Hungarian Academy of Sciences

FERMENTATION OF
THERMOSTABLE ALKALINE PROTEINASE

By

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THESIS

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INTRODUCTION AND AIM OF THE WORK

Fermentation is a long-established process which has expanded over the years to become the basis of biotechnology and biochemical engineering. Enzymes are of supreme importance in biology, since life depends on a complex network of chemical reactions brought about by specific enzymes.

Thermophilic micro-organisms are known for a long time, especially the enhanced heat stability of their spores. They are potentially good sources of extracellular enzymes which are stable against thermal and chemical inactivation.

Microbial proteolytic enzymes play an important role as reagent in laboratory and clinical analysis. They are also find a wide variety of applications in brewing, detergents, food processes, as well as leather industries.

The combination of microbiological and biochemical science with modern technology has made possible the microbial enzymes more attractive candidate in biotechnology.

This research work is therefore focused on the possibility to produce a thermostable alkaline proteinase by local isolate. In order to cover this subject, the scope was given to some aspects as follows:

I. Screening for thermophiles and proteolytic enzymes.
II. Optimization for thermostable proteinase production.
III. Kinetics of bacterial growth in batch fermentation.
IV. Microbial behavior in continuous cultivation.
V. Kinetics of enzymatic caseinolysis.

1. REVIEW OF LITERATURE

1.1. OCCURRENCE OF THERMOPHILIC BACTERIA

Thermophilic microorganisms are wide-spread in nature. They are extensively recorded and studied in different sources as follows:

1.1.1. Food

Thermophilic bacteria are an important group which cause spoilage in the dairy industry particularly in tropical countries where high ambient temperatures provide an environment conductive to their growth. As the protease from thermophilic microbes cause degradation of casein resulting in proteolytic spoilage of heat-treated milk and milk products (Chopra and Mathur, 1984).

Atwal et al. (1974) have reported a higher thermophilic count in pasteurized milk than in raw one. The presence of thermophiles in dried milks has also been reported by Mossel et al. (1974). Further, a heat stable protease was obtained by bacterial group isolated from cooled raw milk as observed by Poffe and Mertens (1988)

From the economic point of view, the proteolytic enzymes has significant role in the industrial field with reference to canned food. The studies of Montville (1983) on the physiology of *Clostridium botulinum* proved that the activity of extracellular proteinase has an important role in the detection of botulinal toxin.

1.1.2. Sel-heating Materials

A large variety of thermophilic microorganisms, actinomycetes, fungi, and bacteria have been reported in composting and other self-heating organic materials (Rothbaum, 1961 and Strom, 1985). Numerous authors have reported the presence of microbiota of solid-waste composting, with major emphasis on *Bacillus spp.* They also stated that, those bacteria are predominate during the earlier thermophilic stages because of its important role as a major active organisms in the composting process (Okafor, 1966; Stutzenberger et al., 1970; Suzuki et al., 1981 and Strom, 1985).

1.1.3. Soil

The microorganisms community; bacteria, algae, fungi, yeast, and protozoa play an important role in recycling the organic matter in soil. They are also sharing in recovering of the soil fertility throughout biodegradation processes for the crop residues because they are having many of enzymatic groups, which can do that, alkaline proteinse (Souter at al., 1992), amylase (Babu and Satyanarayana, 1992), lipase (Sigurgisladottir et al., 1992), xylanase (Dahlberg et al., 1992), cellulase (Jones 1992), and hemicellulase (De et al., 1992).

Many research work have been adopted on the microorganisms from soil. Ramesh and Lonsane (1989) isolated thermophilic bacteria from soil samples collected from municipal waste dump area. Sunna and Hashwa (1990) and Debette (1991) isolated thermophilic bacteria from soil at 60°C on starch nutrient agar.

1.1.4. Hot Spring

In the term of the ecological point of view, the thermophilic bacteria have been studied in the hydrothermal springs in the course of several years ago. Many works have been examined and showed the role of thermophiles in the process of reworking the gas component volcanic area as well as to describe the role of microbiological process in the genesis of native sulfur in the hydrothermal springs (Kuzyakina, 1992).

Because of the importance of these group for the biotechnological applications, numerous works have been carried out in the hot springs, Furuya et al. (1977) and Kristjansson et al. (1986). Takiguchi and Shimahara (1989) and Suzuki et al. (1981) isolated *Bacillus licheniformis* from hot spring in Japan. AEvarsson et al. (1991) isolated a thermophilic *Thermus* strain in a hot spring in Iceland.

Thermophilic bacteria were found in man-made and natural thermal gradients as reported by Brock and Darland (1970), Brock (1967) and Ramaley and Bitzinger (1975). Heinritz et al. (1990) isolated a thermophilic *B. stearothermophilus* from a man-made hot water reservoir.

1.2. MEDIUM FORMULATION FOR ENZYME PRODUCTION

In a microbial culture the individual cells increase in mass by growth and in number by reproduction when the appropriate nutritional and environmental requirements have been met.

1.2.1. Nutritional Parameters

Since the cellular composition and biochemical activities of microbes are function of the growth environments, the composition and concentration of culture media greatly affect the growth and production of extracellular enzymes (Chandra et al., 1980, Okada et al., 1984, Cho et al., 1987, and Fujiwara and Yamamoto, 1987).

Wikstrom (1983) studied the effect of culture medium components on the enzyme production and reported that both microbial protease and their activities are regulated by cultivation factors such as medium composition. The growth medium was modified to increase the level of free Ca^{2+} to be available in the culture during growth of the bacterium when Janssen et al. (1991) studied the effect of medium composition on extracellular proteinase production.

In order to obtain optimum growth of the microorganisms, the most adequate medium, oxygen tension, temperature, and pH must be provided. At the same time the enzyme required may be not constitutive, therefore the necessary inducing agent must be added to the culture media.

The microorganisms have a wide range of carbon and energy source utilization pattern and growth characteristics. In general, low cost, readily bioassimilable carbon and nitrogen sources should be selected. On the other hand, these substrates has to be available at relatively wide range of geographical regions, however, seasonal availability may be a problem in temperate zone regions for those substrates obtained from agricultural crops.

Raw material have been used for growing microorganisms for different purposes, photosynthetic bacteria of genus *Rhodopseudomonas* have been grown on agricultural and industrial wastes (Shipman et al., 1975and Kobayashi & Kurata, 1978). Neutral proteases were also produced using rice bran by *B. megaterium* and *Serratia marcescens* (El-Fadaly, 1985).

1.2.2. Physical Parameters

1.2.2.1. Temperature

Temperature is one of the most important environmental factor controlling and evaluating the activities of microorganisms. The high temperature reduces water density, surface tension, and it also reduced the dielectric constant, viscosity and gas (O_2) solubility of the media. It is also increased water vapour, ionization, solubility of most organic and inorganic compound and diffusion of reactants which make the process more economical. The increased solubility enables a greater amount of reactants to be processed in a given volume shorten the reaction time and leads to higher productivity (Tsuchida et al., 1986, Manachini et al., 1988, and Takami et al., 1989).

On the other hand, the process at high temperature reduced risk of mesophilic microbial contamination (Brock, 1986).

1.2.2.2. Agitation and aeration

In shake flask, because of their operational simplicity, convenience, and low cost, these reactor are widely used. At the same time, oxygen is absorbed primarily in the thin film of liquid thrown up on the flask walls by the centrifugal force generated by the rotation of the shaking machine. So the overall absorption rate is dependent on the frequency of replacement of this layer (Gaden, 1962).

On the other hand, when oxygen concentration is low and the agitation rates is little leads to low mass transfer coefficient. Agitation and aeration therefore have to provide adequate mass and heat transfer and to keep the respective concentration and energy profiles for the growing organisms. These processes is to suspend the cells uniformly throughout the working volume in order to prevent sedimentation and/or flotation, to regulate the temperature and components concentration in the bulk of liquid as well as to maintain high mass transfer rates by the uniform distribution of all reactants.

Many of the culture media contain solid particles (soybean and starch such like the medium used in this study) which must be suspended and dispersed by the mixing action. For example starch containing media have initial viscosity which need high impeller than the others. On the other hand, the assumption of perfect

mixing is generally taken also to imply that the reactor behaves as a single vessel, in distribution of cell ages, and in balance of cell constituents under the rapid growth rate (short time fermentation).

1.3. DEFINITION OF THERMOPHILY

Thermophilic microorganisms have all of the metabolic properties; pathways, metabolic rates, and regulatory mechanisms of mesophilic ones as reported by Ljundahl (1979).

Although there is little supportive evidence for explanation of microorganisms to survive at a high temperature that kill other life form , many theories have been advanced to demonstrate this phenomenon as follows:

1.3.1. Cell membrane

There has been considerable speculation as to the components of the microbial cell that are critical in determining the maximum temperature at which the microorganisms can survive (Farrell and Rose, 1967; Campbell and Pace, 1968). Since thermal death is a first order process, the loss of membrane integrity at a high temperature could be a limiting factor in establishing the upper limit for the growth of microorganisms (Brock, 1967).

1.3.2. Cellular fatty acids

There is a direct relationship between cellular fatty acids composition and growth temperature. Regarding the gram positive bacteria, experimental data of the following workers showed that lowering the incubation temperature for growth generally resulting in an increased proportion of unsaturated fatty acids (Heinen et al., 1970; Yao et al., 1970; and Ray et al., 1971).

In successful trials of Furuya et al. (1977), they found that the lipid constituents are based on ether-linked compounds not to the ester-linked compounds of thermophilic acidophilic bacterium isolated from hot springs. The membrane lipids of extreme thermophiles contain more saturated and straight chain fatty acids than mesophiles which allows the microbe to grow at high temperature by providing the correct degree of fluidity required for membrane function (Zeikus, 1979 and Nordstrom & Laakso 1992).

1.3.3. Cellular protein

Numerous investigations have established that proteins, highly purified enzymes, and protein synthesizing system from thermophilic bacteria are more thermostable than comparable preparation from mesophilic one. These finding suggests that the ability of thermophilic microbes to grow at high temperature is a results of the inherent thermostability of the individual cellular components and is not dependent on any stabilizing factors (Koffler, 1957; Manning et al., 1961; Amelunxen, 1966; and Friedman, 1968).

In addition, extreme thermophiles produce unique polyamines, thermine and thermospermine, which function both to stabilize the protein synthesizing apparatus and to protect macromolecules from high temperature. Increasing the intramolecular interaction in thermophilic enzymes as a results of greater hydrogen bonding and greater hydrophobic and ionic bond interactions, make these proteins more resistant to both thermal and chemical denaturation.

1.3.4. Ribosomes

Studies of the base composition of the ribosomal RNA molecules showed a higher guanine plus cytosine content in *B. stearothermophilus* RNA. On the other hand, the analysis of amino acid composition of total ribosomal protein of 30S subunit ribosomes of *E. coli* and *B. stearothermophilus* showed no appreciable difference between each other. Regarding these results, Saunder and Campbell (1966) and Ansley et al. (1969) pointed out that the differences in thermal stability of isolated intact ribosomes could due be to difference in primary structure of the ribosomal protein or in the interaction between the ribosomal RNA and protein.

The same conclusion was came by Kawai et al. (1980) when they examined 70S sub unit ribosomes in suspending medium with different concentration of Mg^{2+} prepared from *E. coli* and *B. stearothermophilus* as well. In the results of Friedman and Yamamoto (1990) they found good correlation between stability of ribosomes with the maximum growth temperature of bacteria.

1.3.5. Amino acids

The enzymes from thermophilic differ from their mesophilic counterparts by as little as a single amino acid substitution which confers the necessary thermal stability on the protein (Ljungdahl, 1979). The results of Imanaka et al. (1986) proved that the glycine in position 144 of the neutral proteinase of *B. stearothermophilus* was changed to alanine by site-directed mutagenesis giving increased thermostability of the enzyme. While Suzuki et al.(1987) showed that *Bacillus* enzymes reduce the potential of salt bridge formation as their thermostability rises. Amelunxen and Murdoc (1978) have been considered the potential of hydrogen bonding antagonized by the hydrophobic interaction, the most dominant for stabilization of thermophile proteins. They also noted that the large increase of proline, leucine and alanine could lend more hydrophobic bonds to protein with increasing heat stability.

Samal et al. (1991) noted that the higher thermal stability has been attributed to the presence of intra molecular disulfide bonds, hydrophobic interactions, and electrostatic interactions, since many disulfide bonds provide thermodynamic protection to proteins as decided also by Wetzel (1987). Srivastava (1984) found that the enzyme containing sulphur amino acids more thermostable as well as those containing proline and concluded that the content of cystein and proline has a direct relation to the thermostability of enzyme.

Nowadays, in NOVO Nordisk, Denmark, the protein engineering methods are play an important role together with the gene technology to obtain industrial enzymes with improved properties and cost saving as well. The change of amino acids in the enzyme molecule with reference to phenylealanine in the position 139, and serine in 186 were resulted to a new enzyme which has higher enzyme activity as well as higher H_2O_2 tolerancy (Falch, 1991).

1.4. CLASSIFICATION OF PROTEOLYTIC ENZYMES

Hagihara (1960) pointed out that the proteolytic enzymes produced by microorganisms could be fallen into three main groups; acid proteinase with maximum activity between pH 2.0 and 4.0. neutral proteinase which can do the catalysts at pH between 7.0 and 8.0. The third one is alkaline proteinase having their

activity at 9.0 and 11.0.

Proteolytic enzymes were also classified by Hartly (1960), and Priest (1977) into four groups on the basis of the mechanism of action rather than their origin namely; acid, serine, metallo, and thiol proteases.

Dunn (1990) has outlined four mechanistic classes of protease ; serine and cystein proteases which form covalent enzyme complex, aspartic and metallo-proteases class which do not form covalent enzyme complex.

1.5. SCOPE AND FIELD OF APPLICATIONS

1.5.1. THERMOPHILES

In the past decade, the chemical industry moved towards energetic coupling process aimed to decrease the energy expenditures. Approximately one half of the carbone substrate energy which mainly determines the total energy expenditure of microbial biomass production is converted into heat as reported by Heinritz et al. (1990).

On the other side, the energy economic coupling is an increase in reaction temperature which cause disadvantages if the mesophilic microorganisms (30-35°C) are to be used in the energy-economic way, regarding the reaction heat of microbial biomass production. The elimination of the reaction heat by cooling and/or its utilization by using a heat pump requires an additional energy expenditure, the reasons that nominate the thermotolerant and thermophilic microorganisms for biomass production as well as their end products has been recommended as stated by Heinritz et al. (1990).

Thermophiles provide a valuable resource for exploitation in novel biotechnological process and in developing the understanding of how biomolecules are stabilized when subjected to extreme conditions. They also have application in chemical feed stock and fuel production, bioconversion of wastes, enzyme technology, and single cell protein production.

1.5.1.1. Production of Microbial Biomass

Using thermophiles in producing biomass showed many advantages for instance, the decrease of RNA content in the biomass comparing to mesophilic microbes which can be easily used in animal feeding.

The improvement of downstream processing for the recovery of microbial biomass as a results of decreased viscosity has been achieved. The lower cost of cooling is also unnegligable benifit by using high temperature process.

1.5.1.2. Bioremediation

In the field of bioremediation they have considerable application in the removal of heavy metals from waste waters, the biodegradation of toxic pollutants, and the treatment of domestic, agricultural and industrial effluent, as well as in the microbial desulphurisation of coal to reduce sulphur emissions. Thermophiles considered to be as a possible way to improve the metal extraction rates in different leaching operation since they used to leach metals from sulphide minerals by genera: *Thiobacilus, Sulfobacillus,* and *Sulfolobus.*

They have also been exploited for many years in the leaching of metals from low grade ores and more recently in precious metal recovery (Groudeva and Groudev, 1987).

1.5.1.3. Energy and Fuels

In the search for alternative energy resources to replace fossil fuels, they offer potential for the large scale production of ethanol, organic solvents, methane and hydrogen. There is extensive use of gasohol for cars in Brazil, via a fermentation and distillation system because of a real cost of oil rise (Persley, 1990).

1.5.1.4. Management and Recycling of Wastes

Large volume solid wastes are widely distributed and hence difficult to collect to overcome the environmental pollution such as feedlot wastes, i.e. manure; crops yield low value wastes, cereal straw, bagasse, sugar-beet pulp, residual plant materials, and wastepaper as well as city refuse.

These materials could be fermented after pretreatment by either enzymatic, chemical, or heat, since the resulting microbial cells are filtered, washed and dried prior to being used as recycle feed (Grajek, 1988).

1.5.2. THERMOSTABLE ENZYMES

Thermophilic bacteria have provided thermostable enzymes which for application in industrial process, and have application in molecular biology. They have special interesting application in biotechnology regarding their stability and unique kinetic properties.

Enzymes from thermophiles are invariably more thermostable than their mesophilic counterparts. They are also usually resistant to chemical denaturation when examined the stability of enzymes from both thermophiles and mesophiles using SDS. This characteristic makes them potentially suitable catalysts and attractive candidate for the synthesis of organic chemical and for reactions in which organic solvents are used.

The market for industrial enzymes currently is worth about more than US$ 600 million of which proteinase account for nearly 60% of the total industrial enzymes sales as pointed out by Kalisz (1988) and Sundaram (1988). The main area of application of these enzymes summarized as follows:

1.5.2.1. Industrial Biotechnology

1.5.2.1.1. Silver Recovering

Thermostable alkaline proteinase was found extremely effective for recovering silver from used X-ray films, since it can rapidly decompose the gelatin coating on the polyester film. This allowing the silver released to be recovered by a mechanical or chemical process such as filtration, centrifugation or aggregation (Fujiwara and Yamamoto, 1987).

1.5.2.1.2. Tanning of Leather

In the tanning industry, proteinases are used in two different processes; dehairing and cleaning. Proteinases are also used for the bating of hides freeing them from flesh instead of pancreatic powder for economical reasons. This process considered to be an indispensable step in the preparation of pliable and good quality leather since the undesirable pigments are also removed from the skin as well as to make the skin soft and elastic (Aunstrup et al., 1979).

1.5.2.1.3. Laundry and Biodetergent Formulation

When the proteinase is present in the washing solution, it degrades the denatured protein sticking to the textile material to soluble degradation products mainly peptides that can be easily removed.

Enzyme containing detergents have been known since 1913, but their use was limited because the enzyme available were unstable in detergent formulation until around 1965, when a new stable protease produced by *B. licheniformis* was introduced.

