted solution, were suspended in chilled potassium phosphate buffer (0.1 M, pH 7). Moreover, the treatment was carried out on the debris obtained after using the second disruption method.

3.5. Chemical Treatment of Cellular Debris

3.5.1. Chemical solutions

All chemical solutions, Triton X-100 and CHAPS, were prepared in chilled potassium phosphate buffer (0.1 M, pH 7). Two different detergents were used in the treatment, nonionic detergents, Triton X-100, was prepared at a wide range of concentrations (1, 2 and 3%, v/v) whereas that of CHAPS was prepared at concentrations ranging from 10 to 50 mM. Moreover, Triton X-100 was used in the presence of ethylenediaminetetraacetic acid (EDTA) at concentrations of 0, 1, 2.5 and 5 mM.

3.5.2. Cellular debris treatment

The cellular debris obtained after centrifugation was suspended in 25% (w/v) buffer. Different concentrations of Triton X-100 (1, 2 and 3%) and ethylenediaminetetracetic acid EDTA (0, 1, 2.5 and 5 mM) were prepared in the presence of the cellular debris suspension. The samples were placed in ice-bath for 60 min with periodical shaking at every 10 min. The solution suspension was centrifuged (39,000 x g, 20 min.) at 4°C. The esterase activity assay was carried out on the debris and the supernatant after treatment. However, the *P. fragi* esterase activity and protein determination in the supernatant was carried out after the treatment elimination of the detergent from extract. Filtration of the supernatant was carried out using a centricon (Centricon-10 concentrator, Amicon) after washing with chilled buffer for 3 times to eliminate Triton X-100 and EDTA. Micro centrifuge was used in the cold room.

3.6. Protein Determination

The method of Hartree (1972) was used for the protein determination of the samples. Bovine serum albumin (Sigma Chemical Co.) was utilized as the protein standard and standards of various concentrations were run at the same time as the sample to form a calibration curve.

3.7. Esterase Assays

The enzymatic assays were carried out by the incubation of 0.3 ml of 25% debris suspension and substrate (valeric acid 1,000 ppm and ethanol 2,000 ppm) for 24 h at 150 rpm and 11°C. The final volume of the sample was 3 ml by using potassium phosphate buffer (0.1 M, pH 7). Following the experiment period of time, the extraction was taking place by adding 0.5 ml of 300 ppm of internal standard (methyl heptanoate), and then 1.5 ml ethyl ether was added. Five seconds of vortex was taking place. The sample tube was centrifuged for 5 min. at 3000 rpm. The extract layer of ether was concentrated from 1 to 0.1 ml with a stream of nitrogen. The analyses of end products were obtained by gas-liquid chromatography of Varian 3400 using flame of ionization detector. Capillary column of DB-FFAP (J & W Scientific, Folsom, CA) was used with (Length 30 m, ID 0.32 mm and Filling thickness 0.25 μm). The parameters of analyses were detector temperature 240°C, initial injector temperature 50°C and final injector temperature 240°C, 0.5 min, holding in the beginning and 22.6 min, holding in the end. However, the temperature rose 100°C/min. On the other hand, initial column temperature was 40°C and hold for 4.5 min then it was increased at rate of 10°C/min. The final column temperature was 240°C, hold for 0.5 min. The carrier gas was helium at flow rate of 1.5 ml/min. The flow rate of hydrogen and air were 30 ml/min and 300 ml/min, respectively. The procedure has been summarized in Scheme 2.

3.8. Standard Curve

The peak heights of the ethyl esters compounds were identified on the basis of retention time and were related to the concentration and molecular weight of the esters

compounds in the sample by adding known amounts of each compound to the sample before extraction. The known compounds were added in aqueous solution in place of a portion of the 3 ml of 0.1 M phosphate buffer normally added to each sample. The internal standard used in this experiment was methyl heptanoate. The ratio of the ethyl ester peak heights over the internal standard of methyl heptanoate, extracted by ethyl ether and injected into GC, obtained with various concentrations of known compounds added to the sample were then plotted to yield the standard curves were shown in Figure 4. Similar procedure was followed up to obtain the standard curves for methyl esters by using ethyl heptanoate as internal standard (Fig. 5).

3.9. Substrate Specificity

Substrate specificity measurements were carried out using different fatty acids as follows: butyric, pentanoic, hexanoic, heptanoic and octanoic acids with ethanol and methanol. In the beginning, the retention time for each fatty acid should be determined by adding fatty acids in 10 ml diethyl ether with internal standard. Then the sample was injected into GC. By using the spike method to identify peaks, it was performed by spiking the sample with different known concentrations of each compound and reanalysing it to determine the relative increase in size of the peak. The experiment conducted in our laboratory by using different concentrations of fatty acid to ethanol or methanol by the ratio of 1:1, 1:2 and 2:1. However, determination of substrate specificity by the ratio mentioned above was carried out by preparation of (i) stock solution of fatty acids to alcohols, (ii) the 25% cells or cellular debris suspension, and (iii) making assay with different concentration by keeping the ratio. The determination of standard curves for fatty acids ethyl/ or methyl esters were carried out by using the same concentration mentioned above.

Scheme 2. Schematic diagram for determination of esterase activity

Cells or Debris

↓

bioconversion

0.3 ml consisted of a 25% (w/v) cells or debris

+ 1.7 ml of potassium phosphate buffer (0.1 M, pH 7)

+ 1 ml of phosphate buffer contains substrate of

6000 ppm ethanol (final concentration will be 2000 ppm) and

3000 ppm valeric acid (final concentration will be 1000 ppm)

(Different substrates for Specificity), Bioconversion assay for 24 h at 11°C, 150 rpm

↓

extraction

0.5 ml of internal standard (300 ppm) in phosphate buffer

+ 1.5 ml ethyl ether

↓

5 sec of vortex

↓

centrifugation

(1 or 2) x 5 min at 3000 rpm

↓

concentration

Recuperation of 1 ml of the organic phase in the evaporation tube

↓

Evaporation under nitrogen until 0.1 ml

↓

Gas chromatography (GC) analysis

4. Results and Discussion

4.1 Optimization the Production of the Biomass of *P. fragi* with Reference to the Production of Esterase Activity using Ethyl valerate as a Model system

4.1.1. Determination of esterase activity

It was found that the optimum culture growth of *P. fragi* in whey fermentation medium was at 11°C, 150 rpm and the exponential phase began from ≈ 24 h to ≈ 72 h and the stationary phase started from 78 h to 96 h which there was no significant difference in the ethyl valerate production before going to the death phase (Fig. 1). Following *P. fragi* growing in skim milk, gas chromatography analyses revealed the presence of approximately 90 compounds (Cormier et al. 1991). *Pseudomonas fragi* was fermented on a culture medium composed to whey, butyric acid and ethanol. After separation of the supernatant and the cells of *P. fragi* fermentation, the bioconvension medium is composed of specific fatty acids and alcohols using ethyl valerate as a standard rather than ethyl butyrate. The fermentation process was followed by the bioconvension process. Butyric acid is an inducer. In addition ethyl (methyl) butyrate has less molecular weight than valerate ester leading to butyrate ester peaks very close to solvent peak in GC analyses and there is many other impurity peaks coming close to solvent peaks. Ethyl valerate has distinctive and separated peaks, so it was used as internal standard for esters production. High microbial populations noted in the exponential phase of the growth of the microorganism. The growth of bacterial populations is normally limited either by the consumption of available nutrients or by the accumulation of toxic products. The rate of growth declines and growth eventually stops. The culture coming at last point is said to be in the stationary phase. The transition between the exponential and stationary phases involved a period of unbalanced growth at unequal rates (Stanier *et al.* 1990). Raymond *et al.* (1990) also reported the onset of the stationary phase at 24 h of growth for the same strain. The levels of the odor-active metabolites were observed after 60 h of fermentation, although the ideal time to harvest the cells is usually towards the end of the exponential phase before entering the stationary phase of growth. The desired enzyme may not be at maximum activity at the end of the exponential phase of growth (Scopes, 1994). The

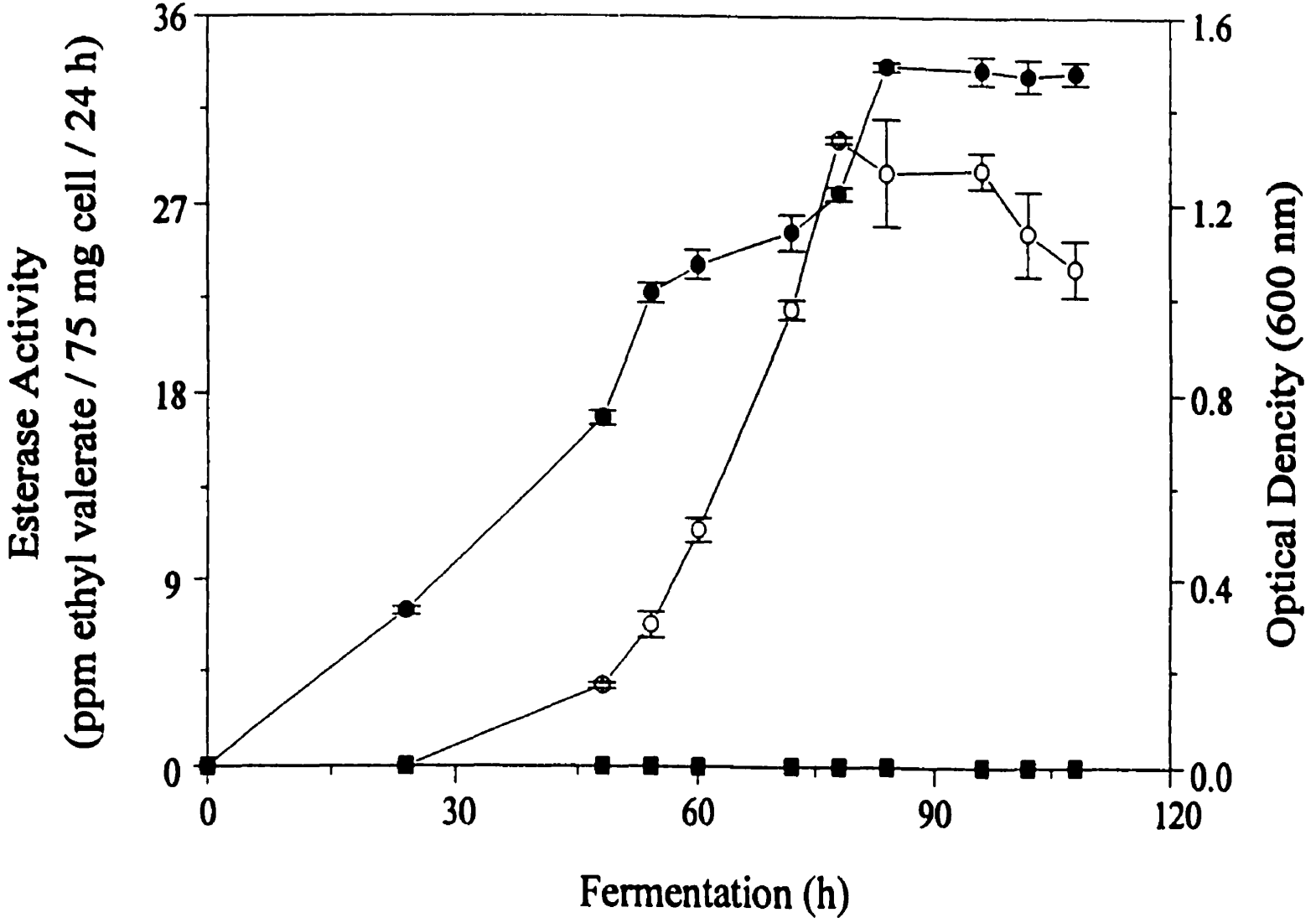


Figure 1. Esterase activity observed during the growth (cultivation) of *P. fragi* in the cells (—o—) and supernatant (—▪—). Optical density (–●–) of growing cell measured at 600 nm.

esterase activity of *P. fragi* present in the fresh whole cells was measured at different time of the fermentation process (Fig. 1). The esterase activity is a determination of specific activity for *P. fragi* esterase of wet weight of whole cells. The values of ethyl valerate (ppm) shown in Figure 1 are the averages of observation from 3 replicates at different times, which had relative standard deviation from 0.48 to 9.04.

The highest esterase activity of the whole cells observed during the growth of *P. fragi* was 30 ppm of ethyl valerate at 78 h (1.5 mg ethyl valerate / 1 g cells) since it was difficult to determine the amount of enzyme in the cells of *P. fragi* (Lamer *et al.* 1996). However, more cells had been produced when the culture had been left to run for a longer period of time, on the other hand the enzyme activity had decreased (Fig. 1). These results are in agreement with those obtained by Raymond *et al.* (1991) who reported that the optimal production of odour-active metabolites of *P. fragi* in whey contributing to the aroma of ethyl isovalerate was observed after an incubation time of 72 h at 11°C and 150 rpm. This was probably due to differences in level of synthesis of lipases and esterases which have been known to be involved in the synthesis of fruity aroma (Nashif and Nelson, 1953; Pereira and Morgan, 1958; Hosono *et al.*, 1974). Although the activity of these enzymes is optimal at 30°C, their synthesis were higher at temperature lower than the optimal growth temperature (Nashif and Nelson, 1953; Hosono *et al.*, 1974). It was important to determine when the enzyme was most active because one can assume that a high production of fruity aroma at temperature lower than the optimal growth temperature was due to a high concentration of enzyme instead of its high activity which is usually temperature dependent, the optimal temperature for esterase activity is 15°C (Cormier *et al.* 1991). The highest esterase activity of growth (30.07 ppm) shown in Figure 1 was produced by supplementing the medium with 0.1% valeric acid and 0.2% ethanol (*P. fragi* cells). The level of esterase activity was found to vary from one culture to another, ranging from 10 to 20%. Percentage recovery of ethyl valerate increased with the higher ester concentration (Fig. 2), showing an exponential trend from 0 and continued up to 50.0 ppm, hyperbolic curve from 50.0 to 125 ppm, and negligible increases in the end of the experiment. The amount of the recovery of ethyl valerate was

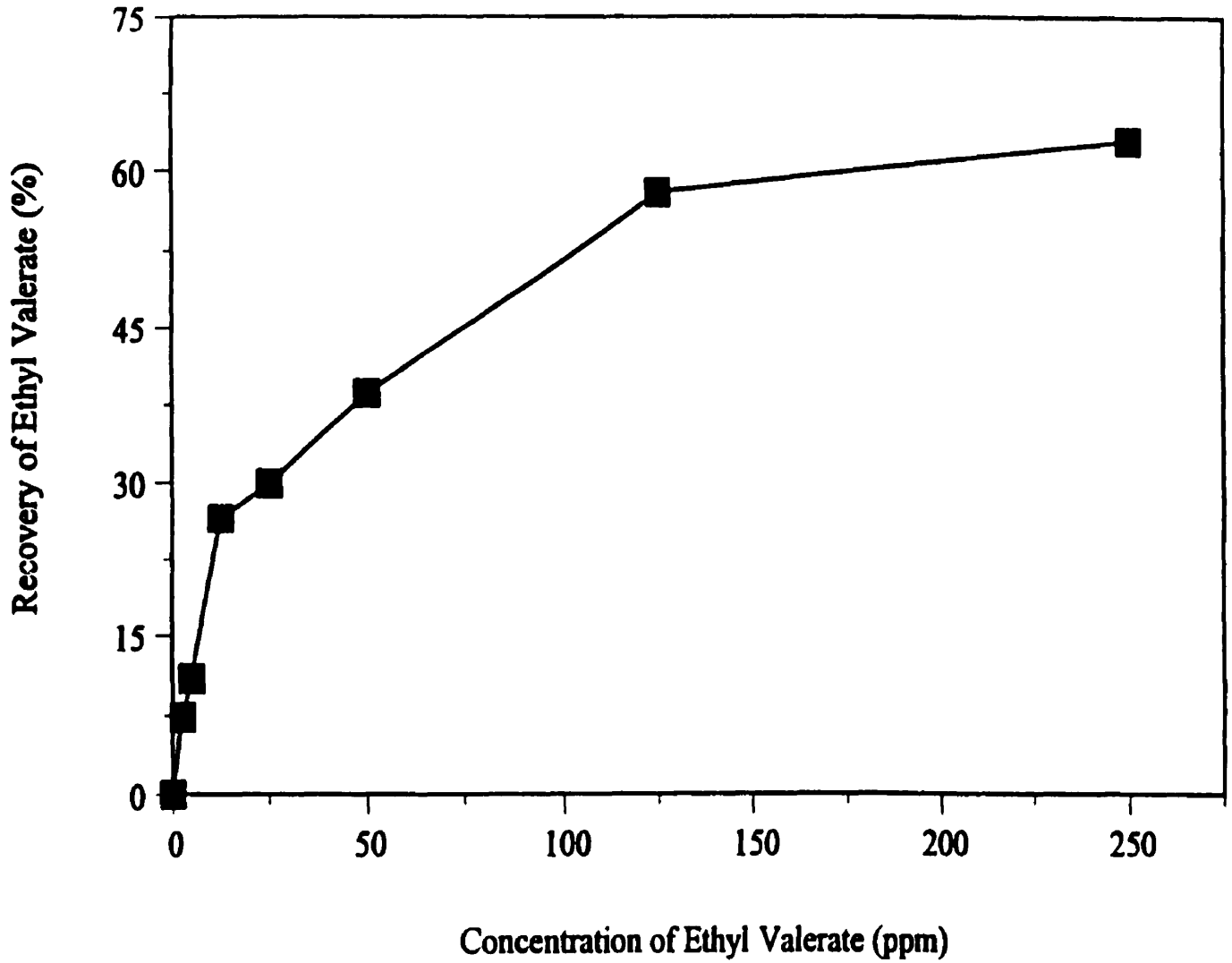


Figure 2. Effect of the concentration of ethyl valerate on the recovery of ethyl valerate.

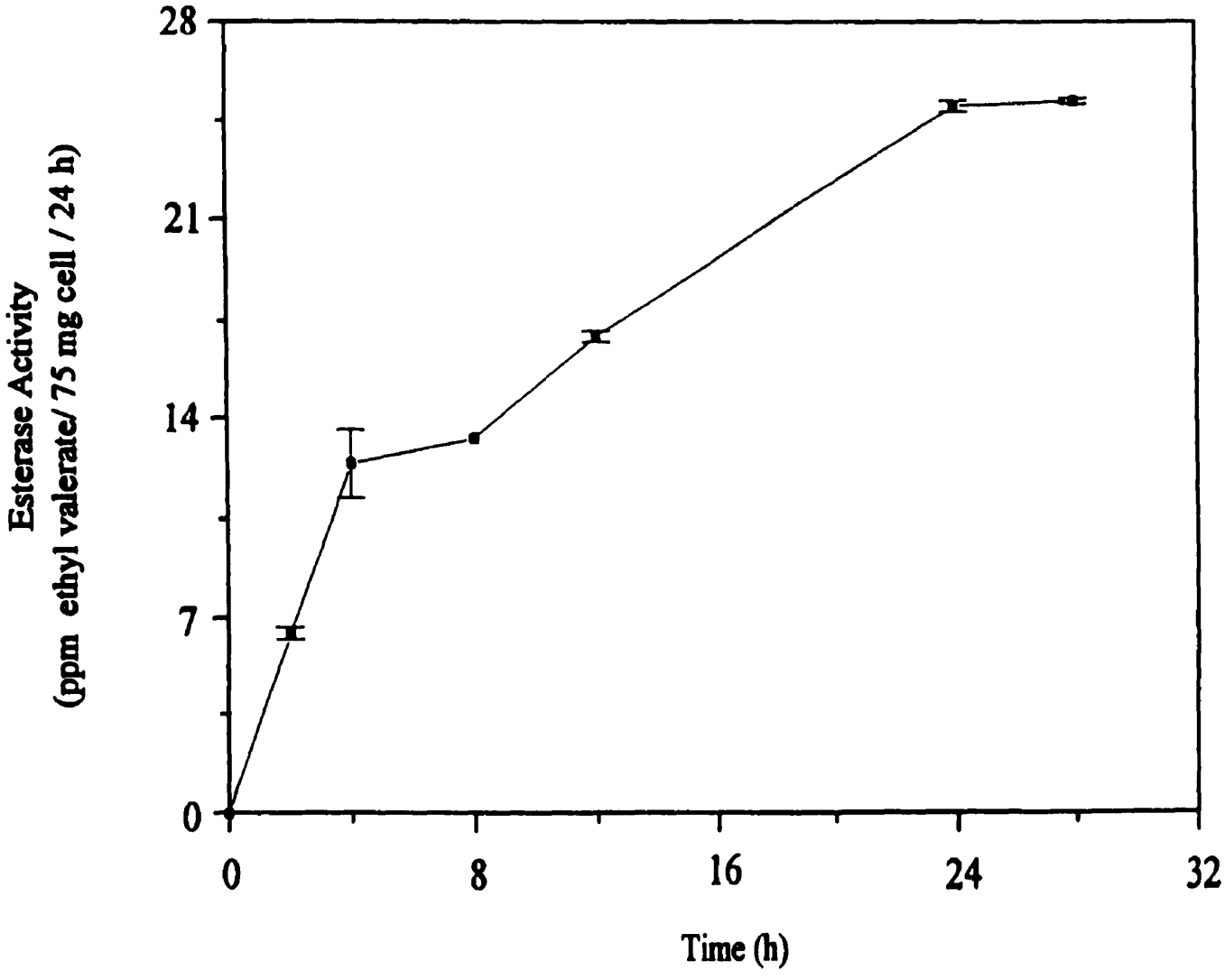


Figure 3. Biogeneration of ethyl valerate by whole cells of *P. fragi* of esterase activity , using 1,000 ppm valeric acid and 2,000 ppm ethanol.

calculated as a percentage of the initial concentration of ethyl valerate before injection on a gas chromatography (GC) with the amount obtained after injection on a GC.

Figure 1 also indicated that the optical density increased during the production of the biomass. These results are in agreement with those of Schuepp *et al.* (1997) who reported the increase in the optical density during the growth of *P. fragi*, in the whey medium, which was also used in this study. The medium was diluted three times to allow the measurement of the optical density by the spectrophotometric techniques. Therefore, the values in the curve should be multiplied by a factor of 3 to compensate for the dilution effect. Figure 1 shows the absence of esterase activity in the supernatant fraction. These findings are consistent with Reddy *et al.* (1970) and Hosono *et al.* (1974) who reported that the *P. fragi* esterase was intracellular. However, Schuepp *et al.* (1997) reported intracellular as well as extracellular lipase activity in *P. fragi*. An extracellular vesicle was collected from the culture supernatant of *P. fragi* cells growth (Myhara and Skura, 1989). Anlysis showed that most of the proteinase associated with one liter of culture could be found in the supernatant with smaller amounts associated with cell contents or extracellular vesicles (Myhara *et al.*, 1990).