A peak of enzyme application occurred in 1969 when 30 to 75 percent of all European detergents and 40 percent of detergents in the USA contained enzymes. The enzymes which are a mixture of bacterial neutral and alkaline proteinases which they are active over the pH range of 6.5 to 10.0 and temperature from 30 to 60°C. (Aunstrup et al., 1979 and Bailey and Ollis 1986).

Samal et al. (1990) examined the stability of the proteinase at 50°C in the presence of laundry detergents of different compositions. Samal et al. (1991) noted that the stability of proteinase at 50°C and pH between 8.0 and 10.0 could considered to be attractive candidate for industrial applications in addition to the use in laundry detergent formulations. The application of alkaline proteinase in washing preparations has so far been the pronounced success of applied enzymology.

1.5.2.2. Food Processing and Feed Manufacturing

1.5.2.2.1. Food Processing

1.5.2.2.1.1. Brewing

Regarding the proteins and their degradation products have a significant role in brewing, since nitrogen fractions in wort have several effect on metabolism and growth of yeast, taste and aroma, foam stability, as well as biological stability of beer. Markovic et al. (1988) used microbial proteinase for barley protein solubilizing. It is also used for purification of the cloudiness in beer which develops when beer is chilled.

Nowadays, the proteolytic enzymes used in this field on the large scale in many countries especialy in Hungary since large amount of beer are produced for the domestic uses as well as for export.

1.5.2.2.1.2. Meat Tenderization

Proteinase from *Bacillus* and *Streptomyces* used in meat tenderisation and fish solubles which is based on the partial hydrolysis of proteins of connective tissues which increased solubility and sequencly shortened the cooking time of protein rich food staff. It is also used in baking industry for reducing the time of dough making and mechanical plasticizing (Kas, 1984).

1.5.2.2.1.3. Synthetic Milk

The group of proteinases have a great deal in biotechnology since the protein hydrolyzates are now produced for various purposes, such as pharmaceutical preparation and human nutrition. At that case the selection of raw materials, the microbes, the enzyme and the technology should be carefully determined by the goal to be achieved.

The proteolytic group are also used for human purposes especialy in the developed countries (USA & Europe) for preparing synthetic milk which was found to be more healthy being contains low ratio of cholesterole.

1.5.2.2.1.4. Baking Industry

Proteinase are also used in baking industry especialy those produced by fungi, primarily from *A. oryzae* and *B. amyloliquefaciens,* particularly in USA. The enzymes used for reducing the time of dough making and mechanical plasticizing. Some properties of the dough are also required such like softness and plasticity in the production of crackers and various biscuits which can be attained by applying proteinases.

1.5.2.2.1.5. Cheeses Ripening

Proteinase produced by *Brevibacterium linens* is well known for using in the technology of surface-ripened cheeses (Zemanovic and Skarka, 1987).

1.5.2.2.2. Feed Manufacturing

The enzymatic digestion of whole fish powder and barley with proteolytic enzyme was found very important since increasing the nutritive values of animal and poultry feeds. They are also used in hydrolysis of soy proteins in the preparation of synthetic milk

and milk substitutes for pigs and calves (Kas, 1984).

1.5.2.3. Molecular Cloning

In vitro amplification of DNA by the polymerase chain reaction, the DNA polymerase I of *E. coli* is inactivated at the temperatures that are required to denature DNA, that is the reason for each round of synthesis required the addition of a fresh aliquot of enzyme which make the process more difficult and the yield is frequently poor as well as the products are heterogeneous in size. These problems were solved with the introduction of a thermostale DNA polymerase (*Taq* DNA polymerase) purified from the thermophilic bacterium *Thermus aquaticus* (Chien et al., 1976 and Saiki et al., 1988b). This enzyme which can survive extended incubation at 95 C, is not inactivated by the heat denaturation step and does not need to be replaced at every round of the amplification cycle.

1.5.2.4. Enzymatic Modifications

Giec et al. (1987) reported that the microbial protein can not be used in food production without, however, some modifications of their physico-chemical properties resulting in functional properties typical for food protein which could be realized by proteolytic preparations.

O'Meara and Munro (1984) examined many types of proteinases to solubilize lean beef tissue at different temperature and pH. They found in this selection procedure that alcalase was chosen as the most appropriate enzyme for 80% solubilizing lean beef tissue at 60°C and 8-10 pH in 24 h.

2. EXPERIMENTAL

2.1. MATERIALS AND METHODS

2.1.1. MATERIALS

2.1.1.1. Microorganisms

Spore forming long rod bacilli were isolated at 55°C from Egyptian soil samples, collected from the farm of Mansoura University, Hungarian compost samples, and Hungarian soil samples.

2.1.1.2. Chemicals and Raw Materials

All the chemicals used in this work were Hungarian made, and the materials used for cultivation of microorganisms were: soybean meal, provided from Komaromi Kòolajipari Vàllalat, and soluble starch, obtained from ASZAR Kemenyitogyar.

2.1.1.3. Media Used

Isolation and Screening Media

Milk agar medium (nutrient agar + 10% sterile skim milk) of Skerman (1967) was used for isolation and first step of the screening programme. For the second step of the screening different media were used as recorded in Table 1. Microbial cultures were maintained on medium No.5 at 4°C. after regular transferring.

Media Used in Laboratory Fermentation

The medium No.3. of Manabe et al. (1985) was taken for enzyme production. The process of medium development of Kuhn et al. (1979) was followed which can be divided in two sections: (1) medium design concerning to determination of essential components; and (2) medium optimization regarding the quantification of medium components.

2.1.2. METHODS

2.1.2.1. BACTERIOLOGICAL PROCEDURES

2.1.2.1.1. Enrichment of Caseinolytic Microbes Prior to Isolation.

Enrichment of natural sources including compost and soil samples for thermophilic proteolytic bacteria was carried out. One g

Table 1. Composition of growth media used for enzyme production.

Component	Concentration ($g/100^3$) of med. no.				
	1	2	3	4	5
Soluble starch	2.0				
Corn steep liquor	2.4				
Soybean meal	1.0		1.0	1.0	
Glucose		3.125		2.0	
Yeast extract		1.250			0.25
Dextrine			0.8		
Peptone				1.0	
Tryptone					1.0
K_2HPO_4	0.1				
$MgSO_4.7H_2O$	0.03		0.03		
$ZnCl_2$	0.03				
$CaCl_2$	0.03		0.02		
$NaHCO_3$	2.0				
$CaCO_3$		0.625			
NaCl		0.33			
KH_2PO_4			1.30	0.1	
KCl			0.05		
Na_2CO_3				0.1	
pH	7.2	7.3	7.2	7.2	7.0

1- Takami et al., 1989.

2- Gibb et al., 1987.

3- Manabe et al., 1985.

4- Fujiwara et al., 1987.

5- Chopra and Mathur, 1984.

sample was dispersed in 50 ml nutrient broth supplemented with 5 ml sterile skim milk as a selective substance. The flasks then were incubated at 55°C. After 24 h incubation period under shaking condition, 5 ml of the culture was transferred into 50 ml of fresh medium (nutrient broth plus 10% skim milk), and after an additional 24 h, 2 ml was inoculated into 50 ml of fresh medium (Allais et al., 1987).

2.1.2.1.2. Isolation and Purification of Proteolytic Thermophiles

Isolation of thermophiles

In order to isolate the proteolytic thermophiles, different serial dilutions were made with vigorous shaking. One ml sample was taken and plated on both milk agar medium (3% agar) and gelatin agar medium (0.4% gelatin containing nutrient agar). After 48 h incubation period at 55°C, separated colonies showed clear zones were transferred to the slope agar. In order to make sure this phenomenon, the plates were flooded with acidic solution (20 ml 1N HCl in 80 ml of 15% $HgCl_2$). The presence of a clear halo around the bacterial colonies indicates that those isolates were proteolytic organisms (Skerman, 1967)

Purification of The Isolates

From the preceding steps, well separated colonies were selected and suspended in sterile water. Purification of the isolates were carried out by two cycles cultivation using the same liquid medium as shown in the preceding paragraph followed by single colony isolation after streaking onto plates. Slope cultures were considered pure when they showed uniform morphological feature by microscopic examinations.

Some morphological and biochemical characteristics of the strains were examined by the methods described by Bajpai and Bajpai (1989).

2.1.2.1.3. Screening for Proteolytic Enzyme

Two different steps were performed in this programme for selecting the high potency isolates from those obtained in the preceding technique.

The first step depends on qualitative measurement of proteolytic activity using casein agar method. The diameter of each clear halo was measured in millimeter as a rough indication for the activity (Srinivasan et al., 1964; Snell et al., 1978; Cohen, 1981 and Montville, 1983).

The second step is adopted with the quantitative measurement of enzyme activity expressed in unit of enzyme per one ml of culture medium (Leanard and Wildi, 1970).

2.1.2.1.4. Inoculum Preparation

Prior to use the maintained cultures, the organisms were transferred two times to a fermentation medium and cultivated two run in 250 ml Erlenmeyer flasks with 50 ml working volume and incubated at 55°C under 300 rpm overnight for each run.

2.1.2.1.5. Bioreactor and Operating Conditions

Batch Fermentation

Biostat M (B. Braun, Melsungen) 2L laboratory fermenter was used for both batch and continuous fermentations as shown in Figures 1 & 2. The operating conditions were as follow :

Working volume	1.2 l
Inoculum size	10% (12 h old culture)
Agitation rate	1000 min^{-1}
Aeration	1.5 vvm
Temperature	50 $\pm$ 0.2°C
pH	7.0-7.2

Foaming was automatically prevented by adding Glanapon DG-100 anti foaming agent. Dissolved oxygen (PO_2) was on-line measured and monitored with an Ingold laboratory electrode.

Continuous Culture Apparatus

An apparatus constructed as shown in Fig. 3 was used. The fermenter vessel and operating conditions were the same as in batch cultivation under the same conditions . Some problems occurred, however, because of precipitation of the medium components in the overflow tube. That problem was almost entirely eliminated by using a construction with a magnetic stirrer Type OP-912/3 (Hungarian made). which was easily applicable throughout the time of the experiments (120 h).

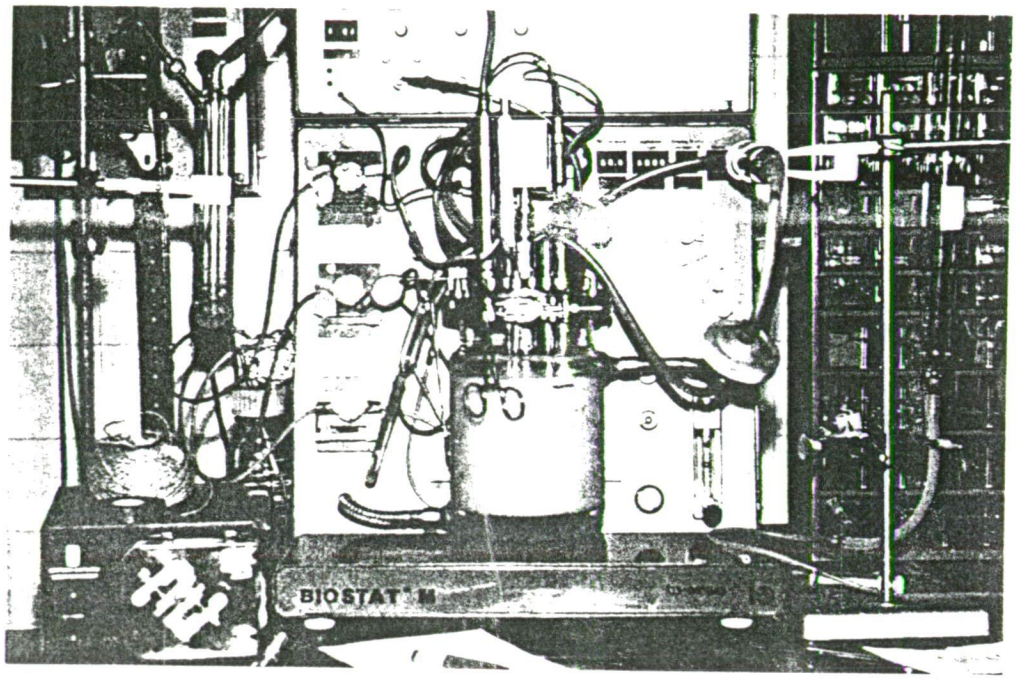
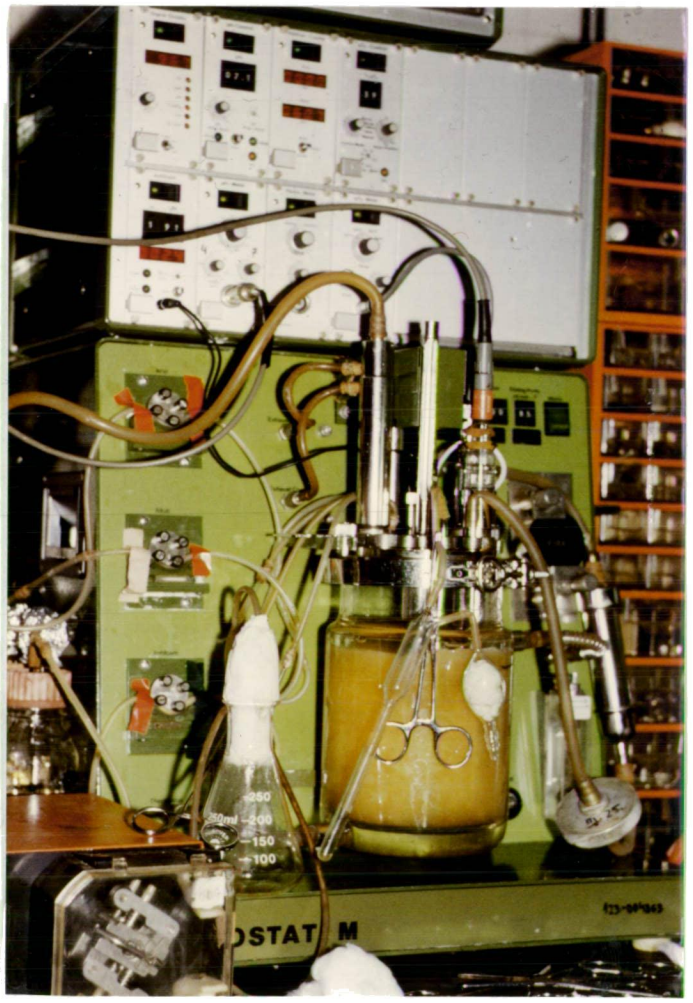


Fig. 1. Biostat M (B. Braun) Laboratory fermentor

Batch fermentation.



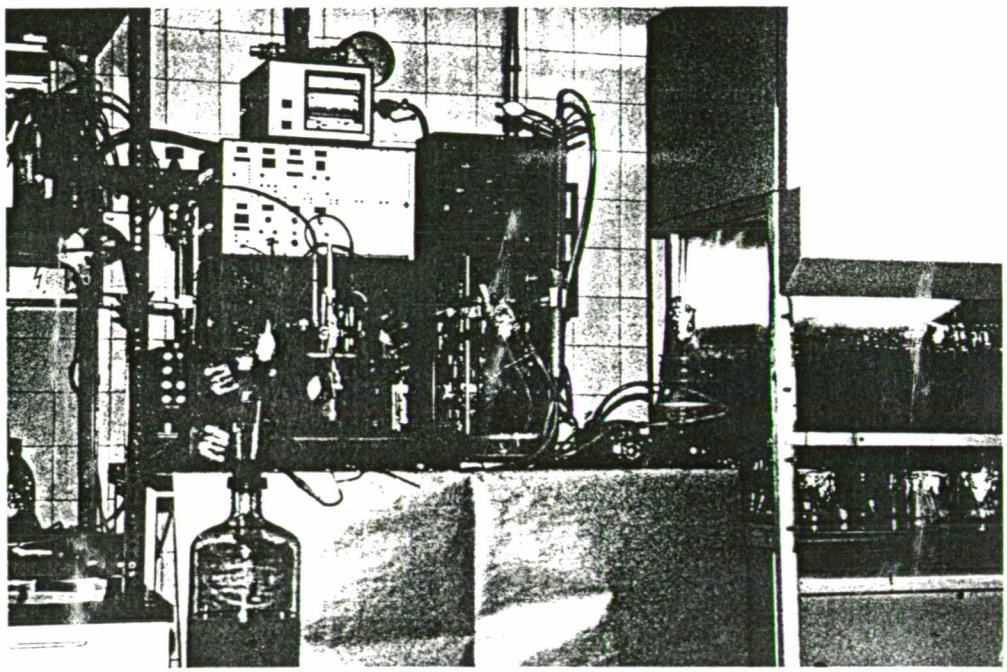
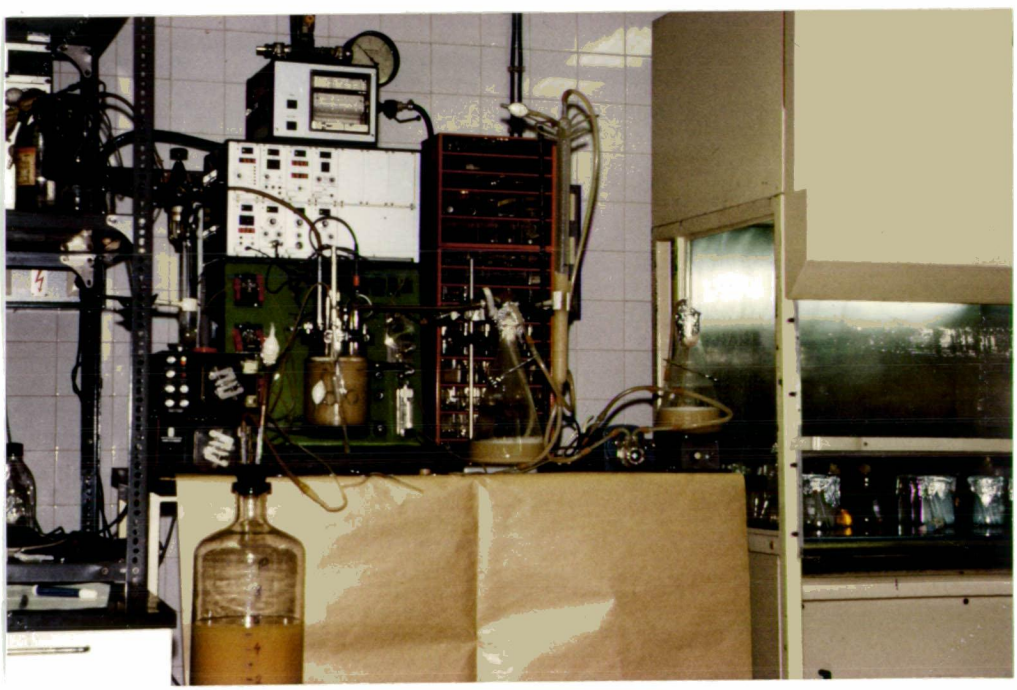


Fig. 2. Biostat M (B. Braun) Laboratory fermentor

Continuous fermentation.