4.1.2. Biogeneration of ethyl valerate

The biogeneration of ethyl valerate was performed by *P. fragi* esterase of whole cells using 1,000 ppm valeric acid and 2,000 ppm ethanol and measured at different intervals varying from 0 to 28 h of incubation (Fig. 3). Using 10% cell suspension, the esterase activity increased with the time reaching a plateau after 24 h of incubation. The relative standard deviations for different reading were between 0.80 and 9.44. Results (Fig. 3) showed that the highest amount of ethyl valerate reached at 11°C after 24 h. The average fatty acid ethyl esters produced in this experiment was 25 ppm/75 mg cells/ 24 hs (Fig. 3). However Lamer *et al.* (1996) studied the same strain of *P. fragi* that the production of ethyl valerate up to a maximum incubation for 8 h. and did not examine the optimization of the biogeneration of ethyl valerate. In addition, under the condition of gas

stripping, the maximal productivity rates of ethyl valerate and of total fatty acid ethyl esters were 26 and 28 ppm h^{-1}, respectively, which occurred after 14 h of conversion (Morin *et al.*, 1994). The increase of the ester production in previous study may be due to the equilibrium changed to produce ester continuously.

4.1.3. Optimization of *P. fragi* cells concentration

Results in Table 1 show that the production of ethyl valerate by *P. fragi* esterase increased by 1.5 times (20.98 to 31.00 ppm) by increasing the cell suspension of *P. fragi* from 10 to 50%. The increase in the production of ethyl valerate was rapid by increasing the concentration of cell suspension from 33% to 50%. However, these results indicate that 10% cell suspension is the most suitable concentration for bioconvension reaction. Moreover, the results also indicate that a further increase of *P. fragi* cells concentration had no real effect on the production of ethyl valerate. This inhibition to the production of ethyl valerate could be related to a higher enzyme concentration in the medium that holds the active site of the enzyme from the available substrate. The decrease in activity could be related to product removal from the bioconversion medium for increasing substrate consumption, preventing product inhibition and improving productivity (Groot *et al.*, 1989; Pham *et al.*, 1989; Liu and Hsu, 1990). The cell concentration of 10% was selected as an optimal throughout the study. These experiments were carried in triplicate. These finding are in agreement with these result obtained by Morin *et al.* (1994) who reported for production of fatty acid ethyl esters by *P. fragi* under conditions of gas stripping by 10% (v/v) cell suspension at 12°C for 48 h and agitating at 150 rpm. However, Lamer *et al.* (1996) suggested that the total amount of ethyl valerate produced after 8 h could be doubled by increasing the cell concentration from 2.5% to 5.0%. They found no significant effect on the production of ethyl valerate beyond 5.0% cells concentration.

In this experimental condition it was not possible to determine the amount of enzyme in the cells of *P. fragi*. Therefore, it was difficult to compare the results with those in the literature. The literature reports clearly that the amount of enzyme in the cells of *P. fragi* could not be determined (Pereira and Morgan, 1958; Reddy *et al.* 1970; Lamer

Table 1. Measurement of ethyl valerate production by *P. fragi* with whole cells.

Cell Suspension (%, w/v)	Ethyl Valerate (ppm)
10	20.98[a] (4.16)[b]
17	20.72[a] (2.03)[b]
23	21.85[a] (0.64)[b]
33	24.42[a] (1.62)[b]
50	31.00[a] (4.58)[b]

[a]Average values of triplicate injections.
[b]Percent relative standard deviation values calculated as: (standard deviation/mean) x 100.

et al. 1996; Schuepp *et al.* 1997). In fact most bioconvension reactions used to produce ethyl esters were mediated by commercial enzyme (Lamer *et al.* 1996). Welsh *et al.* (1990) used various commercial lipases to produce low molecular weight esters in non-aqueous system with an enzyme concentration of 0.01 or 0.02 mg/ml. Langrand *et al.* (1990) performed the ester synthesis with lipase of various microorganisms by using enzyme concentration of 50 mg/ml.

4.1.4. Standard curves

Standard curves were plotted for the ratio of gas-liquid chromatography of ethyl/methyl esters over the internal standard peak area versus different concentrations of ethyl/methyl esters. This method for the quantitative analyses of volatile compounds had been used previously (Bills and Day, 1966).

Plots of the concentrations of ethyl butyrate, ethyl valerate, ethyl caproate, ethyl heptanoate and ethyl octanoate against the ratio of ethyl ester to internal standard (IS) are in Figure 4, and the 1/slope have estimated to be 62.1, 55.9, 52.8, 76.4 and 58.3, respectively. Whereas the 1/slope of methyl butyrate, methyl valerate, methyl caproate, methyl heptanoate and methyl octanoate (Fig. 5) were 68.3, 44.0, 43.5, 36.8 and 48.8, respectively. Known quantities of methyl heptanoate (ppm) and ethyl heptanoate (ppm) were added in the extraction and were used as internal standards for the analyses of ethyl esters and methyl esters respectively. All curves were linear (r^2= 0.98-0.99). These values were used for the calculations of the ester production (ppm).

The gas chromatography technique employed in this study provided sufficient accuracy and relative standard deviation to warrant its use as a quantitative tool. The linearity of response for fatty acid ethyl/methyl esters has been shown in Figures 4 and 5. Each point on the lines in the figures represents the mean of triplicate injections. Relative standard deviation of the technique for other applications has been reported earlier (Morgan and Day, 1965; Bills *et al.*, 1966). It was well known that the use of an internal standard in gas-liquid chromatography overcomes most of the experimental difficulties related to quantitative aspects of the procedure and compensates for variations between

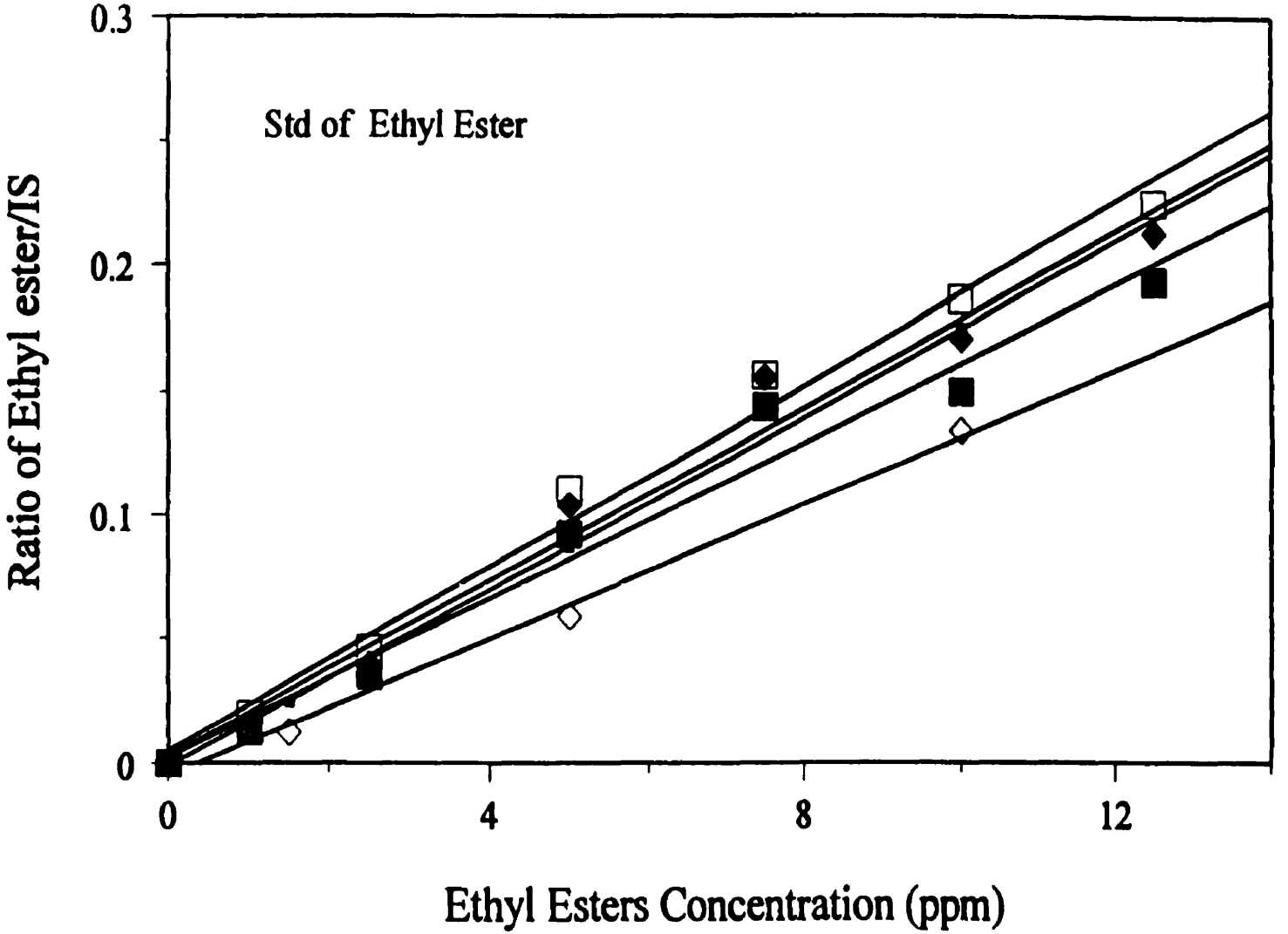


Figure 4. Standard curve of ethyl ester fatty acids concentration of ethyl butyrate (—■—), valerat (—◆—), caproate (—□—), heptanoate (—◇—) and octanoate (—•—) with the ratio of ethyl ester/IS.

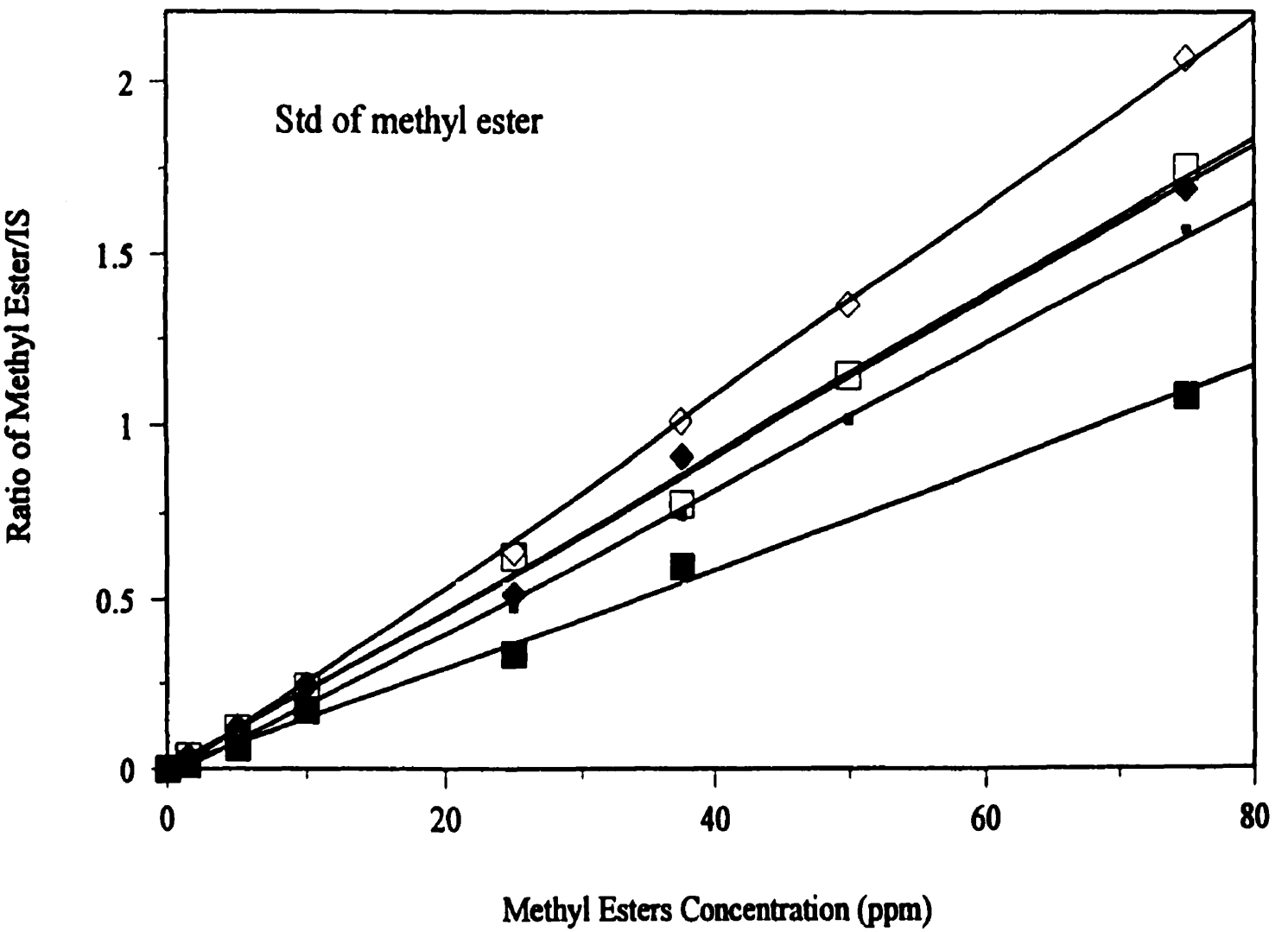


Figure 5. Standard curve of methyl ester fatty acids concentration of methyl butyrate (—■—), valerate (—◆—), caproate (—□—), heptanoate (—◇—) and octanoate (—•—) with the ratio of methyl ester/IS.

runs. It had been shown that, in the absence of an internal standard, the differences were among regression equations of results when different gas chromatographs had been used (Jones-Witters, 1970).

It is difficult to obtain a convenient compound to be used as internal standard for the analyses of by-products extracted from bioconvension by *P. fragi*. However, the gas chromatography results indicated that the methyl heptanoate (Fig. 4) and ethyl heptanoate (Fig. 5) could be used as internal standards for gas chromatographic analyses of ethyl/methyl esters respectively, since the slopes were well quite different. Although many studies on characterization and identification of microbial metabolic products have been performed by gas chromatography, most of them were without internal standards (Farshy and Moss, 1970; Thoen *et al.*, 1972; Carlsson, 1973; Wade and Mandle, 1974; Newman and O'Brien, 1975; Holdeman *et al.*, 1977; Bohannon *et al.*, 1978; Drucker, 1981; Drummond and Shama, 1982). In this study, the gas chromatography data showed relative standard deviations of $\leq 10\%$.

It was considered to be important that the internal standards of methyl heptanoate and ethyl heptanoate were not one of the sample components of ethyl esters and methyl esters, respectively. They neither interfere with the peaks nor reacting with the sample components.

4.2. Disruption of the Cells of *P. fragi*

4.2.1. Cell disruption

From the initial experiments it was possible to identify the physiological state of *P. fragi* that contained the highest enzyme activity. There was no difference in the esterase activity and cell density after 78 h of culture fermentation (Fig. 1). The ethyl valerate content is almost stable between 78 and 102 h.

The obtained results (Fig 1) showed that the 78 h of fermentation was the most suitable time for collection of *P. fragi* and esterase enzyme extraction in term of ester

production by specific activity of the esterase. When the enzyme was in the cytoplasm, or weakly attached to the bacterial membrane; the simple homogenization of the cells was sufficient to recover or extract the enzyme into an aqueous media. This process did not involve solubilization of the membrane itself. Due to the thickness of the cell wall of bacteria, many disintegration techniques were used to disrupt the cells such as lysozyme treatment, grinding with abrasive sand or alumina or by using French press, ultrasonication and glass beads. Certain gram negative bacteria cells were disrupted using a combined non ionic detergent-osmotic shock-lysozyme treatment (Schwinghamer, 1980). Therefore a whole extraction process may be more suitable. For extracting the enzyme using mechanical treatment, there were numerous method of breaking the cells depending on the nature of the cells delegate, moderate or harsh (Cull and McHenry, 1990; Scopes, 1994). Although most of the lipases in microorganisms (on insoluble substrates) were extracellular (Desnuelle, 1972), the esterases of gram-negative bacteria have been often described as intracellular enzyme in different *Enterobacteriaceae* (Goullet, 1978; Goullet, 1980; Goullet, 1981; Goullet and Picard, 1984; Goullet and Picard, 1985), phytopathogenic bacteria (El-Sharkawy and Huisingh, 1971), and in *P. fragi* (Lawrence *et al.* 1967). Different methods or a combination of two methods were used to disrupt the cells. These methods, include glass beads, French press, ultrasonic, glass beads with ultrasonic and French press with ultrasonic.

The homogenization process resulted in the disruption of weak salt on hydrophobic interaction with the membrane (Scopes, 1994). These extracted membranes were removed by centrifugation as a first step in the extraction of the protein. The protein and the esterase activity were then studied in the supernatant and the cellular debris. The crude protein concentration of different extracts obtained after homogenization of *P. fragi* cells, using different disruption methods are given in Table 3. The enzyme may have been released during cell disruptions, and therefore it may be present in extracts.

Once the protein is released into solution, it should remain soluble during further processing, because the process disrupts the weak salt or hydrophobic interactions with the membrane. Removing the extracted membrane by centrifugation was the first step

after extraction (Scopes, 1994).

Table 2 shows the quantities of protein estimated with different extraction methods. The concentrations of bacteria were substantial with all 5 methods. Esterase activity was tested with relatively high protein concentration in the enzymatic assays from 0.7 to 5 g/l. Despite this assay, the tests show the absence of esterase activity in the extract. It may be that the enzyme was partially or completely detached from the cellular membrane, but it was inactive, since the labile enzyme may have been inactivated after being liberated into the solution (Scopes, 1994). Inactivity could also be due to the loss of a cofactor or because the esterase needed the cooperation of another enzyme or one or more particular phospholipid.

4.2.2. Disruption methods

Disruption of *P. fragi* cells with the following methods glass beads, French press, sonication and combination of glass beads with ultrasonic and French press with ultrasonic, results in enrichment of the organelle or membrane fragment preparation with desired protein (Penefsky and Tzagoloff, 1971). Figure 6 shows the results from glass beads, French press and sonication. The esterase activity of cellular debris obtained from the glass beads disruption method (Fig. 6 A) diminished as function of the duration of disruption compared to whole cells of *P. fragi*, 50% of the esterase activity was lost after 1 to 2 min of disruption, and 91% after 6 min of disruption. The loss of activity could be attributed to the denaturation of the enzyme, and harsh treatment, or due to extraction of an inactive enzyme. On the other hand, the esterase activity of freeze-dried debris was decreased by more than 79% within 1 min of disruption.

The disruption of *P. fragi* cells with the French press disruption method (Fig. 6 B) gave a cellular debris without significant lose of esterase activity after 1 to 3 passages but it diminished the esterase activity after 4 to 6 passages. The loss in the activity was less than 1% after 3 passages, and by 35% after 4 to 6 passages. The best esterase activity was therefore obtained after 3 passages even with increased percent of cellular debris suspension in the experiment. Debette and Prensier (1989) reported that *Xanthomonas*

Table 2. Estimated protein concentration (g/l) of different extracts after *P. fragi* cells disruption with different methods

Methods	Time (min)	Protein concentration (g/l)
Glass beads (MSK)	1-4	5.9 - 15.4
French press (FP)	3 times	19.6
Ultrasonication (US)	1-5	0.5 - 10.6
MSK + US	2,4-2,3,4	8.2 - 17.8
FP + US	3 times-2	17.9

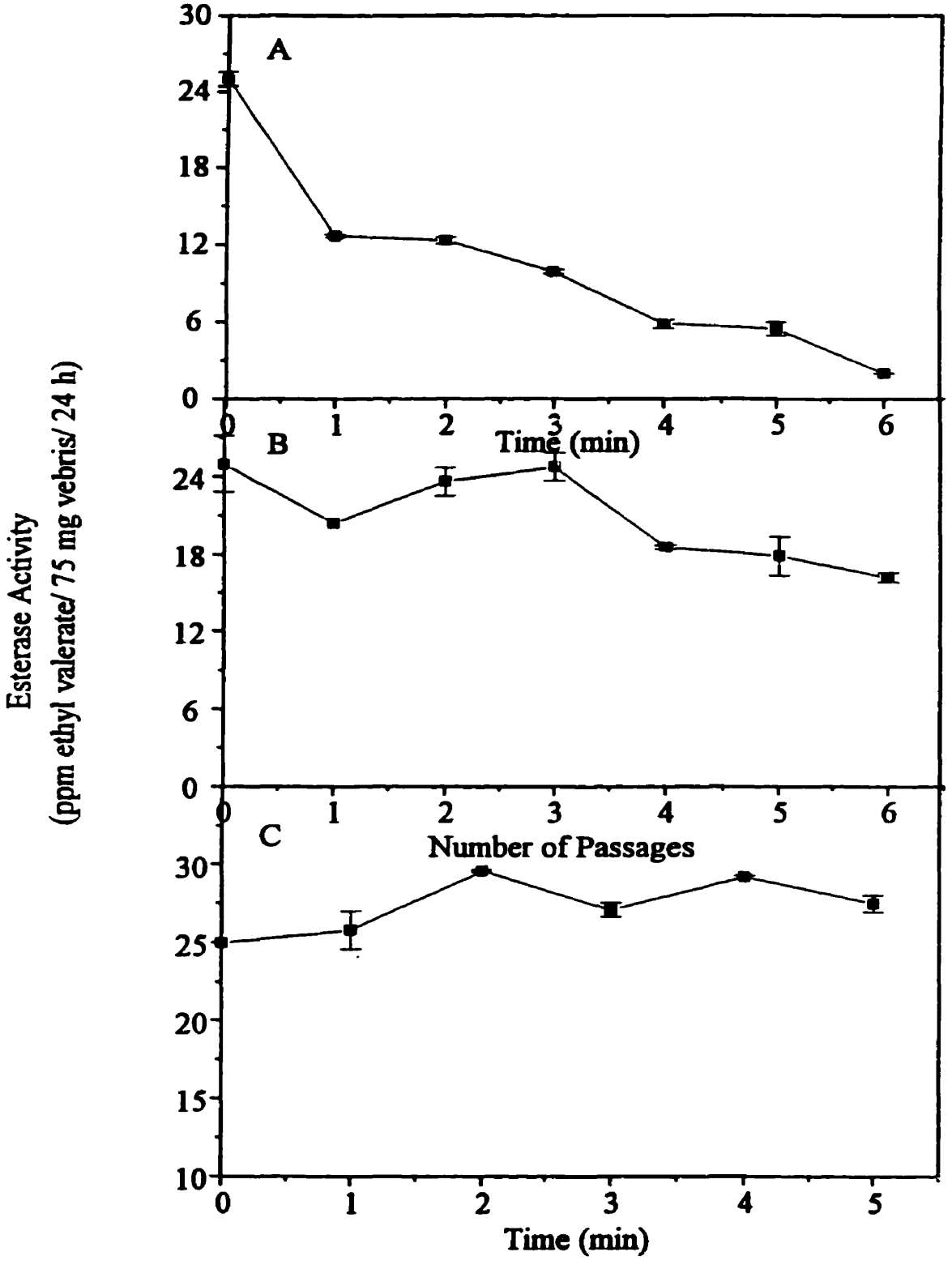


Figure 6. Biogeneration of ethyl valerate by *P. fragi* cells disrupted using A. glass beads, B. French press and C. ultrasonication.

maltophilia broken in a French press cell by using two passages and high enzymatic activity remained in the crude membrane fraction. Moreover, Schuepp *et al.* (1997) has reported that *P. fragi* was subjected to three successive cell disruptions using French press cell for obtaining exo- and endo- cellular of lipases.