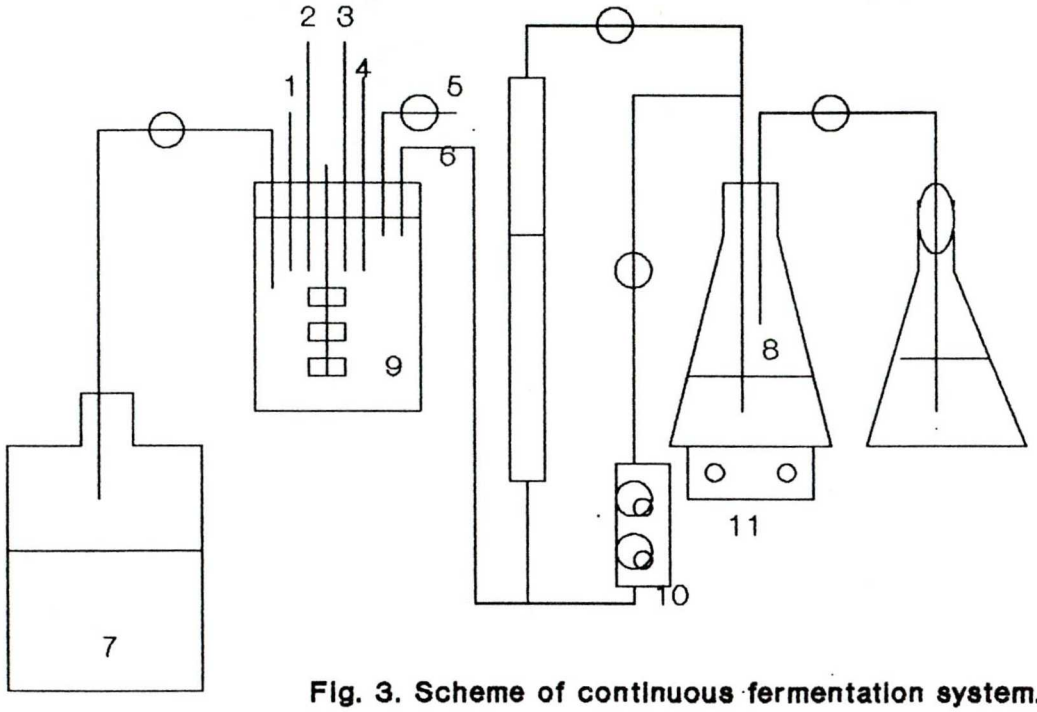


Fig. 3. Scheme of continuous fermentation system.

1- Thermometer pulb.
2- Ingold oxygen electrode.
3- Sterile air inlet.
4- Ingold pH electrode.
5- Sampling tube.
6- Feed inlet.
7- Feed output.
8- Stock of sterile medium (Reservoir)..
9- Constant volume culture vessel.
10- Feed pump.
11- Magnetic stirrer

The medium was inoculated and the culture allowed to grow in the batch mode, before the termination of batch growth, the feed pump was switched on to start the continuous process.

2.1.2.2. ANALYTICAL PROCEDURES

2.1.2.2.1. Microbial Growth Measurement

Bacterial Cell Density

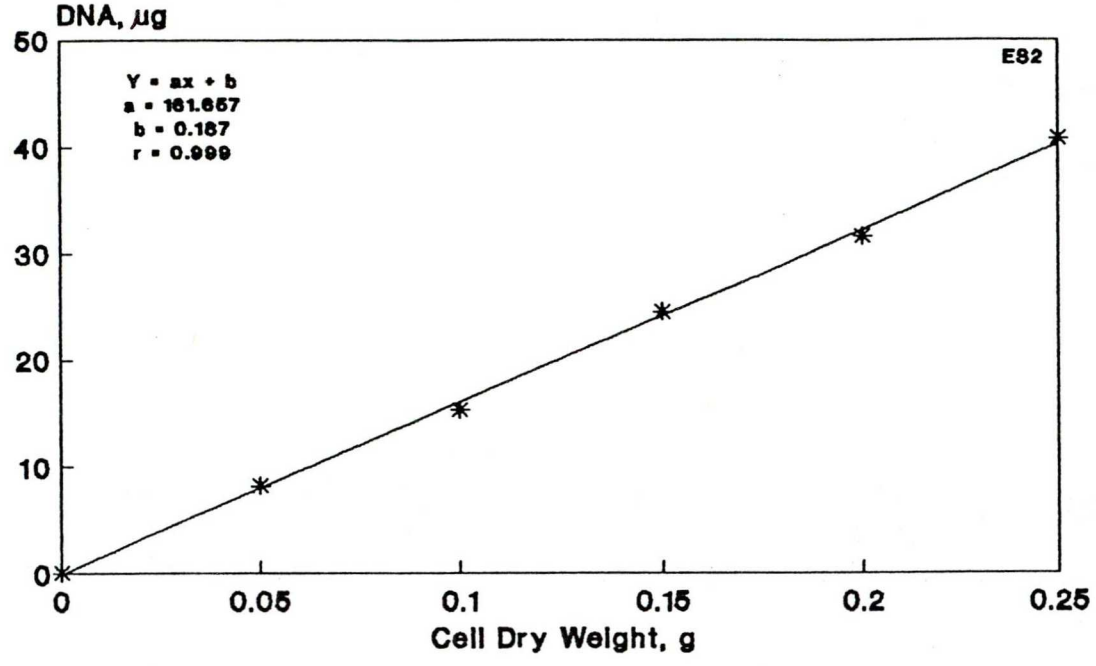
The optical density of microbial population was measured off-line at 620 nm in 1cm cell using Pharmacia LKB ULTROSPEC PLUS spectrophotometer after 25 time water dilution of the culture broth

Cell Dry Weight

Cellular biomass was indirectly measured by means of DNA. The organism was grown on the same medium of maintenance (without agar), followed the same cultivation conditions mentioned above. Samples were removed from the fermenter and quickly cooled just to freezing point to stop the metabolic activity. After thawing, the organism was separated by centrifugation at 4000 rpm, the cells were then suspended in a little distilled water and transferred to a preweighted micro beaker. The suspension were dried to constant weight at 80°C. The DNA content of the samples was measured (see next), and the relation between cell dry weight (CDW) and DNA content was plotted as a standard to be used for in converting the DNA value of the samples taken along the fermentation process. The calibration curves are shown in Fig. 4. for both tested strains.

2.1.2.2.2. Cellular Material Determination

Total nucleic acid content (TNA) of the cellular material was determined according to the method of Fleck and Munro (1962) described by Levine and Cooney (1973). This method applies 1N perchloric acid extraction in water bath at 70°C for 20 min followed by spectrophotometry at 260 nm. For quantitative assay, standard nucleic acid solutions from either RNA (Bakers yeast) or DNA (Chicken erithrocytes) were used. The standard curves are shown in Fig. 5.



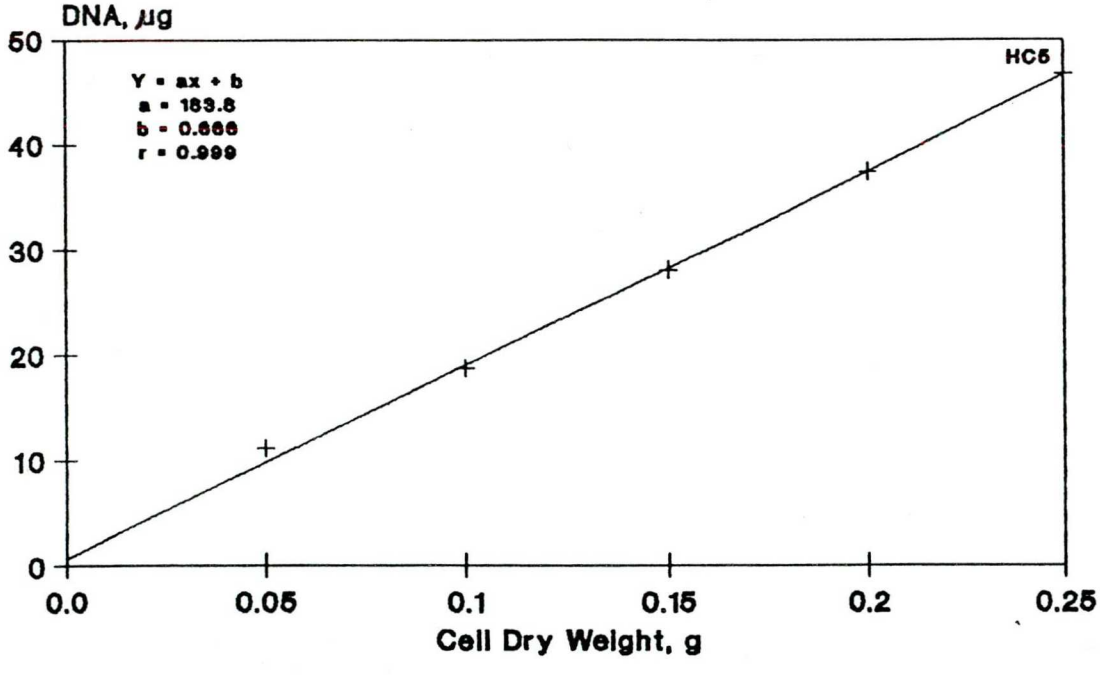
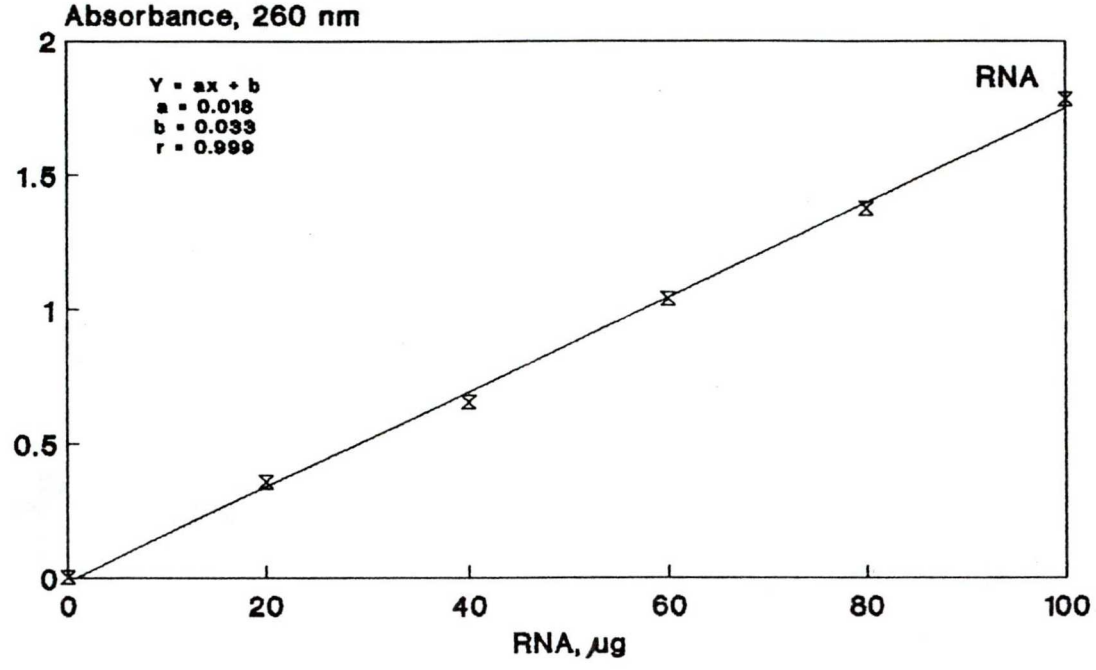


Fig. 4. DNA-CDW calibration curve.



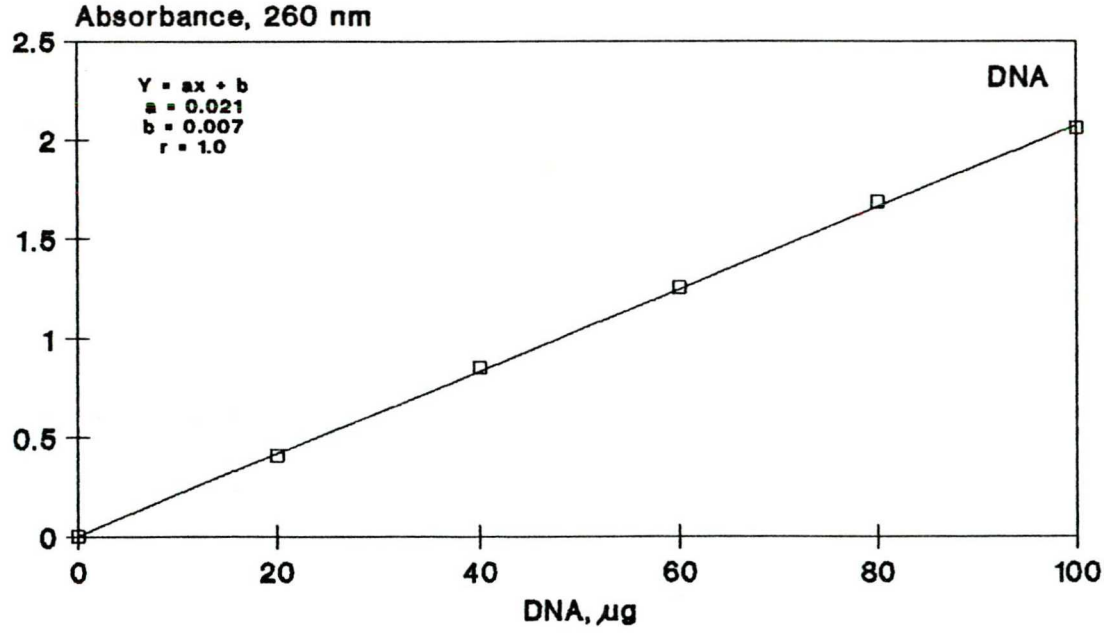


Fig. 5. Clibration curve for microbial nucleic acids.

2.1.2.2.3. Protein Assay

Total protein was colorimetric measured at 280 nm as described by Herbert et al. (1971) using bovine serum albumin standard as shown in Fig. 6.

2.1.2.2.4. Carbohydrate Determination

Total carbohydrate concentration was measured using the colorimetric anthrone method described by Plummer (1987). In this method, 4 ml diluted sample mixed well with 8 ml anthron reagent to be read after 15 min at 540 nm with glucose standard. The standard curve is presented in Fig. 7.

2.1.2.2.5. Evolution of Enzyme Produced

Preparation of Enzyme Solution

About 5 ml portion of cultured broth was taken as interval samples. Centrifugation was carried out at 3000 rpm, High speed centrifuge Type 310 (Made in Poland) for 10 min to remove bacterial cells, cell debris, and residual of the medium components. The supernatant was used as a crude enzyme solution after appropriate dilutions.

Assay of Enzyme Activity

Quantitative assay of proteinase activity was carried out using the modified casein digestion method described by Lupin et al. (1982). The method can be summarized as follows:

One ml of appropriately diluted enzyme solution (10-100 TU/ml, see below). was preincubated at 60°C for 2 min. One ml of similarly preincubated 1% casein solution (Hammerstein quality) prepared in phosphate buffer (pH 7.5) in the case of neutral proteinase (NP) assay or in carbonate-bicarbonate buffer (pH 9.5) in the case of alkaline proteinase (AP) assay was added and incubated for further 10 min. The enzyme reaction was terminated by adding 3 ml of 5% Trichloroacetic acid (TCA) solution. The precipitated undigested casein was separated by centrifugation at 3000 rpm for 10 min. Absorbance of the supernatant was measured against distilled water at 280 nm in 1 cm cell using ULTROSCOPEC PLUS (Pharmacia LKB) spectrophotometer. An UV-absorbing background was taken into account subtracting the absorbance of another test

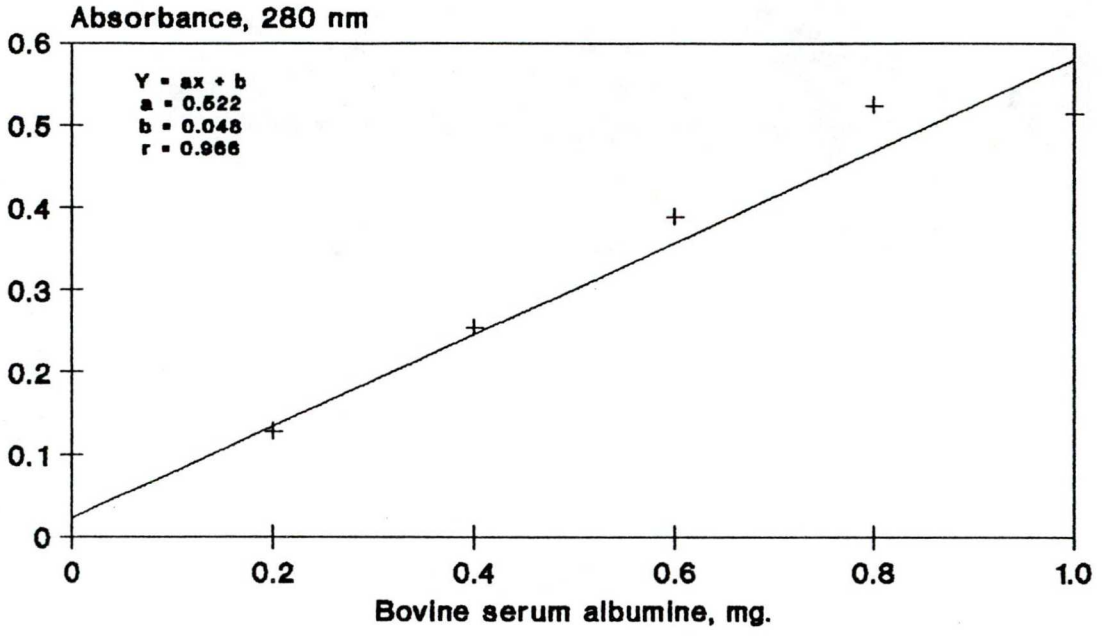


Fig. 6. Linear standard curve for protein assay.

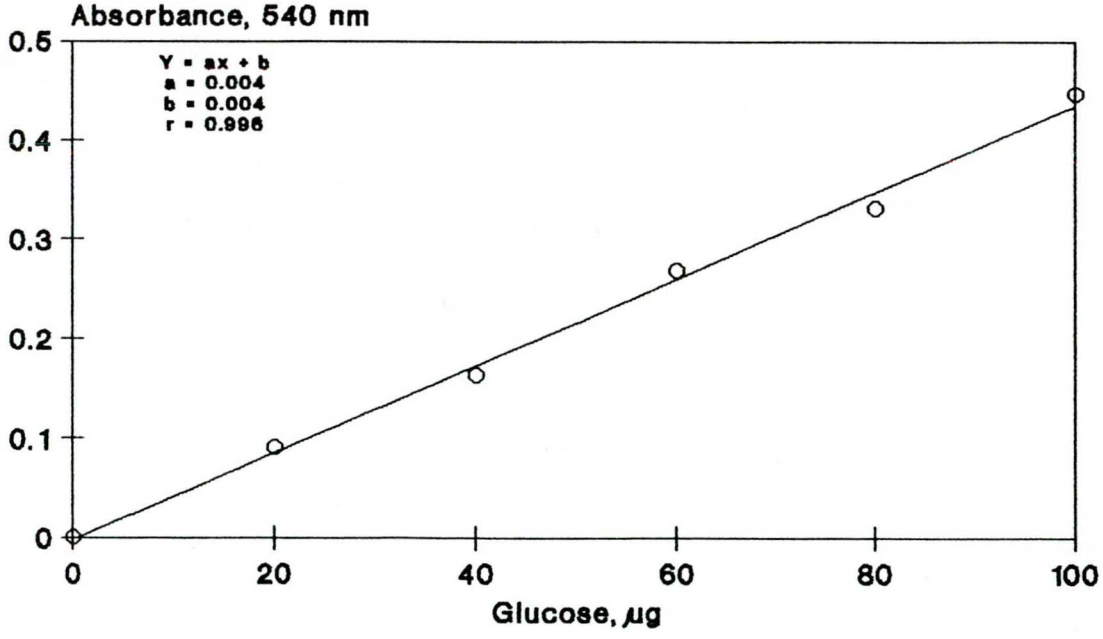


Fig. 7. Calibration curve for glucose assay.

tube which was prepared by the same way in reverse order, not allowing the development of enzymatic breakdown of casein. The resulted reading difference was calibrated to the reading of standard tyrosine solution as can be seen in Fig. 8.

Definition of enzyme unit (TU): One unit of proteinase (TU) was defined as the quantity of enzyme which deliberates 1 μ mol tyrosine equivalent in the form of 5% TCA soluble casein fragments under the reaction conditions mentioned above during 1 minute. In order to give the "concentration" of proteinase, the tyrosine unit (TU/ml) was used.

2.1.2.3. OPTIMIZATION OF CULTURE MEDIUM COPOSITION

Three components of medium (C,N, and P) were selected as factors for the medium optimization. Mineral salts were chosen to be at their original levels. Two design were constructed as follow:

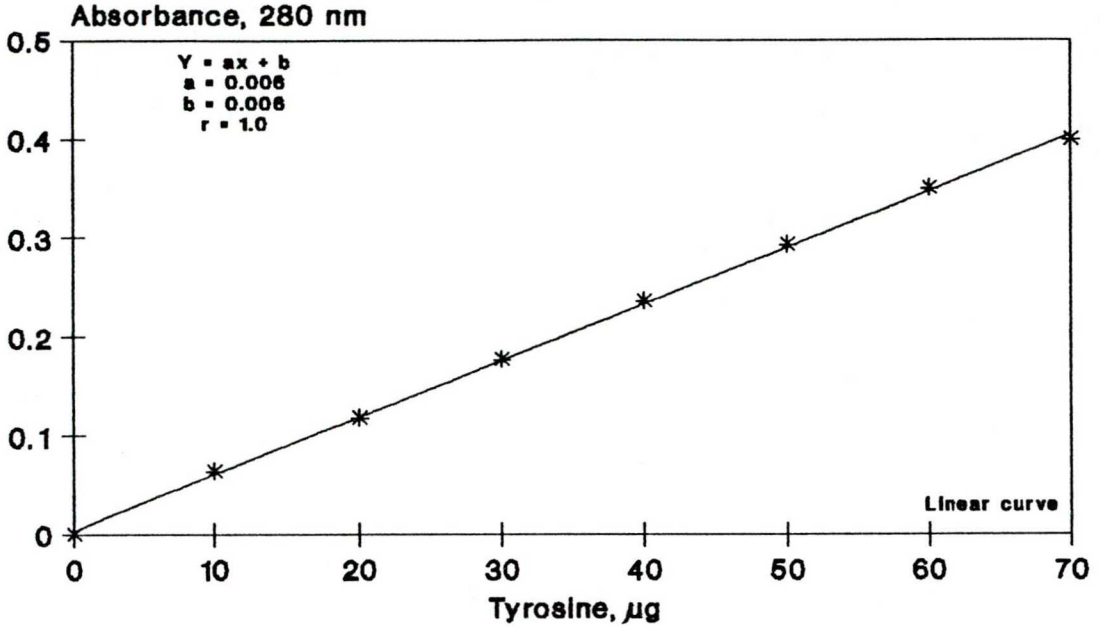
2.1.2.3.1. Experimental Design

First Design

Three factor two level orthogonal factorial design was used. Every components (factors) were tested at two different concentrations. The effect of that factor on the enzyme production rate was evaluated as described by Houng et al. (1989). The proposed model based on linear equation for description the correlation between culture medium composition and the end state of a fermentation through the cell populations density measurement and assay of enzyme activity as well.

Second Design

Two factor three level orthogonal composite factorial design was constructed by which all the three levels occur at the same number of times for the examined factor as described by Silveira et al. (1991). This design is expressed by second order model formulated in a polynomial equation.



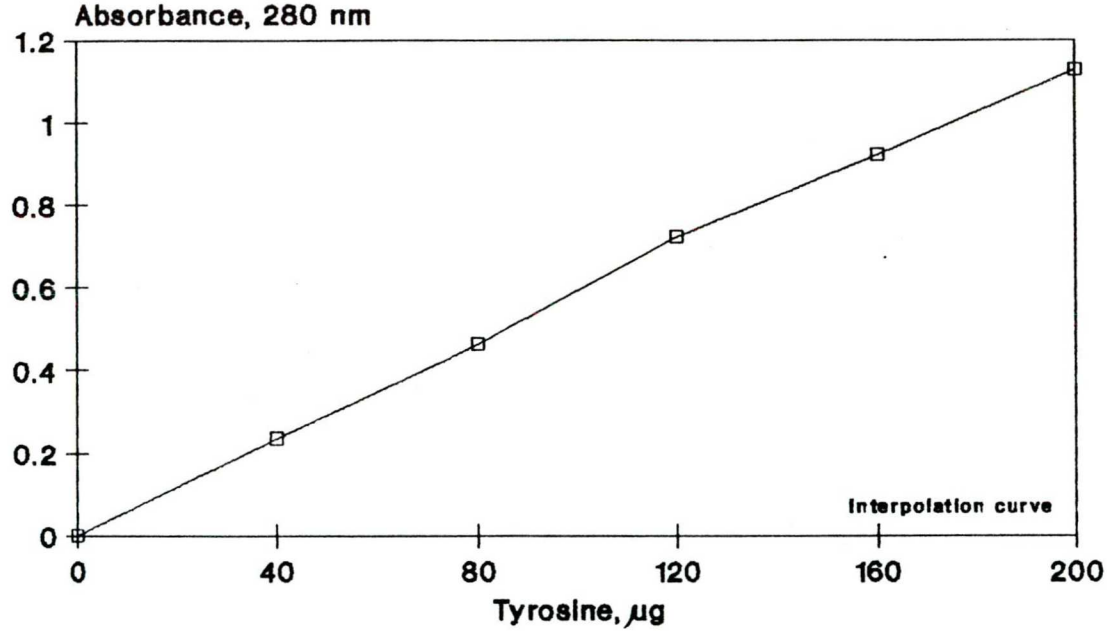


Fig. 8. Calibration curves for proteinase assay.

2.1.2.3.2. Mathematical Analysis

For determination of best fitting curves to sets of data points on the basis of a given function, a computer program package SPSS Microsoft$^{(R)}$ was used allying statistical analysis techniques and multiple regression (Geoffroy & Hollander, 1987; and Anderson et al., 1990).

In order to verify the validity of the proposed model, residual analysis ($E_i = Y_i - \hat{Y}_i$) was realized by examining the set of deviations between the experimental Y_i and the predicted values $\hat{Y}_i$ of the dependent variables (Himmelblau, 1970).

2.2.1. SCREENING FOR PROTEOLYTIC THERMOPHILES

The presence of proteolytic bacterial groups has been reported in a variety of microorganisms as mentioned in the review of literature. The microorganisms can not often produce large quantities of an enzyme until and unless their environment are carefully chosen . In order to produce desired enzyme with appreciate activity as possible, the choice of suitable strains for commercial production should be sought. Therefore, a considerable attention of screening for the proteolytic thermophilic bacteria was realized.

2.2.1.1. OBTAINING STRAINS

Fourteen strains of spore forming facultative thermophilic bacteria with proteolytic activity were isolated from compost and soil samples, collected from Egypt and Hungary.

The enrichment process was taken place, after which the isolation technique was carried out which has been followed by the purification procedures.

2.2.1.2. SCREENING PROGRAMME

Two different steps of screening were performed. The philosophy of screening was to find proteolytic enzyme producing strains with growth ability on cheap and commercial materials. The realized programme was as follows:

2.2.1.2.1. Primary Screening

Based on the visual observation, an experiment was designed to compare the ratio between the diameter of clear zone and diameter of the bacterial colony after 24 h incubation time at 55°C. Nehete et al. (1986) noted that this is a practicable measurement from qualitative point of view.

Results obtained were recorded in Table 2. It can be seen from data that the compost sample contains the most active strains followed by the Egyptian soil samples, but the Hungarian soil samples gave the lowest enzyme activity as clearly demonstrated in Fig. 9. Regarding the graphed data, out of 14 isolates, only ten showed high ratio of colony size to zone of casein hydrolysis represented in seven strains of compost and three soil isolates.

Table 2. Primary screening on milk agar plates.

Isolate Source	No.	Diameter of Growth G (mm)	Halo Z (mm)	Z-G (mm)
ES	1	12	20	8
ES	2	8	15	7
ES	3	6	10	4
HS	4	8	13	5
HS	5	7	13	6
HS	6	12	18	6
HS	7	8	15	7
HC	8	10	20	10
HC	9	2	10	8
HC	10	3	10	7
HC	11	3	15	12
HC	12	3	15	12
HC	13	4	15	11
HC	14	5	15	10

ES : Egyptian soil samples.

HS : Hungarian soil samples.

HC : Hungarian compost samples.

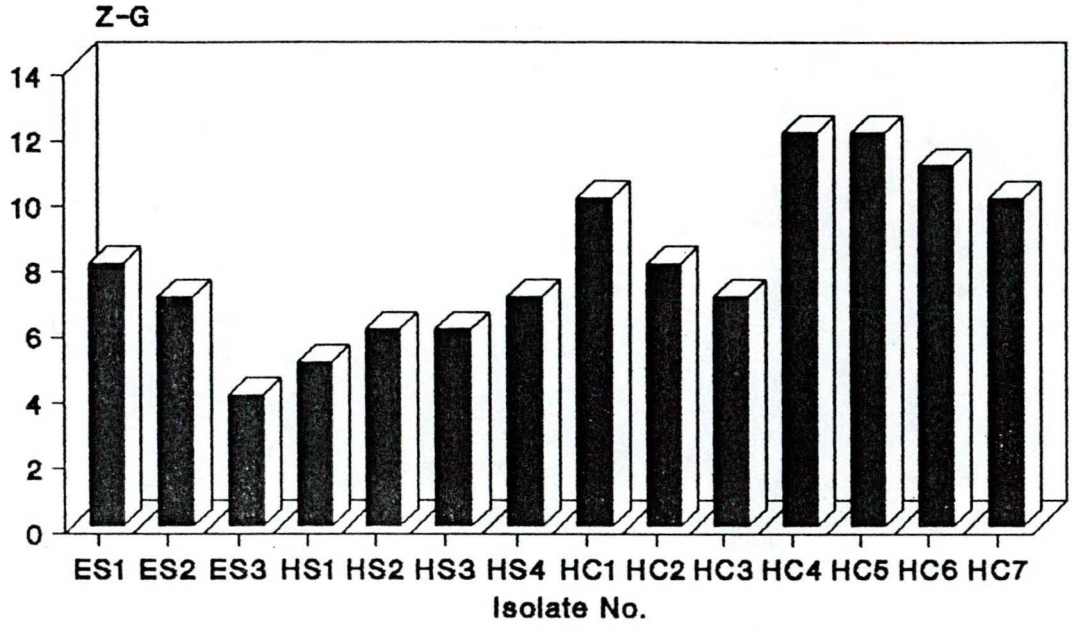


Fig. 9. Primary screening on milk agar plates.

Because of roughly determined values of enzyme activity based on caseinolysis zones criteria which making the interpretation of the results not so precise, the use of shake flask technique was followed for the ten chosen strains for getting quantitative results about enzyme activities as can be seen below.

2.2.1.2.2. Secondary Screening

Some preliminary experiments were taken place using inorganic nitrogen sources such as ammonium and nitrate salts but these did not prove to be satisfactory for proteinase production (not shown here). Therefore, the consideration is focused on the complex and organic nitrogen sources. The selected ten isolates from preceding step which gave high values of Z-G (7-12 mm), were further subjected to more sensitive and reliable estimation of enzyme activity using shake flasks on different recommended media with the compositions given in Table 1.

In this account, proteinase activity of the culture filtrates is described as volumetric activity (TU/ml culture filtrate). Cultivation was carried out in 250 ml Erlenmeyer flask with 50 ml working volume for 24 h incubation period at 55°C and 300 rpm. The proteolytic activity then measured by the modified casein digestion method. The quantitative values of the enzyme activity are recorded in Table 3. It can be seen from the Table that medium no. 3. was superior to other media with respect to alkaline proteinases production.

Medium no. 1 and 2 was neglected for its low activity values with most isolates except HC2 and HC4 strains since their enzyme activities were 132.5 TU/ml and 170 TU/ml, respectively. Similarly, data showed that the medium no. 4 and 5 gave very low values of enzyme activity, less than 100 unit/ml, for all isolates used which make it also out of the consideration. On the other hand, the medium no. 3 of Manabe et al. (1985) showed considerable high activity with about 50% of the isolates. This was the reason to be taken for the continuation of the work.

As expected, the use of starch (C-source) together with soybean in hydrolyzed form (N-source) predisposed the cultures for high titer of enzyme production. The improvement of enzyme production in media containing complex N, and C nutrient sources has been

Table 3. Second screening on liquid media.

Strain No.	Enzyme activity, TU/ml Medium No.*				
	1	2	3	4	5
ES1	55.0	0.0	102.5	92.5	70.0
ES2	00.0	0.0	195.0	21.3	26.3
HS3	52.5	0.0	84.4	0.0	0.0
HC1	36.3	90.0	0.0	15.0	35.0
HC2	132.5	35.0	137.5	50.0	82.5
HC3	0.0	52.5	50.0	0.0	2.5
HC4	6.3	170.0	0.0	36.3	73.8
HC5	0.0	103.1	162.5	15.6	18.8
HC6	90.0	0.0	52.5	0.0	15.0
HC7	105.0	43.0	0.0	0.0	0.0

* See table 1.

observed by many workers (Grueninger et al., 1984 and Takasaki, 1976).

For forthcoming work, the attention was given to only two different groups of isolates; firstly, soil isolates represented in ES2 and compost isolates represented in HC5. Those two isolates were the highest producers among all tested strains on that medium, since they gave 195 and 162.5 TU/ml culture filtrate, respectively. This means that we focused on comparative study for producing proteolytic enzyme by two different isolates obtained from different environmental conditions.

2.2.1.3. STRAINS CHARACTERIZATION

In order to make sure the purity of the isolates, the microscopic examinations illustrates that those isolates all belongs to the spore-forming, long rod-shaped, motile, Gram positive aerobic bacteria. The strains give positive reaction with the catalase test, producers for gelatinase, caseinase, and amylase. The cells were also linked to each other like a chain in a shaking culture and pellicle like growth on the surface culture as well (Fig. 10). The spores were spherical, located at the center of the cells. These characterizations proved that these two isolates are closely related to the genus *Bacillus*.

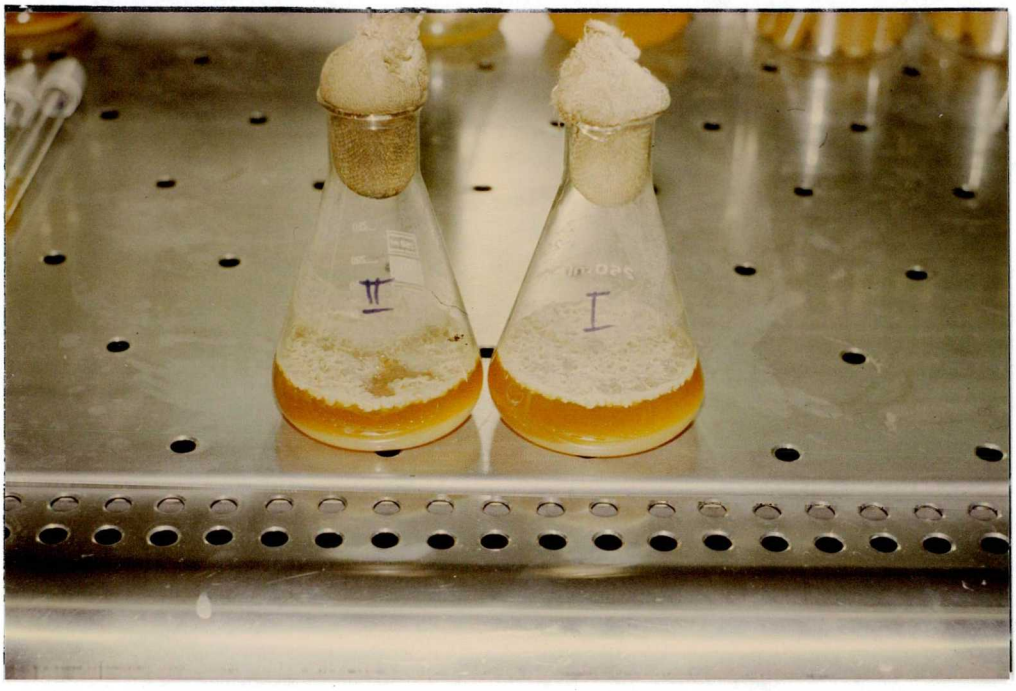


Fig. 10. Pellicle like growth on the surface culture of the two strain, *Bacillus sp.*

2.2.2. OPTIMIZATION FOR ENZYME PRODUCTION

In order to produce the desired enzyme in high quantity, both environmental and nutritional conditions were examined for the two selected strains ES2 and HC5.