The esterase activity of cellular debris obtained from the sonication disruption method (Fig. 6 C) increased as a function of the time of sonication. Compared to the activity of the whole cells before disruption, the esterase activity was increased by 18%, 10% and 16% at 2, 3 and 4 min of sonication, respectively with relative standard deviation of ≤ 8%. Therefore, the sonication method was used for the disruption of the cell through out the rest of the study of the cellular debris. The increase of the cellular debris activity could be due to elimination of numerous cellular components during the sonication. These components include mitochondria, nuclei, endoplasmic reticulum, golgi, vacuolar and lysosomal membranes. When attempting to purify a protein from a membranous structure, the first thing to consider is whether the trouble involved in isolating the organelle/membrane is worth for purification to a substantial degree (Scopes, 1994). Freeze-drying the cellular debris inactivated the enzyme by 90%.

Table 3 shows the activity of the cellular debris after different times of disruption using glass beads and ultrasound methods together. It was found that the cellular debris was slightly enriched with esterase activity after disruption. This is due to elimination of numerous cellular components during the disruption, including protein membranes, which might have concentrated the enzyme in the cellular debris. Table 3 also showed increase in the esterase activity by 4% to 24% as compared to the activity of whole cells with relative standard deviation of ≤ 6%. The enzyme was inactivated by 10% to 84% by freeze-drying. However, the above combination of treatment is time consuming and requires more effort to obtain higher esterase activity than ultrasound alone.

From this experiment it was concluded that the highest activity was found in the cellular debris, even if we considered the effect of concentration due to the elimination of numerous cellular components after centrifugation following the disruption. Esterase

Table 3. Ethyl valerate production by *P. fragi* esterase activity by disrupting the cells using glass beads and ultrasonication.

	Glass beads (min)	Ultrasonication (min)			
		0	2	3	4
Whole cells*	0	25[a] (8.60)[b]	-	-	-
Fresh debris*	2	-	23.49[a] (3.96)[b]	26.02[a] (5.81)[b]	29.50[a] (1.15)[b]
Freeze dry*	2	-	21.36[a] (1.90)[b]	14.77[a] (3.69)[b]	9.44[a] (0.15)[b]
Fresh debris*	4	-	30.55[a] (5.17)[b]	30.30[a] (2.39)[b]	30.40[a] (2.34)[b]
Freeze dry*	4	-	14.69[a] (10.58)[b]	17.71[a] (4.39)[b]	5.32[a] (11.76)[b]

[a]Average values of triplicate injections.
[b]Percent relative standard deviation values calculated as: (standard deviation/mean) x 100.
*Ethyl valerate production is in term of (ppm/75 mg cells or debris/24 h).

activity was assayed in the supernatant and the cellular debris of the disrupted cells. It showed that the activity was still recovered in the cellular debris only. The esterase in *P. fragi* was characterized as the membrane proteins. It was very hydrophobic, and was not detached by these treatments. These data suggested that esterase was membrane bound which is in agreement with earlier reports (Lawrence *et al.*, 1967; Debette and Prensier, 1989; Cadwallader *et al.*, 1992; van der Werf *et al.*, 1995). They reported from gram-negative bacteria that the esterase was intracellular enzyme.

On the basis of these findings (Figure 6 and Table 3), only sonication disruption method gave the best results in term of esterase activity in the cellular debris. It was mentioned above that the esterase activity was increased by 18% after 2 min of disruption as compared to esterase activity in whole cells of *P. fragi*. This is a better result as compared to esterase activity obtained by using glass beads and French press methods, which decreased by 50% and 1%, respectively. In addition, the sonication disruption method was easy to use and economical in terms of time and money.

4.2.3. Treatment of cellular debris

Preliminary results indicated that the esterase activity of *P. fragi* was endocellular (intracellular). The investigation concluded no enzyme activity into the culture supernatant because the cells of the microorganisms excreted it. Therefore, it had to extract the enzyme into an aqueous medium which was the first step of enzyme purification techniques. The literature indicates that certain enzyme can be solubilized using detergent. Triton X-100 was used for permeabilization and lysis of *Pseudomonas pseudoalcaligenes* to increase the production of D-malate (van der Werf *et al.* 1995). Debette and Prensier (1989) were able to extract a cell-bound esterase from *Xanthomonas maltophilia* using Triton X-100 to solubilize the enzyme. In addition α-Terpineol dehydratase from *Pseudomonas gladioli* was also solubilized using the same detergents (Cadwallader *et al.* 1992). For example, *Xanthomonas maltophilia* was considered esterase positive when Tween 80 was the substrate, and lipase positive when Tween 85 and glycerol tributyrate were hydrolyzed (Nord *et al.* 1975, O'Brien and Davis, 1982). On the other hand, Kouker

and Jaeger (1987) claimed that tributyrin and Tween 80 were not suitable substrates for detecting true lipases (triacylglycerol acylhydrolases; EC 3.1.1.3), since both of these compounds were hydrolyzed by esterase (carboxylic ester hydrolases; EC 3.1.1.1).

In cases where the enzymes are structurally associated with insoluble parts of the cells, certain detergent may be used to solubilize the enzymes (Scopes, 1994). Some hydrophobic enzymes have been solubilized in this way. In addition, a α-Terpineol dehydratase was extracted from membranes of *P. gladioli* with a solution of Triton X-100 with sodium trichloroacetate (Cadwallader *et al.*, 1992). The choice of detergent as well as the condition of solubilization are important to provide the maximal solubilization effect and without denaturing the enzyme (Hjelmeland, 1990).

The protein concentration of different extracts, obtained after treatment of the debris by solution of detergent are given in Table 5. It was observed that the substantial amount of protein was found in the supernatant after washing.

The detergent, with or without EDTA, had a real effect on the solubilization of the membrane proteins since the protein concentration increased from 2.6 to 11 g/l by using Triton X-100 and 4.68 to 12.46 g/l by using CHAPS (Table 4) in aqueous extracts. The amount of protein obtained with CHAPS higher than Triton. The protein extracts was assayed for *P. fragi* esterase activity, using ethanol and valeric acid as substrates and the extracts (enzyme) as biocatalysts. Unfortunately the results of bioconversion assay show absence of esterase activity in the protein extracts.

Table 5 shows the comparison of esterase activity of the cellular debris treated with different concentration of CHAPS and Triton X-100 detergents. The esterase activities are higher in the cellular debris treated with detergent in comparison to the activity in the untreated cellular debris and the whole cell. The maximum activity was observed with a 2% solution of Triton X-100 which increase the whole cell esterase activity by 63%. The esterase activity decreased very rapidly as the concentration of ionic detergent of CHAPS increased, and stopped at 50 mM. Where is the important reason is

Table 4. Estimated protein concentration (g/l) of different extracts after *P. fragi* cell disruption and treated debris with detergents and EDTA.

Methods	Time (min)	Protein concentration (g/l) Triton X-100	CHAPS
Glass beads (MSK)	1-4	3.5 – 5.52	4.68-6.47
French press (FP)	1-3 times	5.0 - 11.0	(-)*
Ultrasonication (US)	1-5	2.6 - 4.0	8.30-12.46
MSK + US	2,4-2,3,4	5.0 - 10.0	(-)*
FP + US	3 times-2	5.0 - 11.0	(-)*

*Not determined

Table 5. Ethyl valerate production by *P. fragi* esterase following disrupt of the cells using glass beads (2 min) and washing with detergent.

			Triton X-100 (%, v/v)			Chaps (mM)		
	Cells	Debris	1	2	3	10	25	50
Ethyl valerate*	25 (0)[c]	23.16 (0)[c]	37.02[a] (3.86)[b]	40.96[a] (10.6)[b]	37.51[a] (3.90)[b]	29.56[a] (4.67)[b]	2.63 (0)[c]	0

[a]Average values of triplicate injections.
[b]Percent relative standard deviation values calculated as: (standard deviation/mean) x 100.
[c]One injection
* Ethyl valerate production is in term of (ppm/75 mg cells or debris/24 h).

that nonionic detergents (i.e., Triton) are normally nondenaturing in their action (Bjerrum, 1983), whereas an ionic detergents (i.e., SDS) partially inactivated the enzyme (Debette and Prensier, 1989) or even at low concentration denatures proteins (Helenius and Simons, 1972). However, CHAPS is an ionic detergent. It seems that this detergent denatures the enzyme. No esterase activity was detected in the supernatant. Therefore, the experiments were continued using Triton X-100 at a 2% solution (v/v). Triton X-100 was found to be the most suitable detergent for solubilization of the enzyme. Triton X-100 has been extensively used for the solubilizing the insoluble macromolecules such as membrane protein (Helenius and Simons, 1975; Hearing *et al.*, 1976; Tanford and Reynolds, 1976).

Table 6 contains results for the cellular debris obtained after 3 passages through the French press and after different concentrations of Triton X-100 and EDTA. Compared to untreated cells, there may be more than a 33% loss of esterase activity by using 2% Triton X-100 and 2.5 mM EDTA. It may be noted that the highest ethyl valerate was produced with 1% Triton X-100 and 1.0 mM EDTA, but this may be ignored because of very high relative standard deviation (23.39) of the value. In addition, it is noticed that the esterase activity using 2% Triton X-100 was highest with 2.5 mM EDTA. No esterase activity was found in the supernatant.

When the cellular debris were obtained by disruption with glass beads (Table 7), the lost of esterase activity was 20% as compared to esterase activity in untreated whole cells of *P. fragi*. The esterase activity of cellular debris, however, increased by 6% after using 2.5 mM EDTA. When the cellular debris were obtained by disruption with ultrasonic (Table 7), the gain of esterase activity was 4% in comparison to untreated whole cells, and 11% after treating with 2.5 mM EDTA and 2% Triton rather than treating with 2% Triton only. Debette and Prensier, (1989) reported that EDTA-Triton X-100 was needed to solubilize of the remaining esterase activity (20% of the whole activity of their strain). Esterase activity studied in Triton X-100 extracts was probably produced by a set of membrane proteins which remained tightly bound with lipid molecules. The binding with Triton X-100 tends to bring the membrane proteins into solution as water-soluble protein-lipid-detergent complexes (Helenius and Simons, 1975).

Table 6. Ethyl valerate production by *P. fragi* esterase by disrupting the cells using French press and detergent.

Triton (%, v/v)	EDTA (mM) 0.0	1.0	2.5	5.0
0	25[a] (2.30)[b]			
1	14.03[a] (1.60)[b]	18.60[a] (23.39)[b]	15.45[a] (1.23)[b]	14.94[a] (3.58)[b]
2	14.49[a] (0.45)[b]	14.68[a] (1.63)[b]	16.51[a] (0.61)[b]	13.78[a] (8.09)[b]
3	11.50[a] (2.13)[b]	12.73[a] (1.77)[b]	12.28[a] (0.12)[b]	11.58[a] (5.75)[b]

[a]Average values of triplicate injections in (ppm/75 mg debris/24 h).
[b]Percent relative standard deviation values calculated as: (standard deviation/mean) x 100.

Table 7. Ethyl valerate production by *P. fragi* esterase (whole cells) by using disruption methods and detergent.