2.2.2.1. EFFECT OF ENVIRONMENTAL CONDITIONS ON GROWTH AND ENZYME PRODUCTION

A logical starting point in examining the fermentation process kinetics would be to start with kinetics for simple cell growth and examine how the physical and chemical environments affect these kinetics. It could also show what kind of correlation exist between growth and product formation. The most important factors can be studied are the temperature and medium pH.

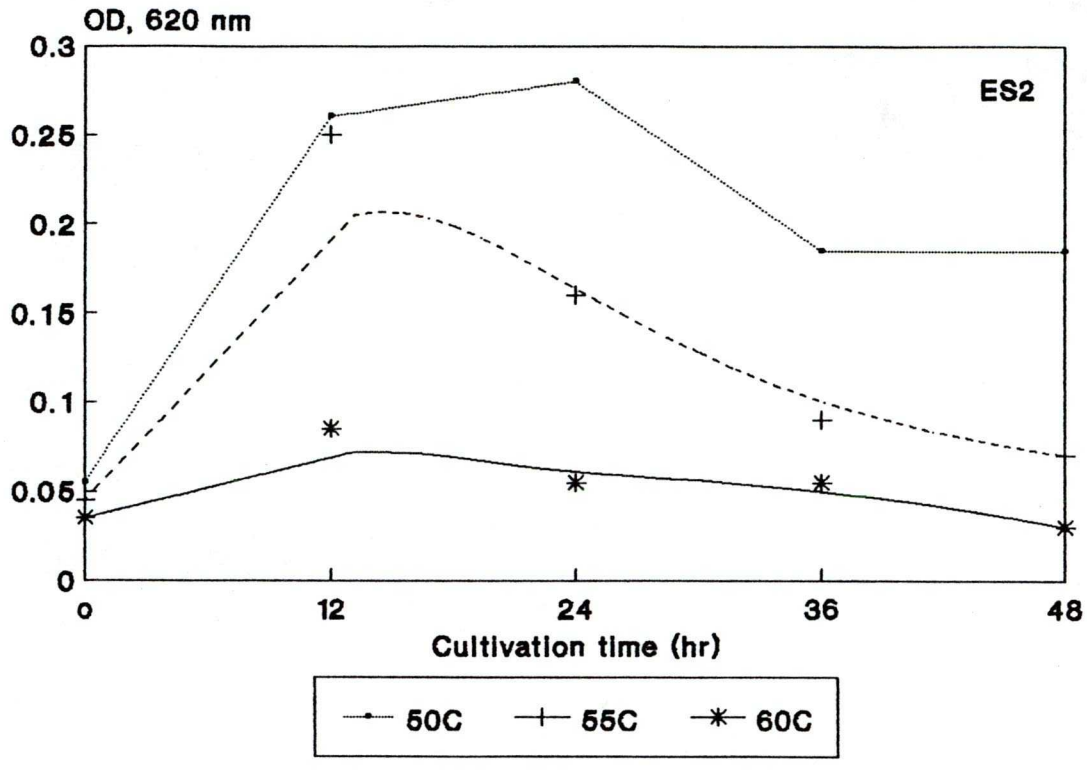
2.2.2.1.1. Effect of Temperature

In living organisms all physico-chemical processes responsible for the functional activity of cells, to a greater or lesser extent, are dependent on temperature. This is because of the microbial growth and product formation are the results of a complex series of chemical reactions which are greatly affected by the temperature.

To determine the optimum temperature of the growth and enzyme production, shaking flasks experiments were realized at different temperatures; 50, 55, and 60°C. Both neutral and alkaline proteinases were examined at the three temperatures used, and data obtained for the two strains are illustrated in Figures 11-13.

Data shows that 50°C was the most favorable in respect of growth and enzyme activity for ES2 strain while 55°C was the best for HC5. The maximum growth of ES2 strain at 50°C was reached after 24 h but at 55°C this time was reduced up to 50 %, while opposite results were found with HC5 strain, it showed its maximum growth after 24 h at 55°C but only 12 h was recorded for 50°C as shown in Fig. 11.

The plotted data can showed also that the kinetic behavior of ES2 growth was rather higher at 50°C than at 55°C since the microbial density in the inoculum size was more or less the same at both cases, while the opposite observation was noticed with HC5



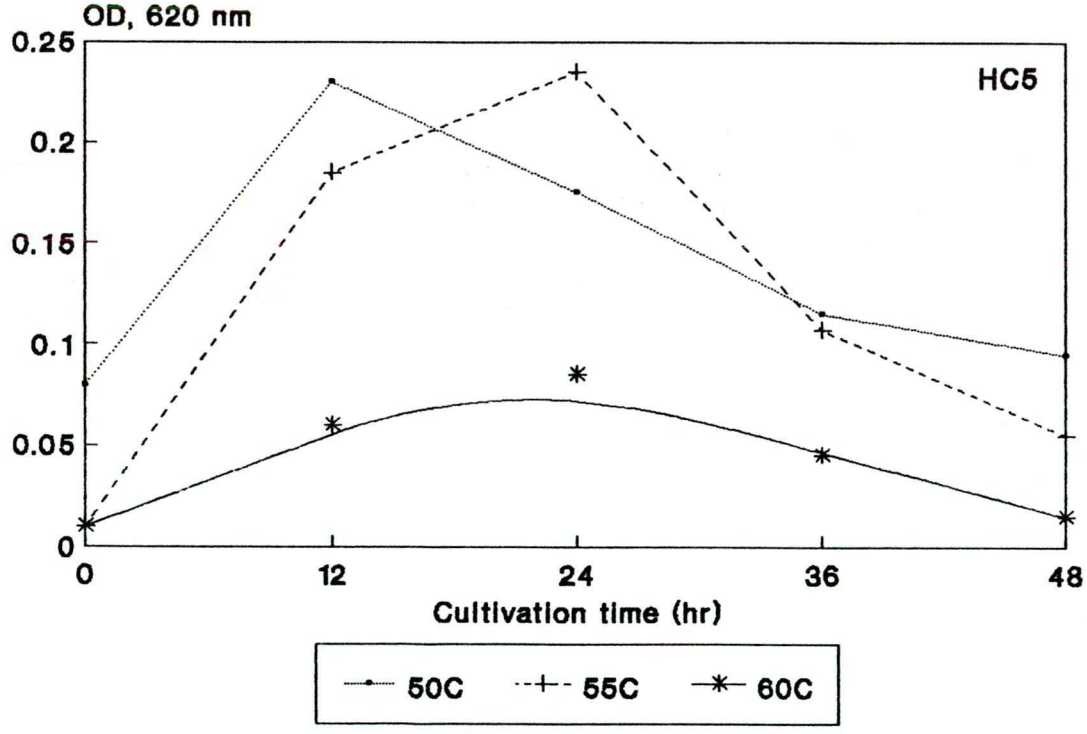
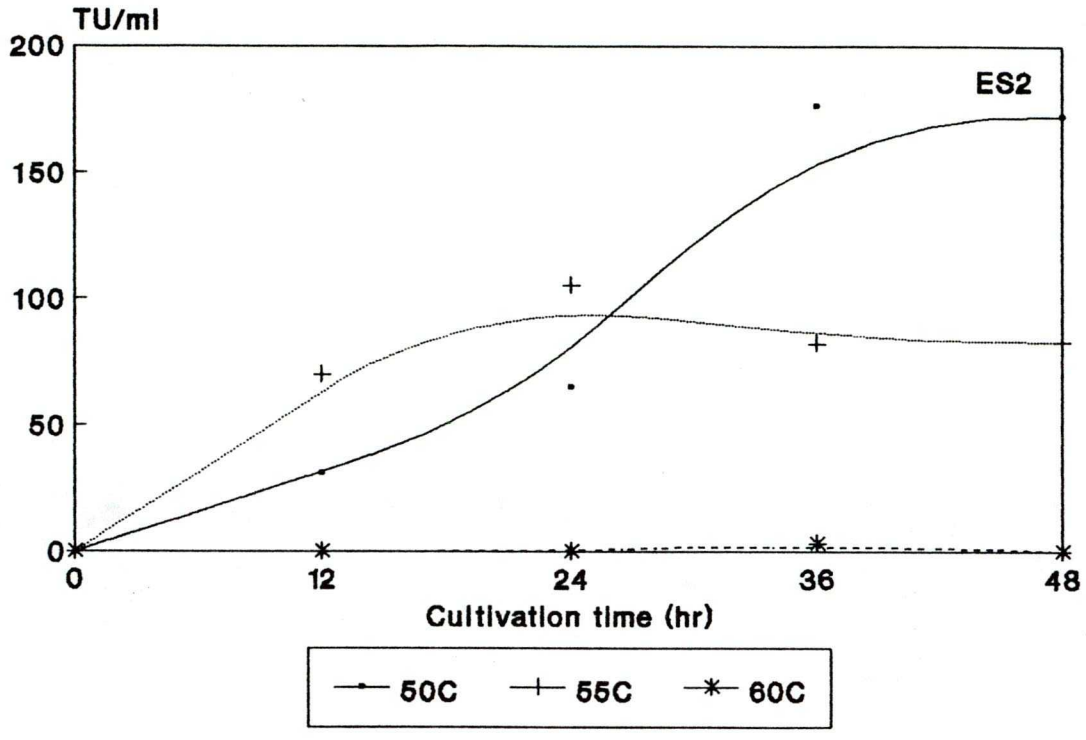


Fig. 11. Effect of temperature on microbial growth.

since the cell density in the inoculum size was more less in the case of 55°C than 50° C in spite of the OD reading was the same in both cases after about 15 h of cultivation starting point. Owing to that the microbial growth depends upon a complex sequences of enzymatic reactions whose rates are individually related to the growth temperature, the decline of bacterial growth were observed at 60°C as can be seen in the same Figure. Topiwala and Sinclair (1971) noticed that increasing of cultivation temperature may appreciably change the physical properties of the culture medium and hence indirectly affect the cell metabolism. The other effect of temperature is lowering solubility of oxygen (at 70°C is 5% of that at 20°C). So the greater yields at lower temperature are attributed to higher solubilities of oxygen, which assure that at high temperature above growth optimum, thermophiles are not killed but, their functions and activities are preserved regarding the low level of oxygen required for the metabolisms.

Product formation by microorganisms is also dependent on temperature in a manner similar to growth. However, the temperature optimum for growth and product formation are not necessarily the same. At 55°C the final neutral proteinase activity was half of the value obtained at 50°C after 36 h with ES2 strain while the opposite observation was found with HC5 strain as clearly shown in Fig. 12. Data regarding the alkaline proteinse activity illustrate that ES2 strain showed only a 25% decrease at 55°C after 36 h when HC5 gave 60% increase after 24 h at the same temperature as illustrated in Fig. 13. Skjenstad et al. (1992) found that the growth curves at 55, 60, and 65°C were very similar, but the proteolytic activity reached a peak at 60°C. They also found that the enzyme activity at 65°C was less than 20 % compared with 60°C.

On the other hand, data showed that neither neutral nor alkaline proteinase activity was observed at 60°C, corresponding to poor growth. That is may due be to the highly selective effects on metabolic pathway such as repression of particular protein syntheses which could be exerted by high temperature as found by Pirt et al. (1961). Nevertheless these results proved that the two strains used in this work can be considered as thermophilic bacteria according to the definition of thermophily, viz. all the



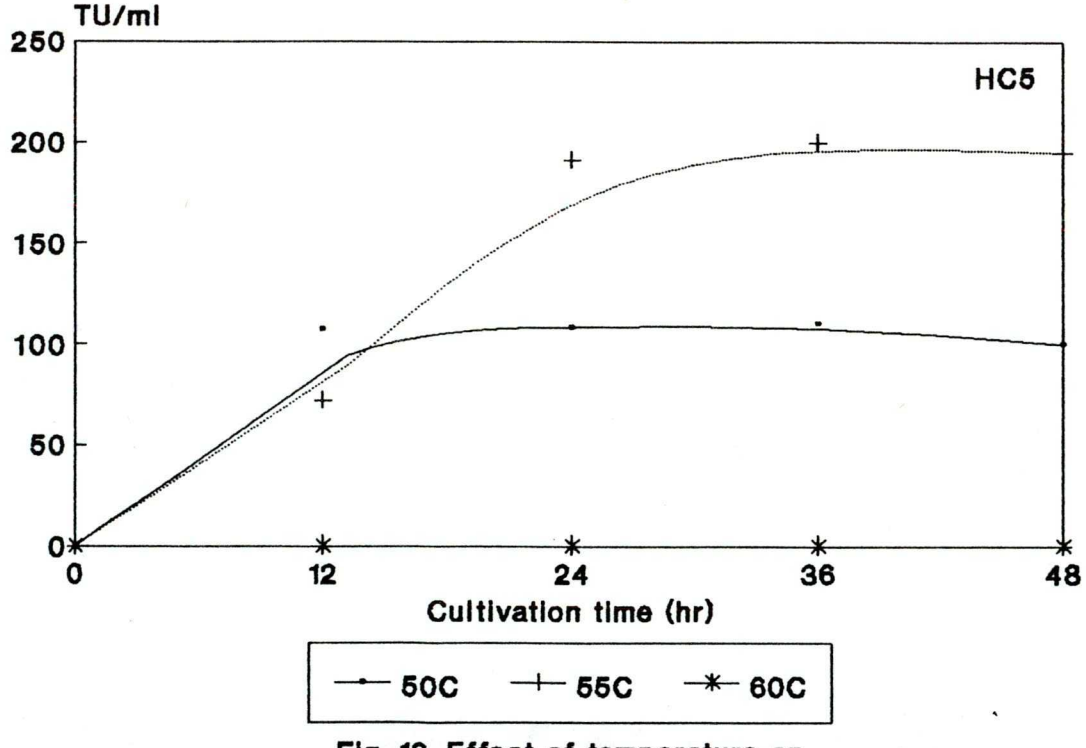
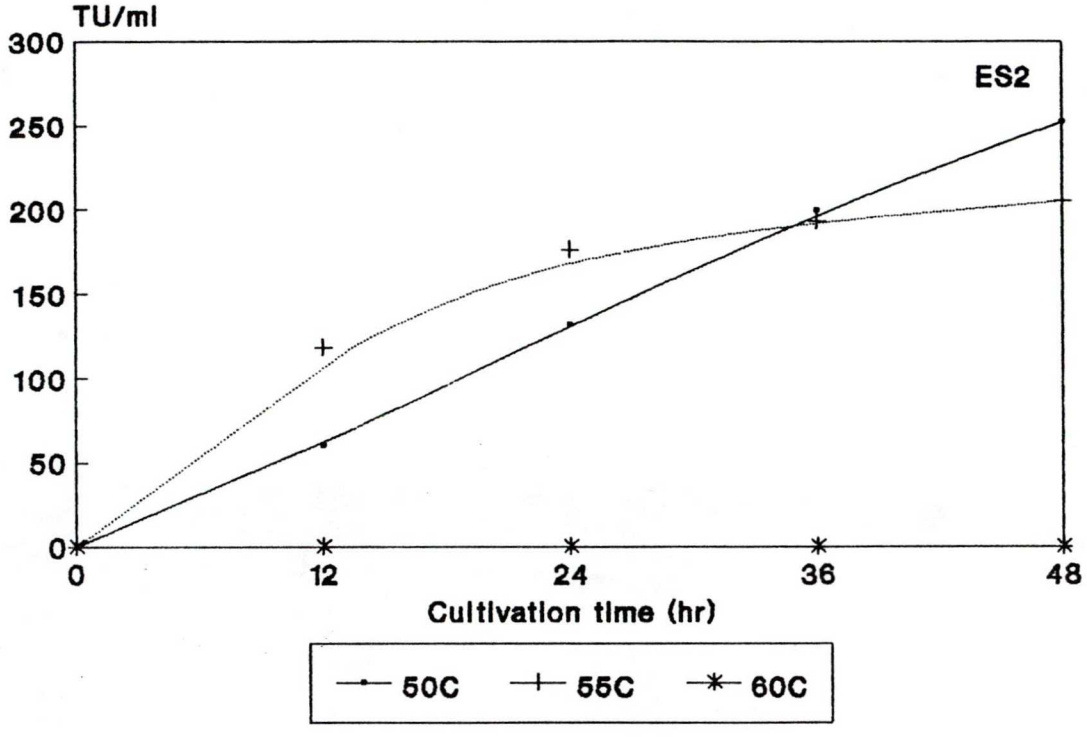


Fig. 12. Effect of temperature on neutral proteinase excretion.



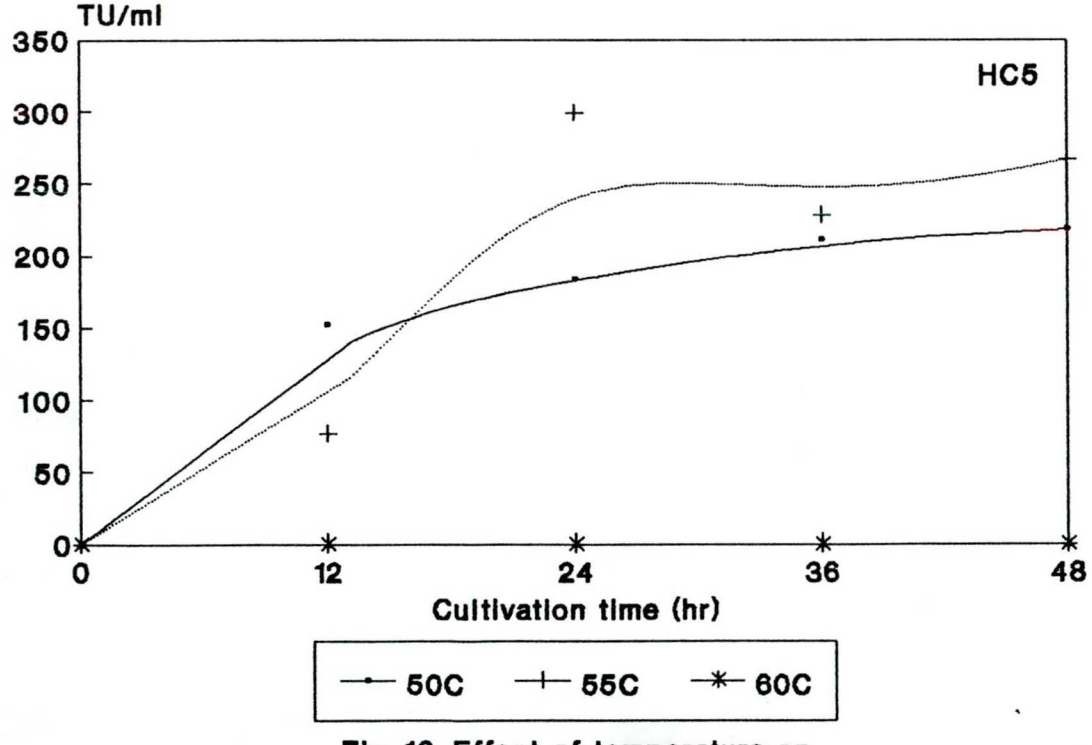


Fig. 13. Effect of temperature on alkaline proteinase production.

organisms are thermophiles wherever their optimal growth exist in the thermobiotic temperature range, over 45°C as reported by Sonneleitner (1984).

2.2.2.1.2. Effect of pH

Data mentioned above showed that the alkaline proteinase activity is produced in appreciate yield much higher than the activity of neutral one. So the scope of the forthcoming studies is focused only on the alkaline proteinase.

The value of medium pH is an important parameter affecting growth and product formation. The effect of pH on growth has been based on the behavior of enzymes. Since enzymes are protein composed of amino acids, they exhibit Zwitterion behavior, i.e. they have an acid, base, and neutral form. However, most organisms are active over a pH range of 3-4 pH units. Initial pH of growth medium was adjusted to various levels with 0.1 M HCl solution prior to inoculation the shake flasks.

Data showed that the increasing the initial pH resulted in an almost linear and steep decrease in the final activity produced as shown in Fig. 14. for the two selected strains. The final pH of the culture broth at every cases was in the range of (8.0-8.2). The decline of pH level have been observed by many workers, Al-Awadhi et al, (1988) stated such behavior with respect to medium pH is typical of the majority of neutrophiles. This phenomenon could be explained as a result of the deamination of the organic amino-compounds which released from the pretreated soybean (nitrogen source) which used for the bacterial growth.

This observation indicates also that the little rise in the pH considered to be the final values after the neutralization between the deaminated organic compounds and the organic acids obtained from the breakdown of the starch used as a carbon source in the cultivation medium.

On the other hand, these results could also suggest that the two strains used in this studies are thermoneutrophiles bacteria according to Brock (1978) who pointed out that this group of microorganisms has a pH optimum between 5.8 and 8.5 pH unit.

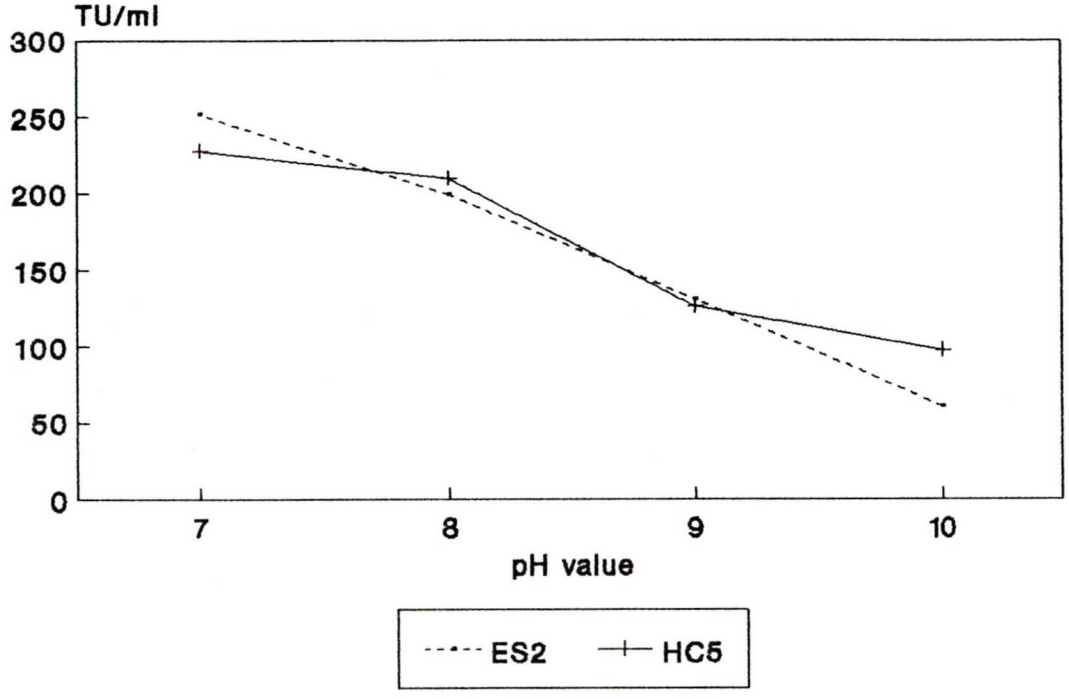


Fig. 14. Influence of initial pH of culture medium on the alkaline proteinase production

2.2.2.2. EFFECT OF CULTURE MEDIUM COMPOSITION

Numerous studies were carried out to investigate the influence of medium components (carbon, nitrogen, and phosphate). Medium composition was optimized both determination and quantification of the essential components in order to be applicable for and to meet the requirements of tested strains for growth and desired product formation.

Two different models were suggested but only one was used in this work because it does not need too much computing time, memory size, and correctly describes the evaluation of the formulation of culture medium used. The assumption for description of microbial batch culture can be based on linear equations for the relationship between the microbial population density, product formation and the interaction between both nitrogen and carbon-energy sources.

Two different design were applied, the first is linear term with interaction between carbon and nitrogen sources (CN), and the second is quadratic term with the interaction (CN). The parameters of the models were obtained from a particular set of experimental data which can be summarized as follows:

2.2.2.2.1. The First Design

In order to choose an adequate concentration range, three different factors were taken into account; carbon, nitrogen, and phosphate sources. A three factor, two level, first order orthogonal composite factorial design was realized. To find the maximum response, eight different treatments with three replicates were performed considering C= 5, N=9 and P=13 g/l as the working point of culture medium composition as shown in Table 4. in respect of alkaline proteinase activity as an objective function after 24 & 36 h cultivation time for each examined strain. Data obtained were calculated by multiple regression and formulated in equation 1 & 2 (24 h) and 3 & 4 (36 h) for both used strain ES2 & HC5, respectively.

Table 4. Treatments of first design.

Treatment	Medium components g/L		
	C	N	P
1	6	11	15
2	6	11	11
3	6	7	15
4	6	7	11
5	4	11	15
6	4	11	11
7	4	7	15
8	4	7	11

Three replicates for each.

Table 5. Gradients of product formation

	ES2		HC5	
	24h	36h	24h	36h
ΔY/Δ C	-40.8	-36.1	28.5	18.4
ΔY/Δ N	19.2	33.3	00.4	-13.2
ΔY/Δ P	-24.5	-14.8	-3.3	-6.4

$$Y = -183.1 + 115.3\,C + 105.9\,N - 24.5\,P - 17.3\,CN \quad \text{----------[1]}$$

$$Y = -842.9 + 219.9\,C + 106.7\,N - 3.3\,P - 21.3\,CN \quad \text{----------[2]}$$

$$Y = 168.05 + 3.28\,C + 55.16\,N - 14.77\,P - 4.37\,CN \quad \text{----------[3]}$$

$$Y = -1017.54 + 284.50\,C + 134.69\,N - 6.37\,P - 29.57\,CN \quad \text{------[4]}$$

According to the steepest ascent step, the increasing rate of the product formation has been calculated and obtained data are recorded in Table 5. The new working points of culture medium formulation were also estimated as shown in Table 6. By comparing the new and starting working points, data shows that phosphate source has no significant effect on the enzyme production which can be omitted from the model. On the other side, regarding phosphate serves as the constructing material of cellular components such as cyclic AMP, nucleic acids, phospholipids, nucleotides, and coenzymes, keeping its level at 10 g/l as constant in every experiments has been taken. The importance of phosphate was also considered by Yoon et al. (1989) on amylase fermentation by *Bacillus sp.*

2.2.2.2.2. The Second Design

In order to achieve higher rate of enzyme production, further optimize with two factor, three level, second order orthogonal composite experimental design was followed as described in Table 7. The treatment of the central point of the design was performed in eight replicates and the other treatments were only carried out in two parallels. After statistical treatment of data, the comparison of model prediction and experimental observations of enzyme activity by ES2 strain are illustrated in Fig. 15.

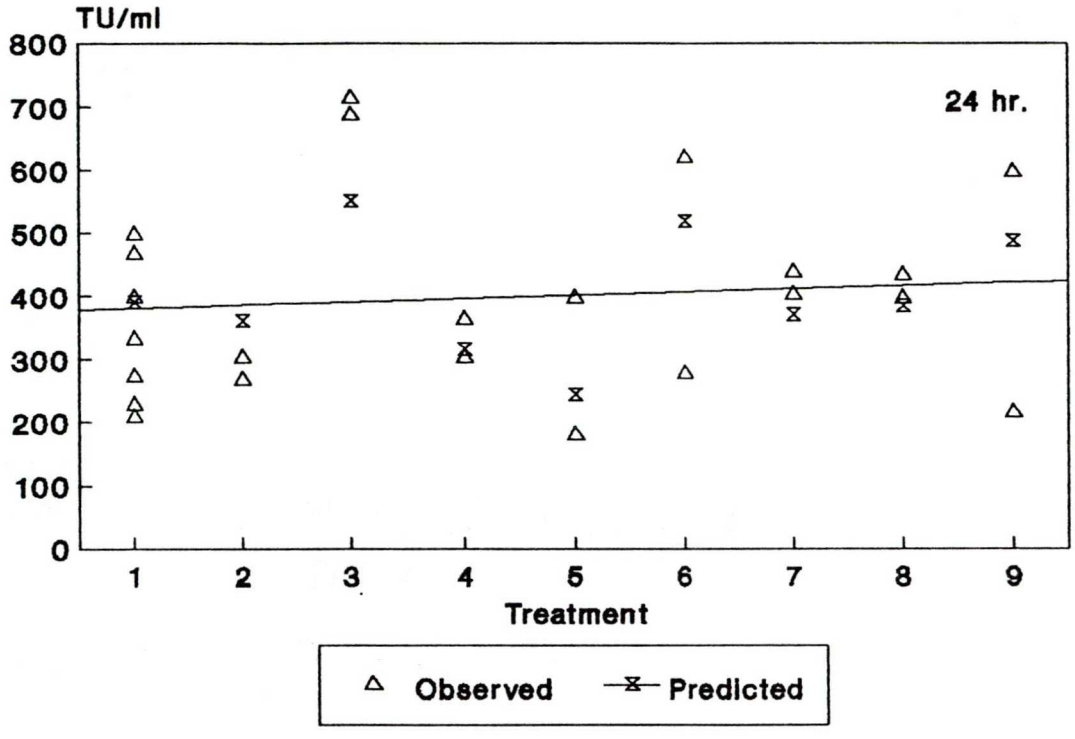
Plotted data showed relatively straight line with nonsignificant positive slope after 24 h with all treatments examined, while a straight line with sharp negative slope in enzyme production was observed after 36 h. After the mathematical analysis, it could be concluded that the treatment No.1 has given the highest value of the enzyme activity being 571 TU/ml culture filtrate after 36 h of cultivation period.

Table 6. Formulation of culture medium

Medium Components g/l	Starting Working Points	New Working Points ES2 24h	ES2 36h	HC5 24h	HC5 36h
C	5	3.0	3.0	7.0	7.0
N	9	9.9	10.8	9.0	7.6
P	13	11.8	12.2	12.8	12.3

Table 7. Treatments of second design.

Treatments (Replicate)	ES2 C	ES2 N	HC5 C	HC5 N
1 (8)	3.0	10.8	7.0	7.6
2 (2)	3.0	9.0	7.0	6.0
3 (2)	3.0	12.6	7.0	9.2
4 (2)	2.0	10.8	5.5	7.6
5 (2)	2.0	9.0	5.5	6.0
6 (2)	2.0	12.6	5.5	9.2
7 (2)	4.0	10.8	8.5	7.6
8 (2)	4.0	9.0	8.5	6.0
9 (2)	4.0	12.6	8.5	9.2



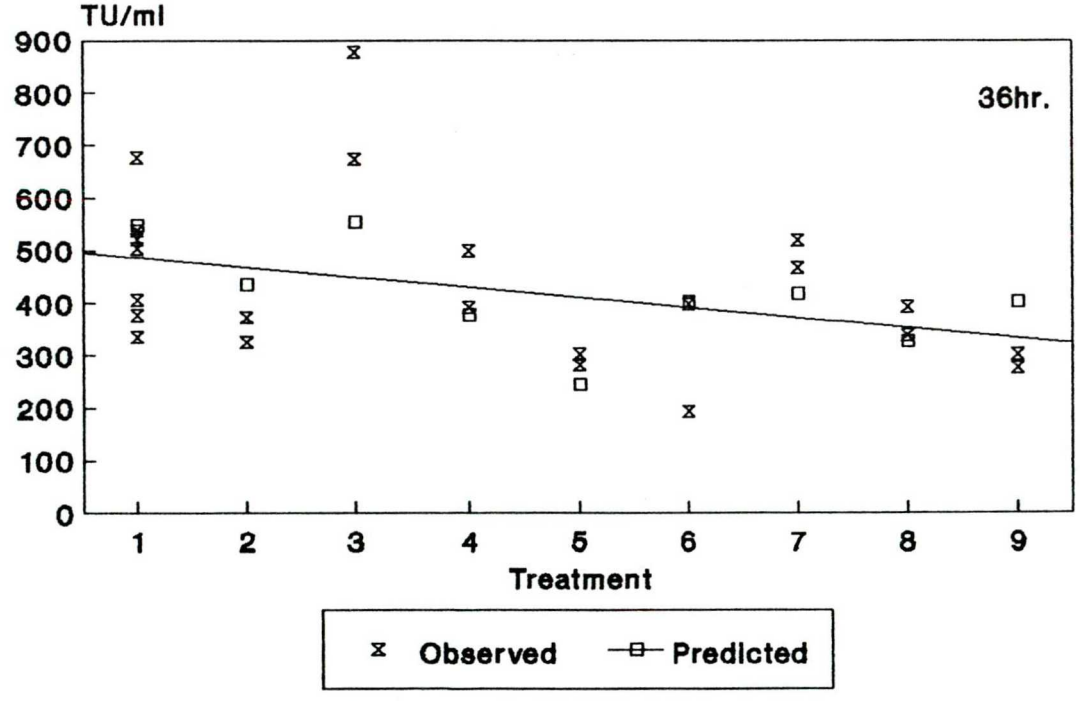
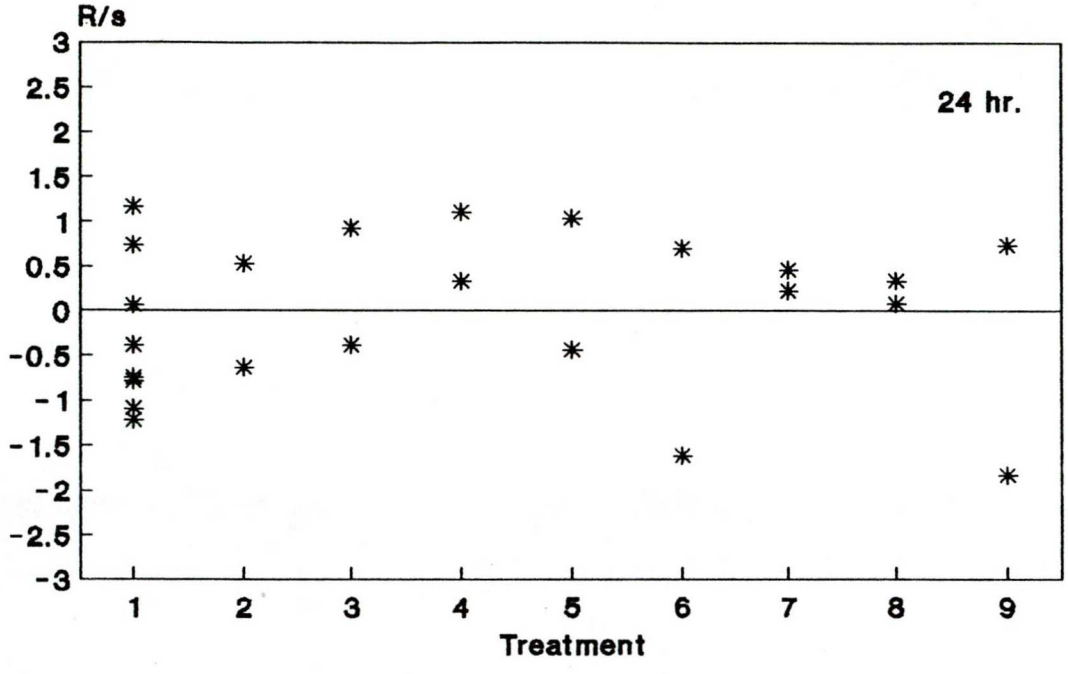


Fig. 15. Comparison of model and experimental observations (alkaline proteinase, ES2 strain).