Disruption Methods	Triton (%, v/v)	EDTA (mM) 0.0	1.0	2.5	5.0
Glass beads	0	25 (0)[c]			
	2	18.88[a] (3.68)[b]	21.99[a] (8.25)[b]	19.93[a] (8.42)[b]	19.12[a] (0.39)[b]
Ultrasonic	0	25 (0)[c]			
	2	23.41[a] (2.72)[b]	18.20[a] (10.30)[b]	26.05[a] (8.56)	23.54[a] (6.29)[b]
French press and Ultrasonic	0	25 (0)[c]			
	2	20.05[a] (0.12)[b]	20.62[a] (9.21)[b]	24.10[a] (6.35)[b]	21.68[a] (9.55)[b]
Glass beads and Ultrasonic	0	25 (0)[c]			
	2	16.84[a] (0.72)[b]	17.08 (0)[c]	16.5 (0)[c]	14.91[a] (9.79)[b]

[a]Average values of triplicate injections in (ppm/75 mg debris/24 h).

[b]Percent relative standard deviation values calculated as: (standard deviation/average) x 100

[c]One injection

Observations were similar when the cellular debris were obtained by combined treatment of French press and ultrasound (Table 7). The esterase activity was lost by 4% after treating the cellular debris with 2% Triton X-100 and 2.5 mM EDTA as compared to esterase activity in untreated whole cells of *P. fragi*. But the esterase activity of cellular debris (0 mM EDTA) increased by 20% after treating with 2.5 mM EDTA. Results were close to that with the combined glass beads and ultrasound treatment (Table 7). The esterase activity was lost by 20% after treating with detergent, but it was regained by 19% after treating with 2.5 mM EDTA. However, the above mentioned treatments may partially denature the enzyme.

Miozzari *et al.* (1978) reported that, after treatment of *Saccharomyces cerevisiae* with Triton X-100, only molecules with a molecular mass below 70 kDa were released from the cytoplasm into the supernatant, whereas when *S. cerevisiae* cells were incubated with ether, 70% to 80% of the total protein was released into the supernatant (Breddam and Beenfeldt, 1991). On the other hand, *Escherichia coli* cells release only 10% to 20% of the total protein into the supernatant after incubation with either Triton X-100 or toluene (Jackson and DeMoss, 1965; Naglak and Wang, 1990). Electron microscopy revealed that the morphology of the cell wall of *E. coli* was not affected after incubation with Triton X-100 (Schnaitman, 1971). However, a complete efflux of K^+ into the supernatant was observed after the incubation of *E. coli* cells with phenol (Heipieper *et al.*, 1991). This indicates that *E. coli* cells become completely permeable after treatment with detergents or solvents, but no lysis takes place. Lysis of cells can however be advantageous when cells need to be disrupted. Chemical agents can therefore be very effective in releasing intracellular enzymes (Harrison, 1991).

Debette and Prensier, (1989) reported that the addition of Triton X-100 resulted in enzymatic activity apart from the cellular debris. Triton X-100 can be used to extract the enzyme from the debris, it was more efficient at a high concentration, whereas the bile salt had no effect. Finding from this study using 2% Triton X-100 is in agreement with the above mentioned study. This nonionic detergent (Triton) does not inhibit malease or other

enzymes (Helenius and Simons, 1975). In contrast to many solvents such as the extraction with organic solvents can denature the enzyme (Breddam and Beenfeldt, 1991; Cadwallader *et al.*, 1992).

However, it was important to verify the effect of the detergent, such as Triton X-100 and / or EDTA, and their interference with the enzymatic activity. Therefore, another experiment was conducted by eliminating Triton X-100 and the EDTA present in the aqueous extracts. Filtration and washing of the extracts using membranes having a cut-off pore size of 10,000 did not allow proteins to pass, but allowed Triton and EDTA to pass. These aqueous extracts, having identical concentrations of protein before and after filtration, showed no esterase activity.

After these trying experiments, it was so difficult to find a method that permits to dissociate the endo-cellular esterase of *P.fragi*. The enzyme seems to be the membrane debris associated to the bacterial cells.

4.3. Stability of Esterase Activity

Figure 7 shows the plot of the esterase activity of whole cells of *P. fragi* measured in terms of ethyl valerate production. It decreased with the time of storage at 4°C almost linearly reaching a minimum after 8 days. Therefore, it was decided to carry out all assays within 24 h.

4.4. Determination of Esterase Specificity, *in vivo* and *in vitro*

4.4.1. Substrate

Short chain fatty acids substrate of butyric, pentanoic, hexanoic, heptanoic and octanoic acids with ethanol and methanol were used through this study in bioconversion reactions which were catalyzed by *P. fragi* esterase to produce fatty acid alcohol esters. The esterase production by *P. fragi* was estimated through bioconversion reaction by determining the quantity of fatty acid ethyl esters in extracts. The amount of esters was

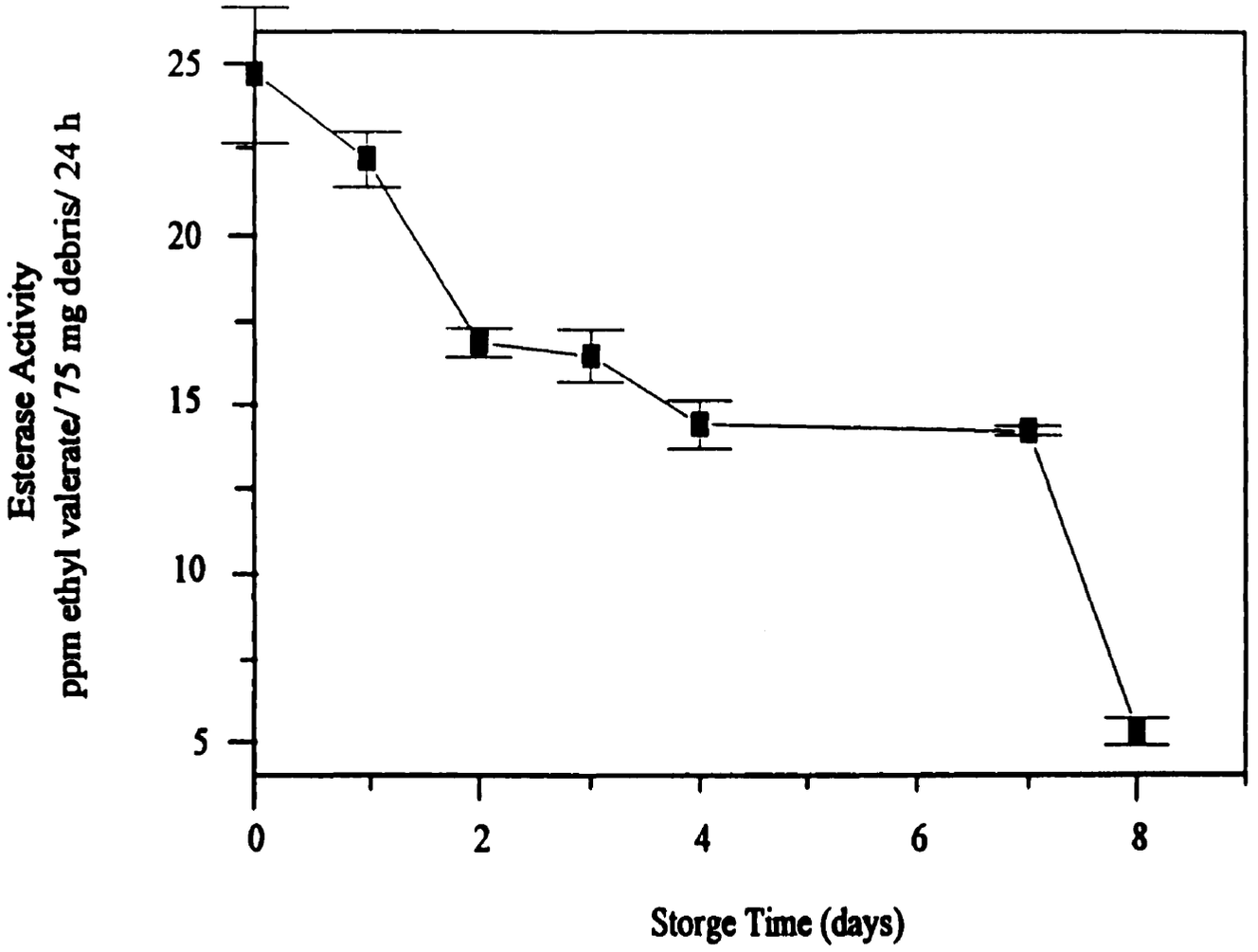


Figure 7. Effect of time of storage at 4°C on ethyl valerate production by whole cells of *P. fragi*.

determined by gas-liquid chromatography analyses.

The determination of bioconvension reactions can be moniterd by the level of subsrate consumption. The number of carbon atoms present in the short chain fatty acids might have influenced the conversion yield (Langrand *et al.*,1990). However, the enzyme specificity is expected to affect the conversion yield, such as the *Mucor miehei* lipase is very active on long chain fatty acids while the *Aspergillus* lipase is more active on short chain fatty acids (Rangheard *et al.*, 1989). An enzyme immobilization can be used to catalyze the synthesis of ethylpropionate and various other esters in nearly anhydrous hexane (Carta *et al.*, 1991). Moreover the carbon number of alcohol substrate should be taken into consideration in the process of bioconversion. For example, with the *Aspergillus* lipase the yields decreased by increasing the number of carbon atoms in the alcohol, but with *Mucor miehei* the yields were higher (Langrand *et al.* 1990).

4.4.2. Substrate specificity

The substrate specificity was studied to understand the behavior or the activity of the esterase enzyme with different substrates. The *P. fragi* esterase was observed to be responsible for esterification reactions between free fatty acids (Butyric, Pentanoic, Hexanoic, Heptanoic and Octanoic acids) and alcohols (Ethanol and Methanol). These substrates are important from the industry points view. Nowedays, there exist a commercial demand for these esters. For example, ethyl butyrate is a constituent of natural aroma of fruits like those from strawberry, pineapple and passiflora caerulea, and has an special relevance in food processing as a flavor enhances (Manjón *et al.*, 1991).

Substrates were used in different concentration from 0 to 2,000 ppm, and the ratios of fatty acids to alcohols (Fa/AL) used were 1:2, 1:1 and 2:1.

Since the enzyme could not be dissociated from the membrane, whole *P. fragi* cells were used in this study. They contained the entire enzyme intact. The cellular debris were obtained after disruption by the ultrasonication disruption method.

Plots of the effect of esterase activity of whole cells on the substrates of fatty acid concentrations and ethanol used a ratio of 1:2, 1:1 and 2:1, respectively, and with methanol with same ratios are in Figures 10 and 11, respectively

Plots of the effect of esterase activity of cellular debris on the substrates of fatty acid concentrations and ethanol used a ratio of 1:2, 1:1 and 2:1, respectively, and with methanol with same ratios are in Figures 12 and 13, respectively

It may be noted that the above graphs were based on the standardized values with reference to an arbitrary value of 25 ppm for fatty acid ethyl ester and 58 ppm for fatty acid methyl ester. It was determined by reference assays, (made with the reference method: valeric acid 1,000 ppm with ethanol and/or methanol 2,000 ppm), in order to compare between different experiments throughout this study.

4.4.3. Effect of substrate ratio

The substrate ratio was defined as the ratio of fatty acid to alcohol. The analyses of the whole cell (Figures 8, 9) with three different ratios showed that a ratio of 1:1 of fatty acid to ethanol was the best in term of esterase activity production of fatty acid ethyl ester. Similar results were observed (Fig. 9) for the same ratio of fatty acid to methanol. On the other hand, the specificity in cellular debris with fatty acid to ethanol showed that highest activity were with a ratio of 1:2 for butyric and caproic acid (Fig. 10 A), 2:1 for valeric acid (Fig. 10 C) and 1:1 for heptanoic and octanoic acids (Fig. 10 B). For the cellular debris specificity also the ratio of 1:1 of fatty acid was most appropriate, except a ratio of 2:1 (Fig. 11 C) for octanoic acid to methanol.

The level of saturation of ester production with cells occurred when the fatty acid to ethanol was 1:2. The production of esters seems to reach a maximum level of synthesis with about 28 ppm of ethyl valerate (Fig. 8 A) and similar finding was obtained with the cellular debris (Fig. 10 A). However, the production of fatty acid methyl ester with both whole cells and cellular debris were similar as follows: When the ratio is 1:2 (Fig. 9 A), the enzyme has broken heptanoic acid to produce methyl heptanoate (17 ppm) and methyl

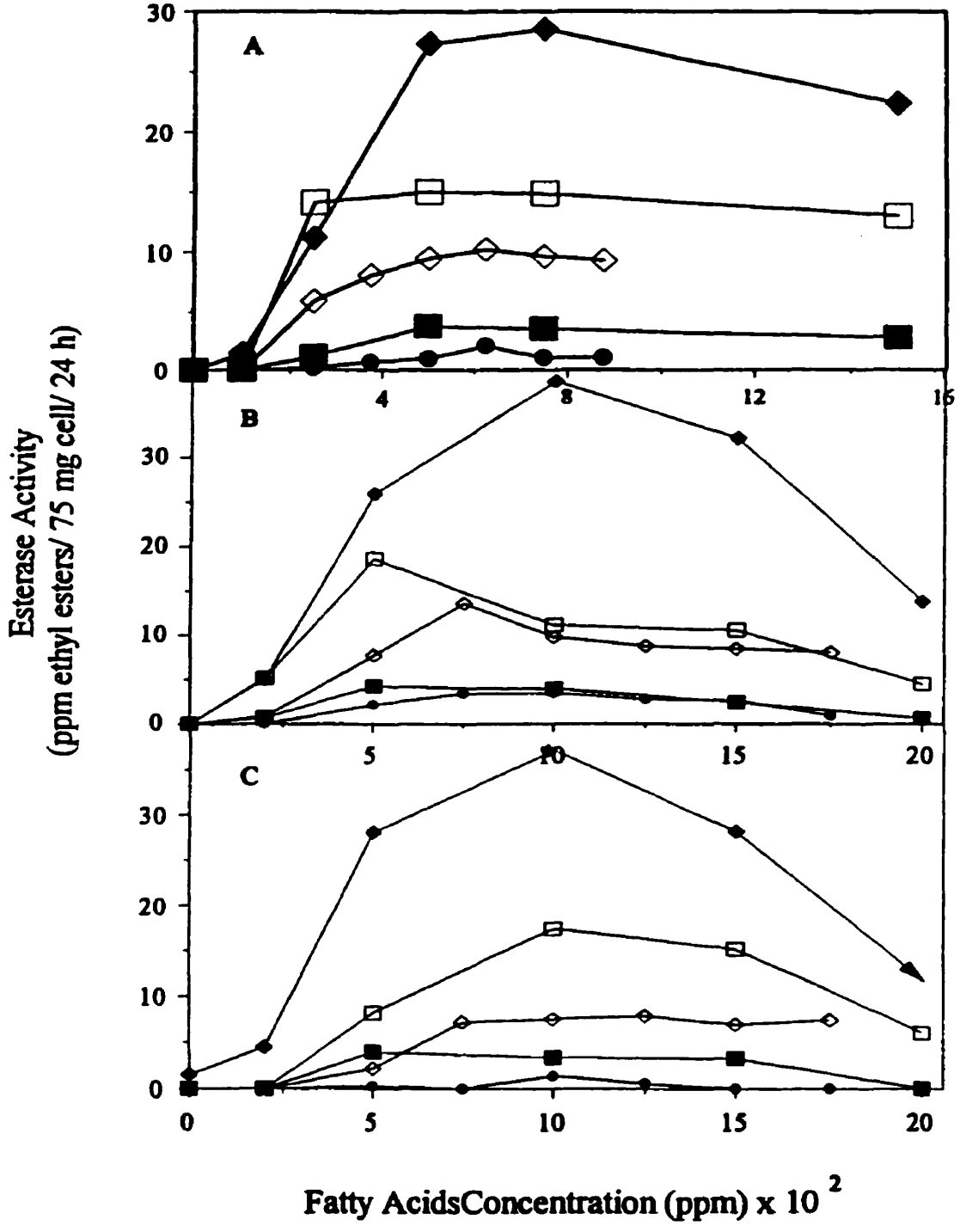


Figure 8. The effect of fatty acid concentration on the esterase activity of *P. fragi* cells using fatty acids, butyric (—■—), valeric (—◆—), caproic (—□—), heptanoic (—◇—) and octanoic acids (—●—) to ethanol on a proportion of A 1:2, B 1:1 and C 2:1.

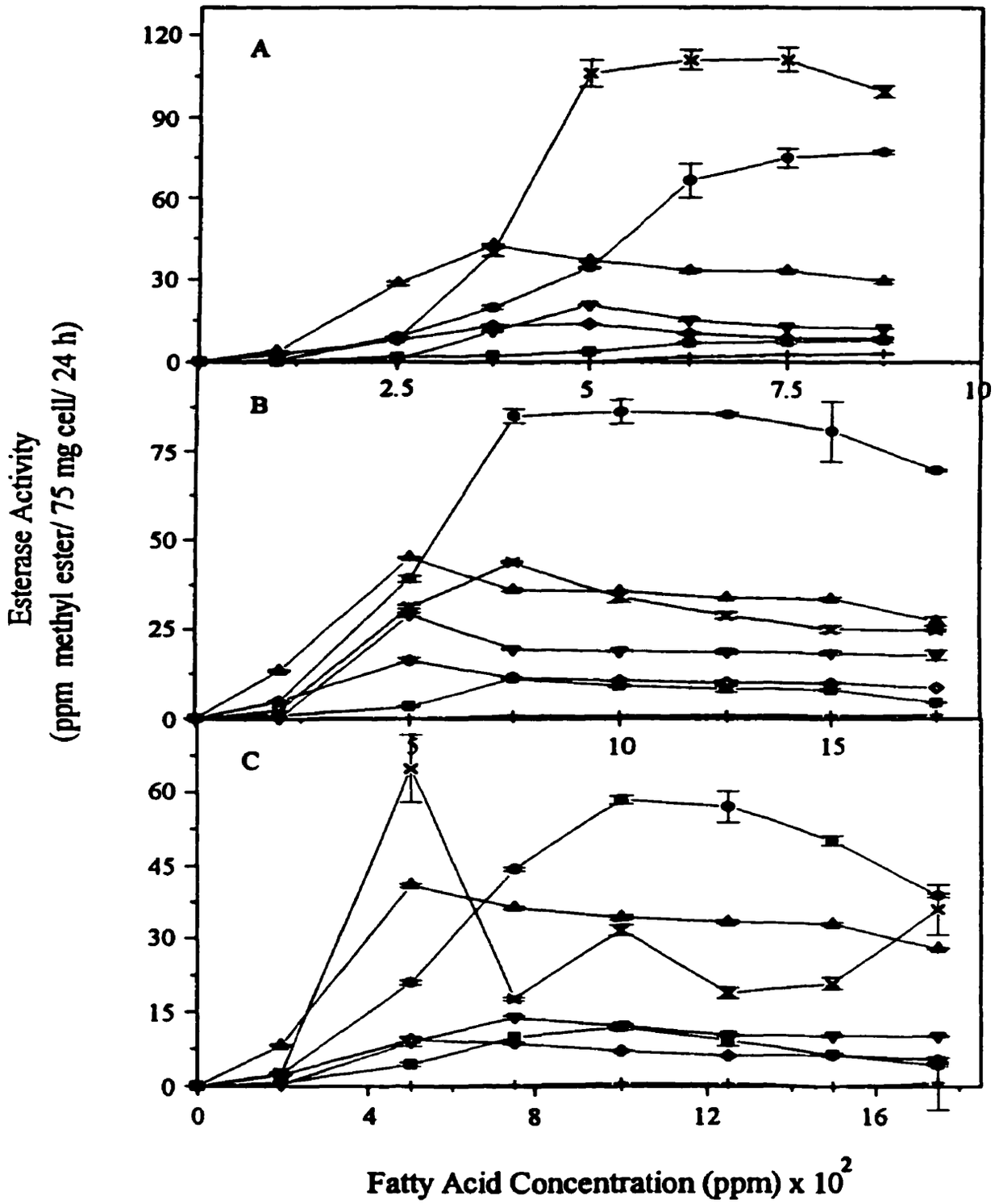


Figure 9. The effect of fatty acid concentration on the esterase activity of *P. fragi* cells using fatty acids, butyric (—■—), valeric (—●—), caproic (—▲—), heptanoic[methyl heptanoate (—▼—), methyl valerate (—◆—)] and octanoic acids[methyl octanoate (—+—), methyl caproate (—✕—)] to methanol proportion of A 1:2, B 1:1 and C 2:1.

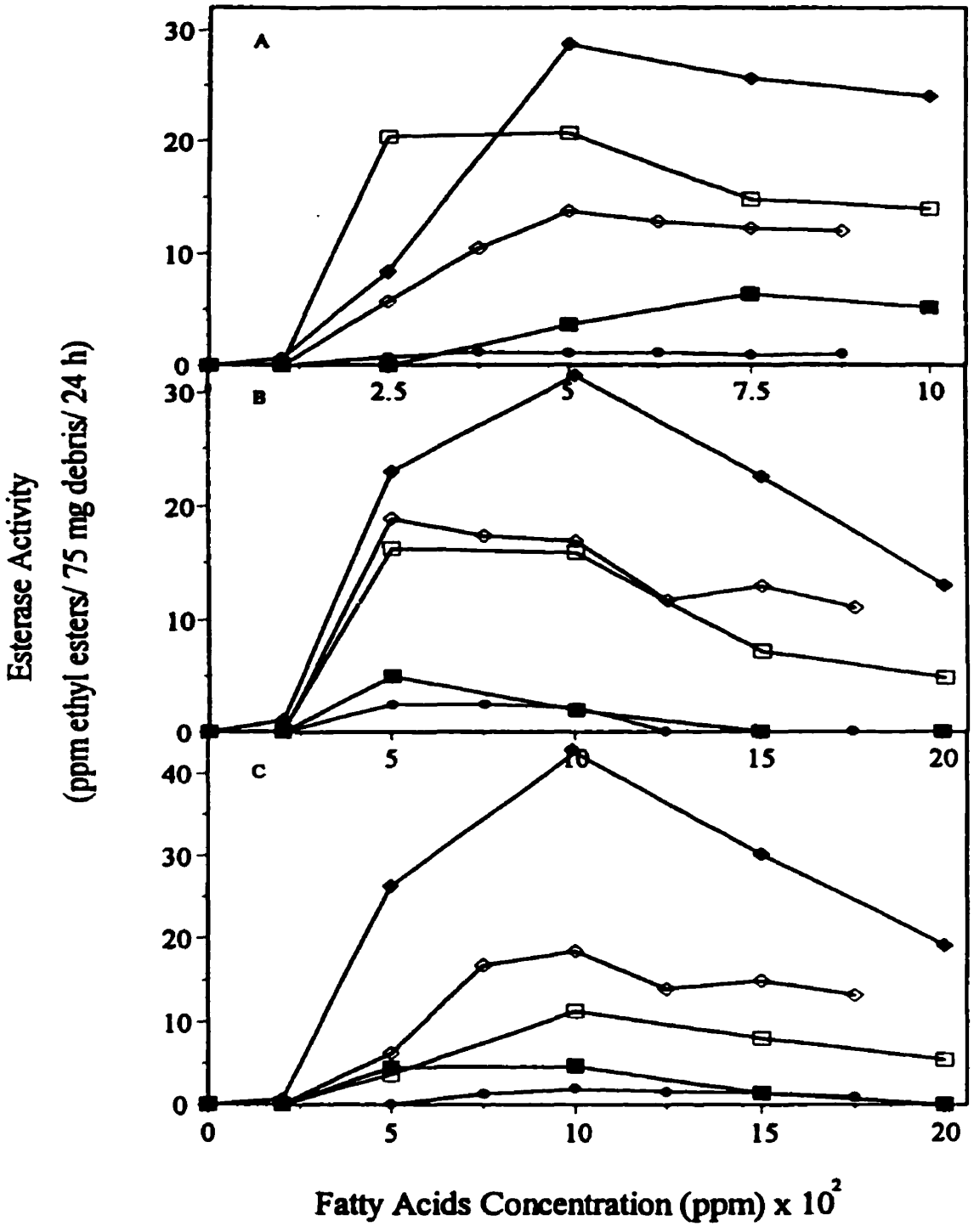


Figure 10. The effect of fatty acid concentration on the esterase activity of *P. fragi* debris using fatty acids, butyric (—■—), valeric (—◆—), caproic (—□—), heptanoic (—◇—) and octanoic acids (—●—) to ethanol on a proportion of A 1:2, B 1:1 and C 2:1.