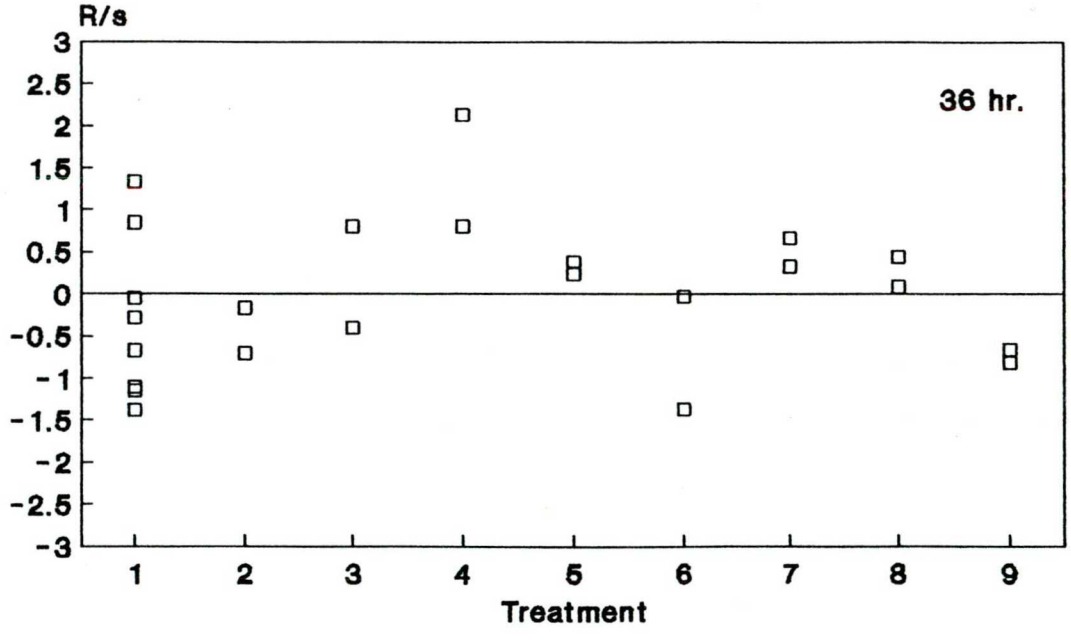


Fig. 16. Standardized residual plot (alkaline proteinase, ES2 strain).

Similarly, concerning the HC5 strain, graphed data in Fig. 17. illustrated a straight line with positive slope for enzyme activity produced at both 24 and 36 h. The higher values were observed after 24 h in contrast to the case of ES2. Again the same phenomenon between the two strains was also found considering the treatments run since the treatment No. 1 showed the highest value after 24 h being 540 TU/ml culture filtrate.

After statistical analysis of the data, testing of the model adequacy and testing the significant of the model coefficients, the experimental results were then fitted with the second order polynomial equations; 5 & 6 (24 h) and 7 & 8 (36 h) for ES2 & HC5, respectively.

$$Y = 388.92 + 52.39\,(N-10.8) + 27.64\,(C-3) - 23.6\,(N-10.8)(C-3) + 20.23\,(N-10.8)^2 - 47.9(C-3)^2 \quad [5]$$

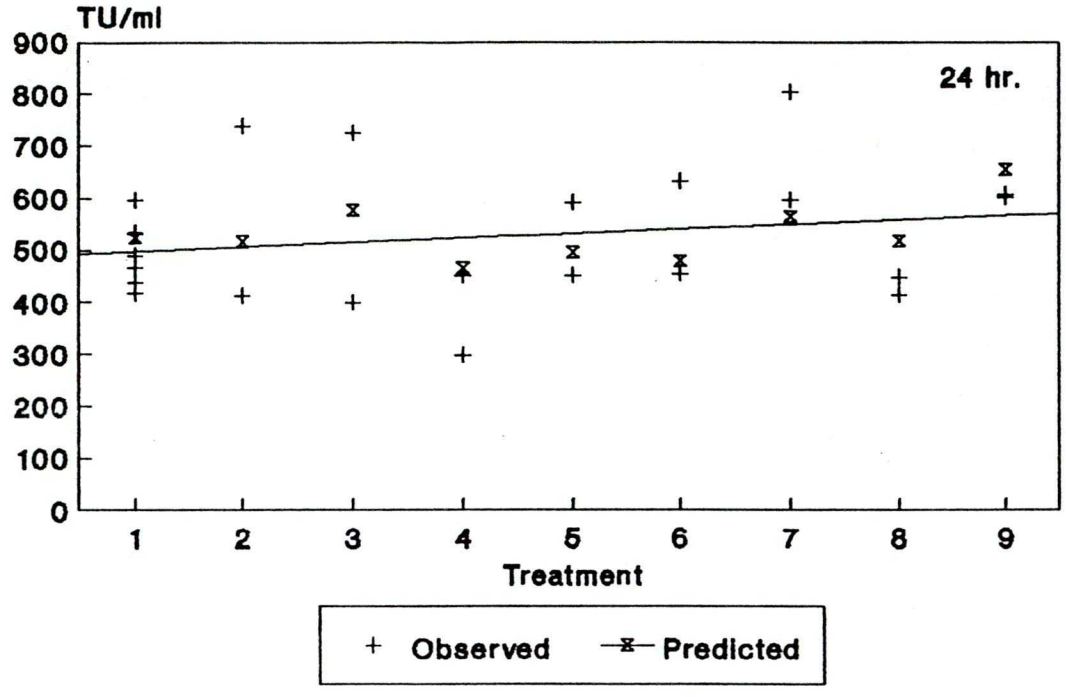
$$Y = 523.54 + 18.875\,(N-7.6) + 32.92\,(C-7) + 15.828\,(N-7.6)(C-7) + 8.56\,(N-7.6)^2 - 4.289\,(C-7)^2 \quad [6]$$

$$Y = 546.38 + 32.69\,(N-10.8) + 20.37\,(C-3) - 11.27\,(N-10.8)(C-3) - 16.45\,(N-10.8)^2 - 149.8\,(C-3)^2 \quad [7]$$

$$Y = 312.79 - 12.18\,(N-7.6) + 30.88\,(C-7) - 7.047\,(N-7.6)(C-7) + 42.22\,(N-7.6)^2 + 10.73\,(C-7)^2 \quad [8]$$

Applying residual analysis ($E_i = Y_i - \hat{Y}_i$), both examined times were quite fit the model with all examined cases. The number of different treatments were plotted against the values of standardized residual ($Y_i - \hat{Y}_i / S_r$) derived by computer programme as could be depicted in Figures 16 & 18. The Figures clearly bring out that the model represent the experimental data adequately, since the values of standardized residual lies in the acceptable range between +- 3.

From the results discussed, it could be stated that ES2 showed higher yield coefficient after 36 h than at 24 h. On the contrary, the opposite criterion was obtained by the HC5 strain. Since the stationary point of this surface is a saddle, the maximum of the function over the domain estimated by composite design are g/l C = 3.03, 8.5 and N = 11.78, 10.2 for ES2 & HC5, respectively.



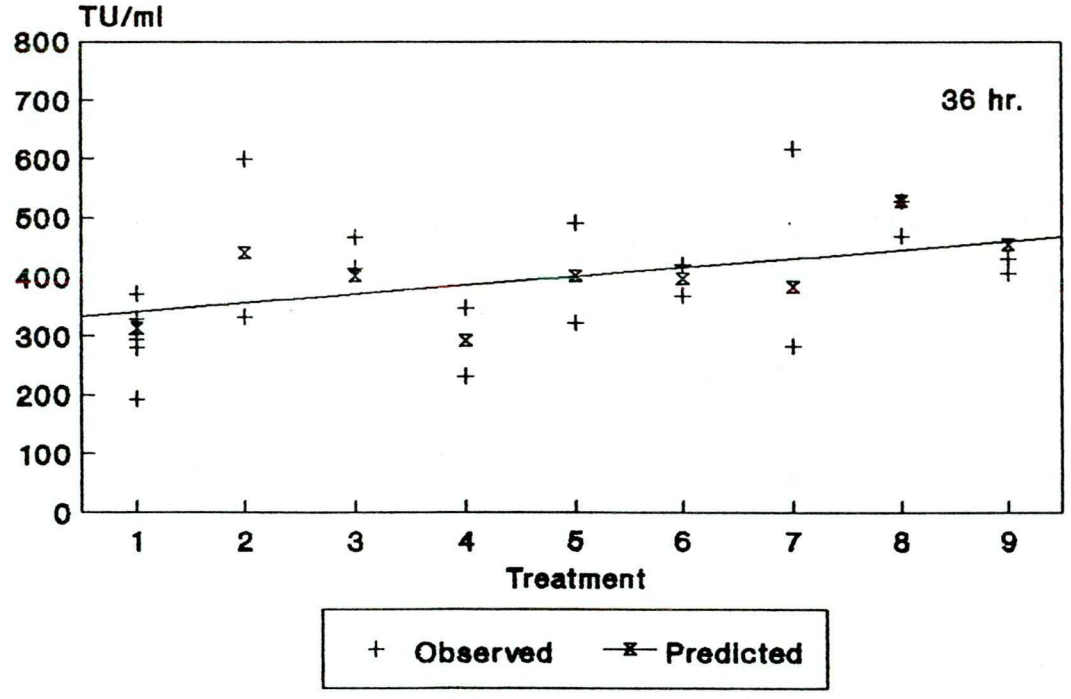
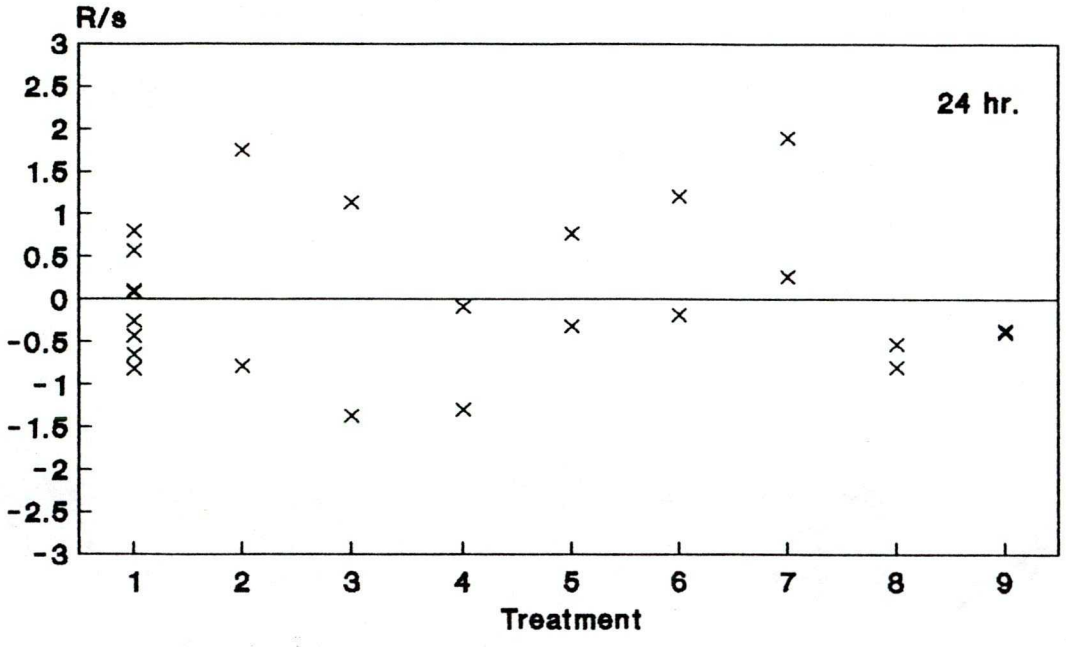


Fig. 17. Comparison of model and experimental observations (alkaline proteinase, HC5 strain).



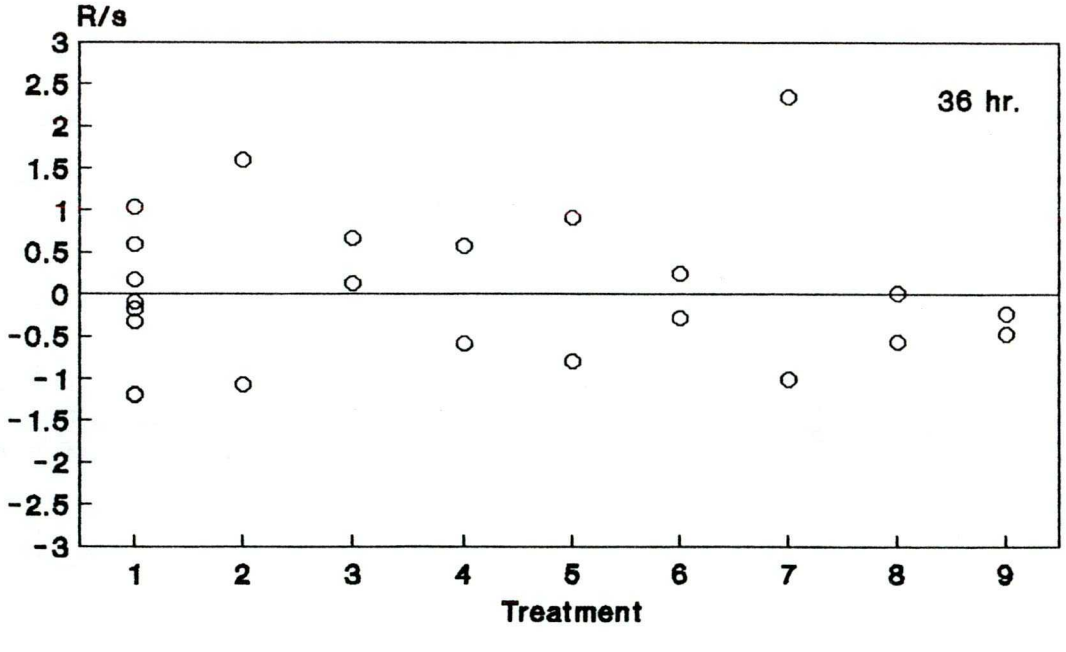


Fig. 18. Standardized reidual plot (alkaline proteinase, HC5 strain).

Although this result considered to be as a sub optimum of the process, it is quite enough for optimizing the formulation of culture medium as an exploratory experiment toward the fermentation processes as would be seen in the next studies.

2.2.3. KINETICS OF BATCH FERMENTATION

In recent years enzymes are of great importance with the development of biotechnology. Proteinase is one of the most widely used enzyme in industry and a lot of work have been done on its fermentation technology.

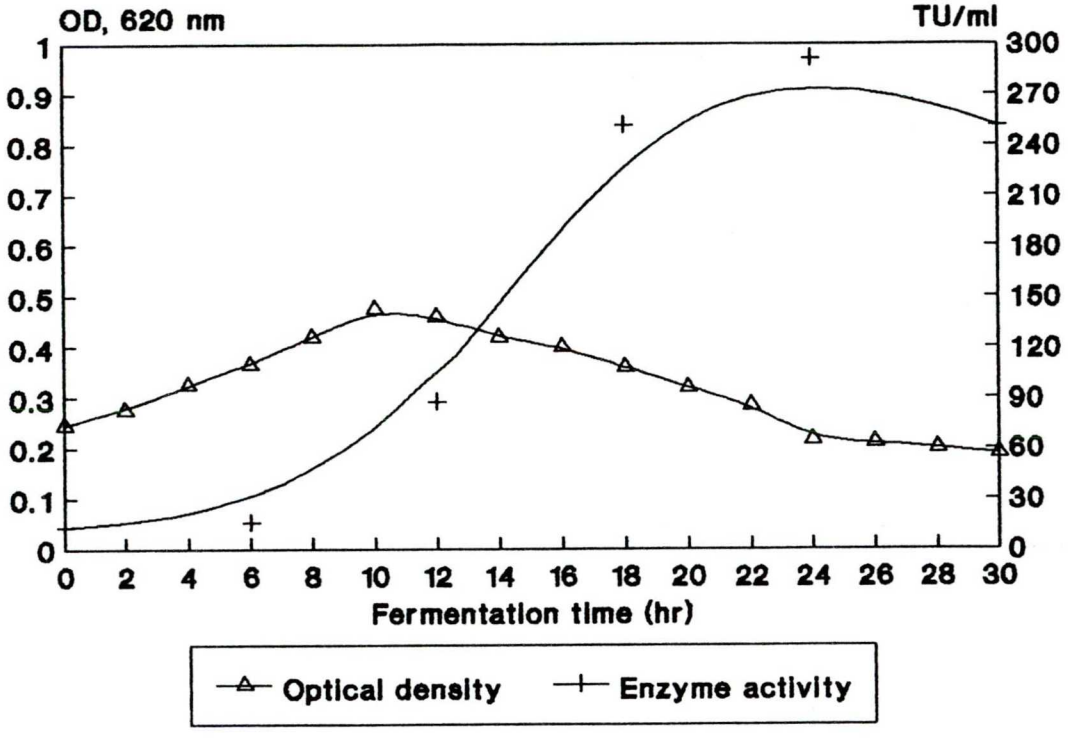
Applying the medium composition obtained from the preceding chapter, laboratory scale cultivation experiments in BIOSTAT M reactor were carried out to examine the time course of the growth and proteinase production. Batch fermentation without pH-control were first done, then controlled runs had taken place with the two tested strains.

2.2.3.1. BATCH FERMENTATION WITHOUT pH-CONTROL

Microbial growth (OD) and enzyme production are measured and the enzyme yield during the short period fermentation (30 h) were recorded. The maximum activity decreased after 24 h for ES2 strain, but in case HC5 was reached to 30 h as depicted in Fig. 19 & 20. Plotted data showed that in spite of using the same inoculum size in both cases, a different growth kinetic was observed since the kinetic of HC5 growth was in a higher speed than of ES2. As the nutrient is depleted or becomes sufficiently small and/or with the excessive toxic products sufficiently large, the rates of cells growth and division become smaller and the number of viable cells gradually decreases as shown in the same Figures after about 10 h. The same observation was found by Saidel (1968).

Although the enzyme activity hardly increased during the first stage of batch growth, proteolytic activity in the culture continued to increase linearly until and after the cessation of cell growth. The same results were also found by Emanuilova & Toda (1984).