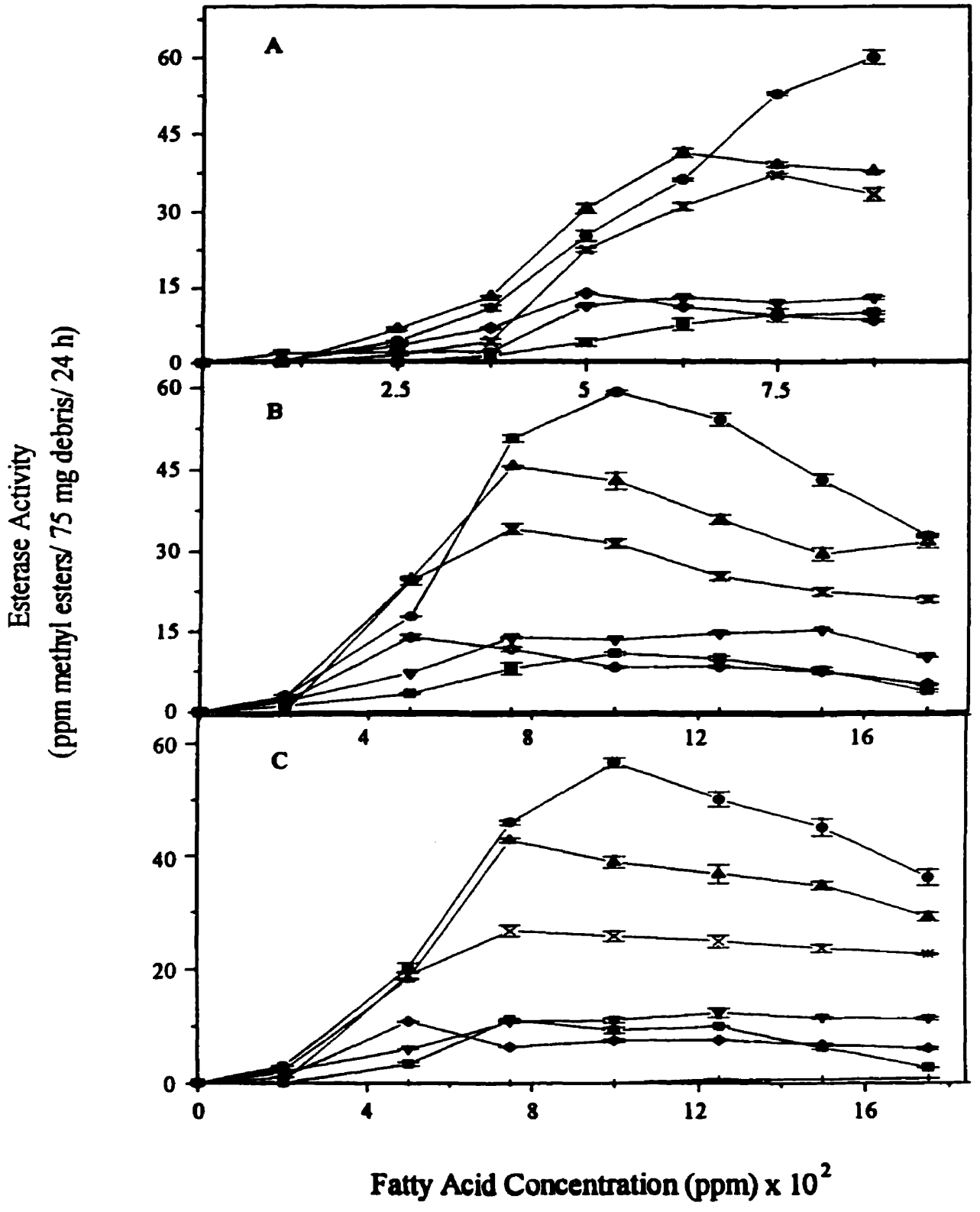


Figure 11. The effect of fatty acid concentration on the esterase activity of *P. fragi* debris using fatty acids, butyric (—■—), valeric (—●—), caproic (—▲—), heptanoic[methyl heptanoate (—▼—), methyl valerate (—◆—)] and octanoic acids[methyl octanoate (—|—), methyl caproate (—✕—)] to methanol proportion of A 1:2, B 1:1 and C 2:1.

valerate (13 ppm). However, the bioconversion assays of octanoic acid yielded a very low amount of methyl octanoate while it produced relatively high amount of methyl caproate (100 ppm). Moreover, the enzyme catalyes an assay of valeric acid with methanol to produce methyl valerate (75 ppm) with cells (Fig. 9) and 60 ppm with cellular debris (Fig. 11 A).

Results with ratios of fatty acid to ethanol of 1:1 and 2:1 indicated that the production of esters goes to a maximum of 30 to 40 ppm for ethyl valerate with the cells or the cellular debris and then decreases (Fig. 8 B, 8 C, 10 B, 10 C) when there is as much or more fatty acid than ethanol. Similar observations have been noted with cells and cellular debris for the same ratio of fatty acid to methanol. Methyl valerate production reached to 60 ppm. These results are in agreement with those of Lamer *et al.* (1996), who reported that the substrate ratio had an influence on the esterifying activity. Increase in the substrate concentration at constant ratio had no effect on the ester production. Welsh *et al.* (1990) used a molar acid and ethanol in a ratio of 1:1.55 to produce ethyl butyrate, using lipase from *Pseudomonas fluorescens* in non-aqueous system.

4.4.4. Effect of acid chain length

The number of carbon atoms, in the short chain fatty acids used in this study, generally influenced the conversion yield with the exception of butyric acid. In general the effect is similar on ethanol and methanol tested. However, the valeric acid yielded the best results among all 5 fatty acids tested in this study (Fig. 8, 10) and (Fig. 9, 11) with a relative standard deviation $\leq$ 10%. Octanoic acid gave the lowest activity or almost not transformed into ethyl octanoate. In addition, the best rates of transformation of fatty acids to fatty acids methyl esters were caproic and valeric acids. The rate is 11% at 500 and 1,000 ppm (Fig. 9, 11).

The bioconversion assays from valeric acid to ethyl valerate were still low. The best rate for caproic acid was 8.1% at 250 ppm for cells and cellular debris in the ratio of 1:2 (Fig. 8 A, 10 A). When valeric acid was used, the best results were between 4% to

6%. Results from the method used by us are inferior to those obtained by Morin *et al.* (1993) using gas stripping method, and reported that 76% of valeric acid was transformed to ethyl valerate when the ester was eliminated. Langrand *et al.* (1990) reported that the number of carbon atoms in the short chain fatty acids influenced the conversion yield with *Aspergillus* and *C. rugosa.*

4.4.5. Substrate concentration effects

Substrate parameters such as concentration, size, and/or presence of side chain groups have been shown to affect large molecular weight ester synthesis. With cell specificity study, the maximum production of esters was obtained from low fatty acid concentrations at 250-500 ppm and concentration of ethanol at 500-1,000 ppm. When the fatty acid to ethanol ratio was 1:2 (Fig. 8 A) and in concentrations of fatty acid at 750-875 ppm to concentration of methanol at 1,500-1,750 ppm for the same ratio of fatty acid to methanol (Fig. 9 A). In addition, In comparison to ratio of 1:1 was at 500-1,000 ppm of fatty acid to concentration of ethanol 1,000-2,000 ppm (Fig. 8 B) and at concentration of fatty acid at 1,000 ppm to 1,000 ppm of methanol (Fig. 9 B). Moreover, the concentration of fatty acid to alcohol ratio was 2:1 at 1,000 ppm of fatty acid and 500 ppm of ethanol (Fig. 8 C) and 1,000 ppm of fatty acid and 500 ppm of methanol (Fig. 9 C). All of them reached the plateau and decrease of the esterifying activity. Similar results were obtained with cellular debris. The bioconversion assay was higher while the maximums obtained were similarly identical (Fig. 8, 9). Lamer *et al.* (1996) reported that there was a decrease in the esterifying activity at a substrate concentration higher than 0.05 M valeric acid and 0.1 M ethanol. Welsh *et al.* (1990) reported that the ethyl butyrate and isopentyl butyrate syntheses maintained high yields (> 80%) up to the substrate concentration of 0.3 mol/l, and then the yield decreased even with higher substrate concentrations. Carta *et al.* (1992) showed the inhibition of ester synthesis by immobilized lipase of *Candida cylindracea* at ethanol concentrations higher than 0.2 M.

It can state that there was no different between the cells and cellular debris with respect to the profiles of the curves or the maximums obtained.

5. Conclusion

This work had for its long objective to find a method of extraction for intracellular esterase of *Pseudomonas fragi*, and to study the specificity of the enzyme with respect to substrates. It was found that the optimum culture growth of *P. fragi* was at 11°C, 150 rpm and the exponential phase begin at 24 h to 72 h. The stationary phase of growth started from 78 to 96 h. The highest esterase activity of the whole cells was at 78 h in bioconversion assay.

Different methods or a combination of two methods were used to disrupt the cells. These methods, include glass beads, French press, ultrasonic, glass beads with ultrasonic and French press with ultrasonic. The esterase activity was increased by 18% in a comparison of condition to whole cells control by disruption the cells with sonication. Therefore, ultrasonication disruption method was used for cells disruption through out this presented study.

The detergents of Triton X-100, CHAPS and EDTA were used to solubilize the enzyme. Triton X-100 (2%) and EDTA (2.5 mM) were the best concentration used in term of esterase activity.

The enzyme is not excreted into the medium of the culture or the extracts but it is strongly bound up with cellular membrane. In effect, the methods of extraction by neither mechanical means nor by solubilization of the enzyme with the use of detergents succeeded to liberate the enzyme into the aqueous phase.

The fresh cells of *P. fragi* should be used in the first day of production because the effect of time of storage on the ethyl valerate production by the whole cells. The freeze-drying inactivated the enzyme.

Through out this study, the specificity of *P. fragi* esterase enzyme was carried out

using whole cells and cellular debris. Valeric acid was the fatty acid the most easily consumed by esterase with respect to the other fatty acids tested. With methanol, the transformation obtained was higher than using ethanol. Generally, the most favorable fatty acid / alcohol ratio for the bioconversion (producing fatty acid alcohol ester) was 1:1. However, the rate of transformation remained [weak ($\leq$ 11%)] in all cases. In addition, no difference was revealed with respect to the specificity of the substrate between the cells and cellular debris.

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