It is evident from the Figures that, a rapid death of the organisms has take place, which would be the most logical explanation for the undershoot of population density and the overshoot of dissolved oxygen concentration (po_2). The same conclusion was obtained by Matsche & Andrews (1973). Al-Awadhi et al. (1988) stated that the culture enters the decline phase where the cell population decreased at a high rate indicating



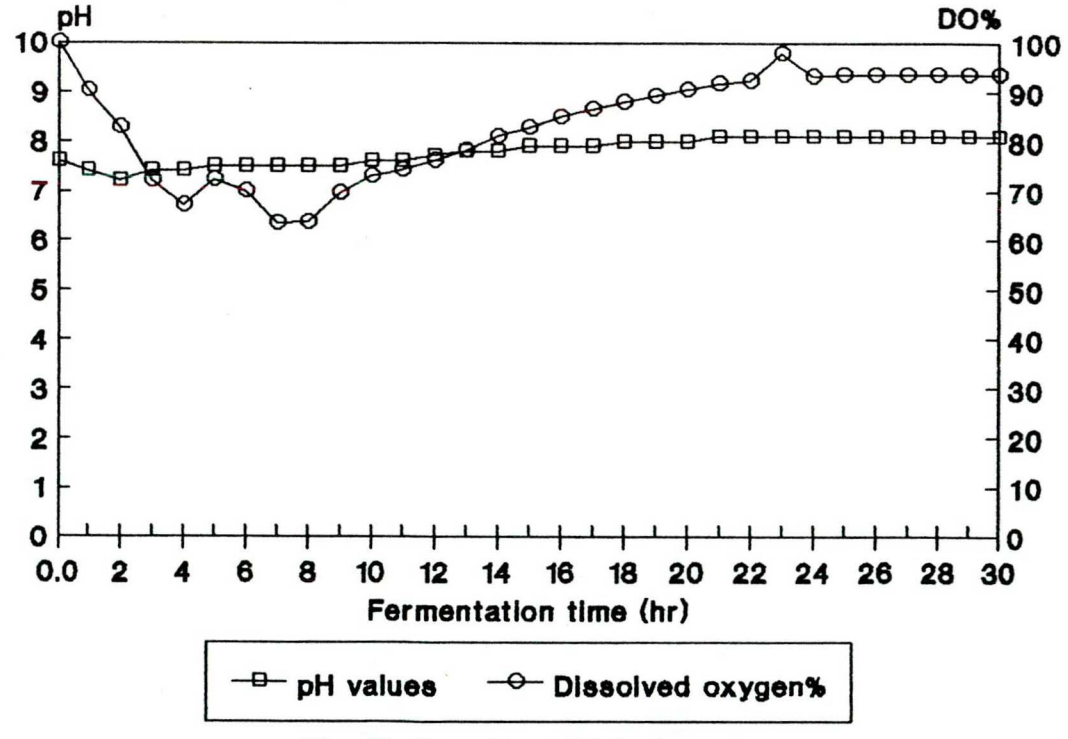
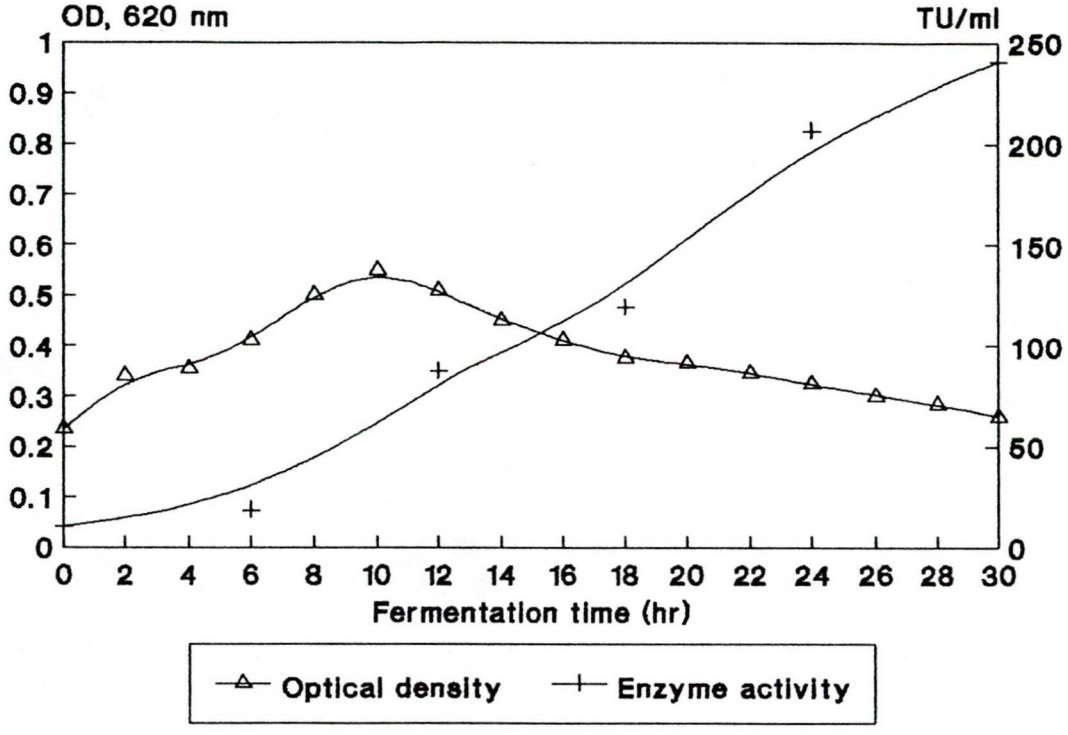


Fig. 19. Growth of ES2 strain in batch culture.



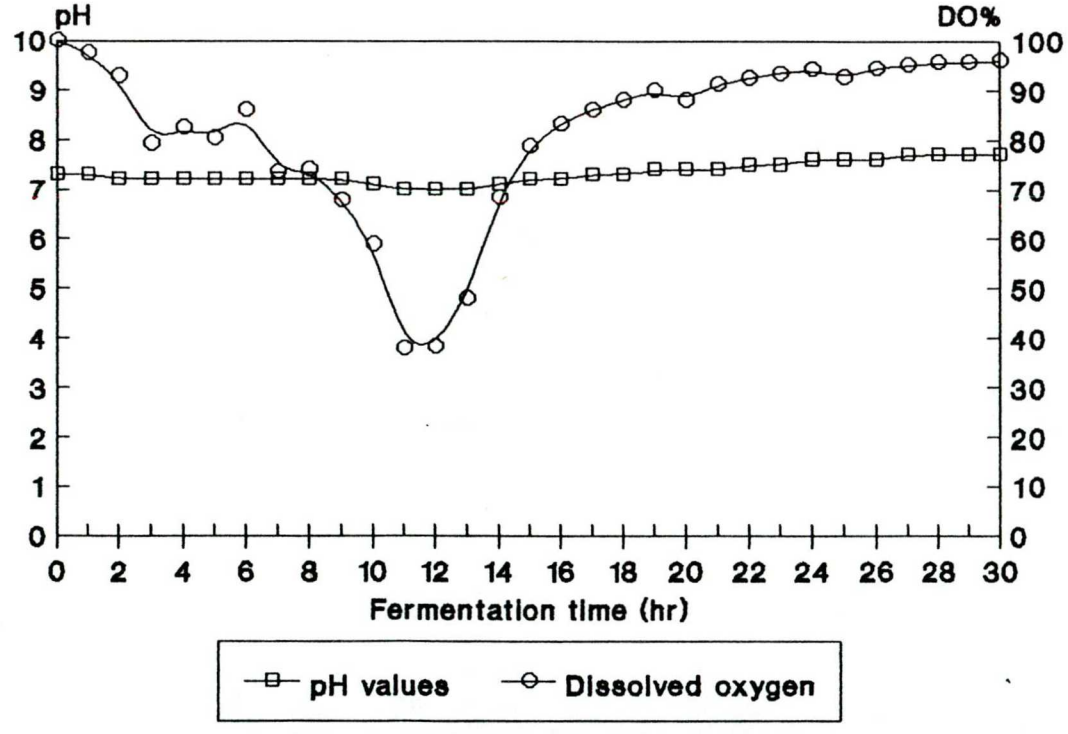


Fig. 20. Growth of HC5 strain in batch culture

simultaneous death and lysis as important features of carbone substrate starved batch culture.

The decline graph of the microbial growth can also be explained by the autolysis process suggested by Dixon and Webb (1964) who noted that if there is no enough energy supply, the whole system runs down by breakdown of the unstable essential substances and by the predominance of catabolic enzyme processes, which is leading to autolytic of the cell. However, when this process has gone sufficiently far, it becomes irreversible for the synthetic reaction can not be resumed in the absence of the essential catalysts, and this what is really meant by the death of the cell.

The reading of dissolved oxygen (PO_2) and pH values during the cultivation period were also followed. No significant changes in pH were observed during the fermentation in both cases as depicted in the same Figures for the two tested strains. A very little decline in pH was observed during the growth phase followed by an increase in its values near and beyond the termination of the growth phase. Initial pH of culture medium was decreased by o.1 to 0.2 pH unit during the first 12 h of cultivation, after that the increased by 0.6 unit was observed in case ES2 and 0.4 unit by HC5 strain in which the microorganisms going to its lowest density. These data could also proved the role of phosphate source as a buffering effect outside the cell beside its role inside the cell, as noticed by Yoon et al. (1989).

Regarding the dissolved oxygen concentration decreased based on the uptake of oxygen by the viable cells. A significant difference between ES2 and HC5 was found considering the (PO_2) as another growth monitor illustrated in the same Figures. The level of (pO_2) rapidly decreased during 7.5 h for ES2 being 40% saturation level, but to 65% saturation level by HC5 strain after 11.5 h was found.

2.2.3.2. BATCH FERMENTATION WITH pH CONTROL

In order to follow the microbial growth kinetics, some experiments were carried out with pH control (pH 7). The pH was automatically kept constant by adding 20% sulfuric acid.

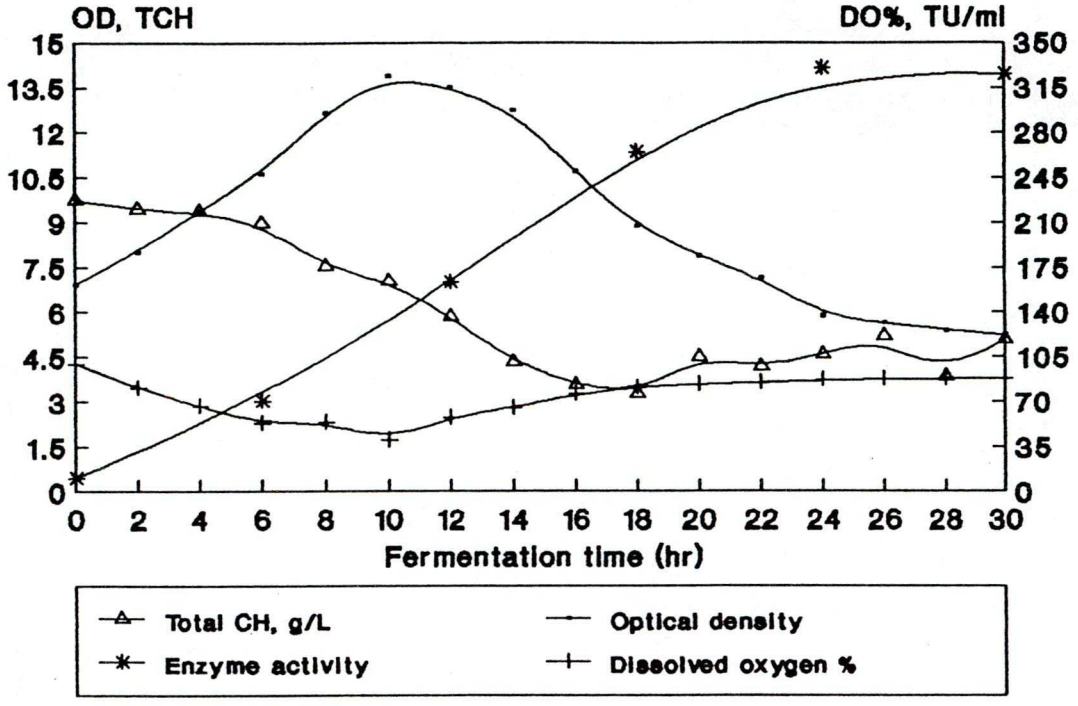
2.2.3.2.1. Growth Monitoring

From the fact that the population does not increase until the enzyme system of the microbe adapt to the new environment, the lag phase which depends on the character of the preceding medium and cultivation conditions has to be exist in the bacterial growth curve. In these experiments no lag phase was observed because of using the same culture medium in the same composition and physiological state in preparing the inoculum used, together with satisfactory inoculum size as well. Primrose (1987) was noted that a prolonged lag phase before growth commences should be found and also unduly prolonged fermentation period will be needed. Comparing to the results obtained in the preceding chapter, the growth phase was completed after about 8 h for both strain used.

The stationary phase can also be detected starting after 8 h until 14 h for ES2 strain, but HC5 showed little longer stationary phase from 8 h until 16 h of fermentation time. After that the decline phase was take place as shown in Fig. 21 & 22. The lowest dissolved oxygen tension about 40% saturation level was reached also at that time for ES2 and about 70% was observed by HC5 followed by a slow gradually increasing in (PO_2) and simultaneous decrease in the growth proved the correlation between the growth and oxygen consumption rate.

Bringing a large quantity of seed cultures into the fermenter maintaining the microbes concentration at a high level leads to considerably accelerate the consumption of carbohydrates with special reference to glucose. The concentration of glucose remaining in the culture was the balance between glucose production by hydrolysis of starch with microbial amylase and the glucose consumption by the cells. It is noteworthy also that whereas the total cell mass decreased after 12 h there was a remarkable further carbohydrate consumption indicated the energy requirement of the proceding enzyme synthesis and or excretion.

During that period it can be also seen that the enzyme production rate substantially increased and the continuation of the production has take place after the decline phase to its peak being 330 & 296 unit/ml culture filtrate after 24 h and 30 h for the two tested strains respectively. These data are in good agreement with the results obtained by Cantero (1990) and Moon and Parulekar (1991) who also observed that enzyme production was



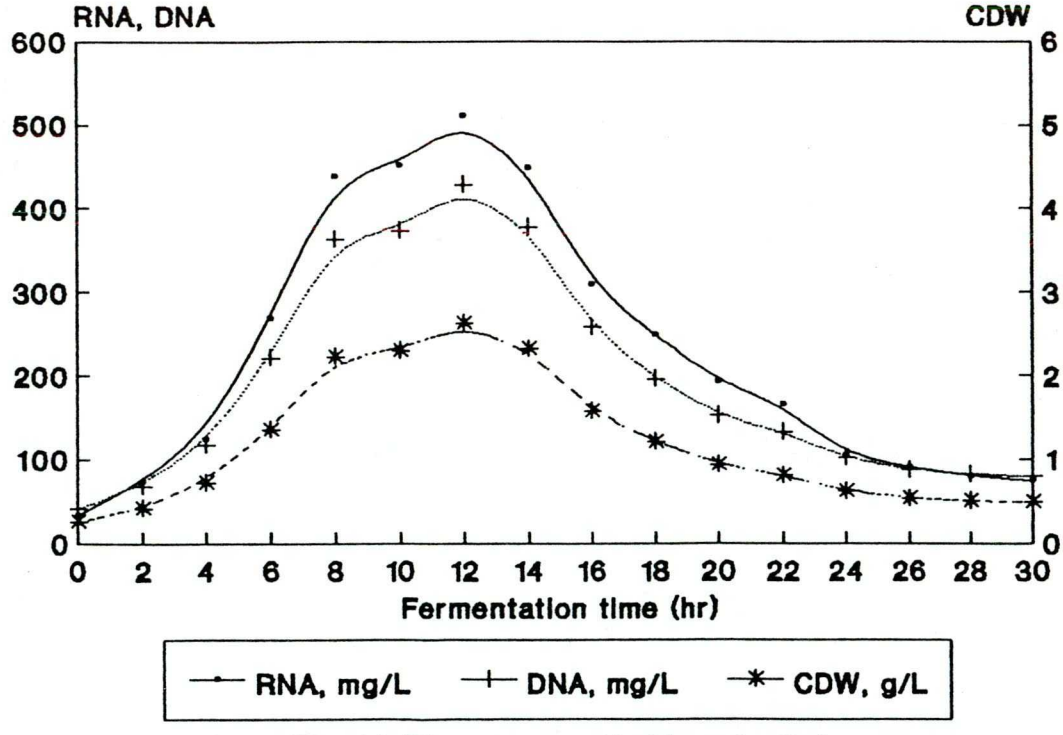
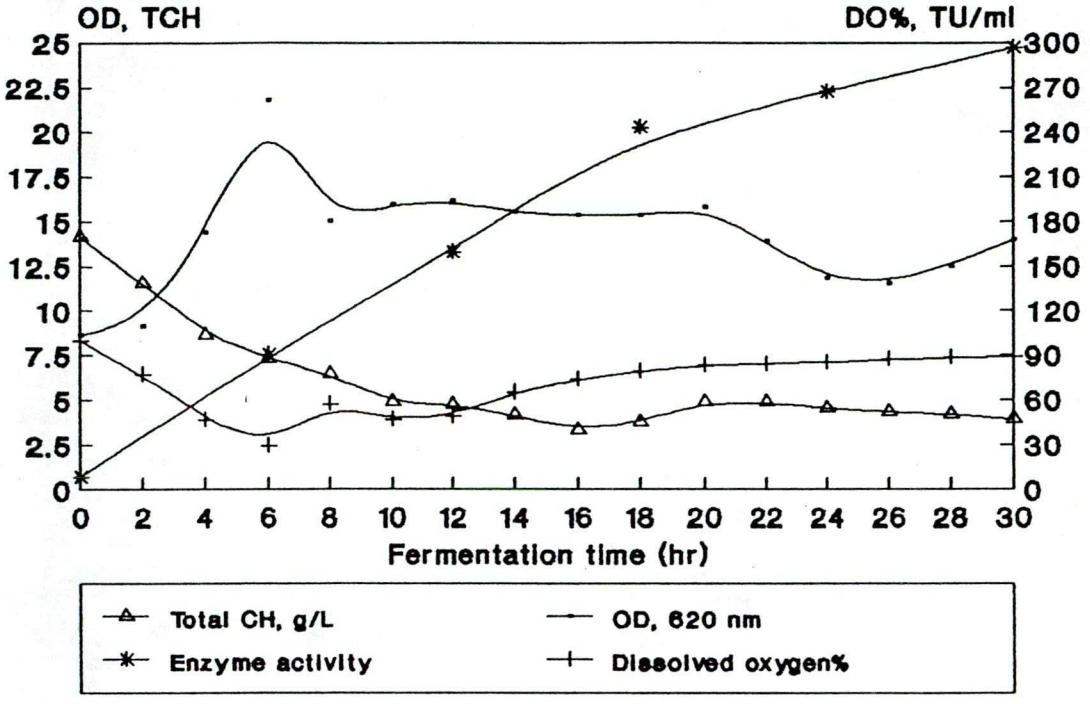


Fig. 21. Time course of pH-controlled batch fermentation of ES2 strain.



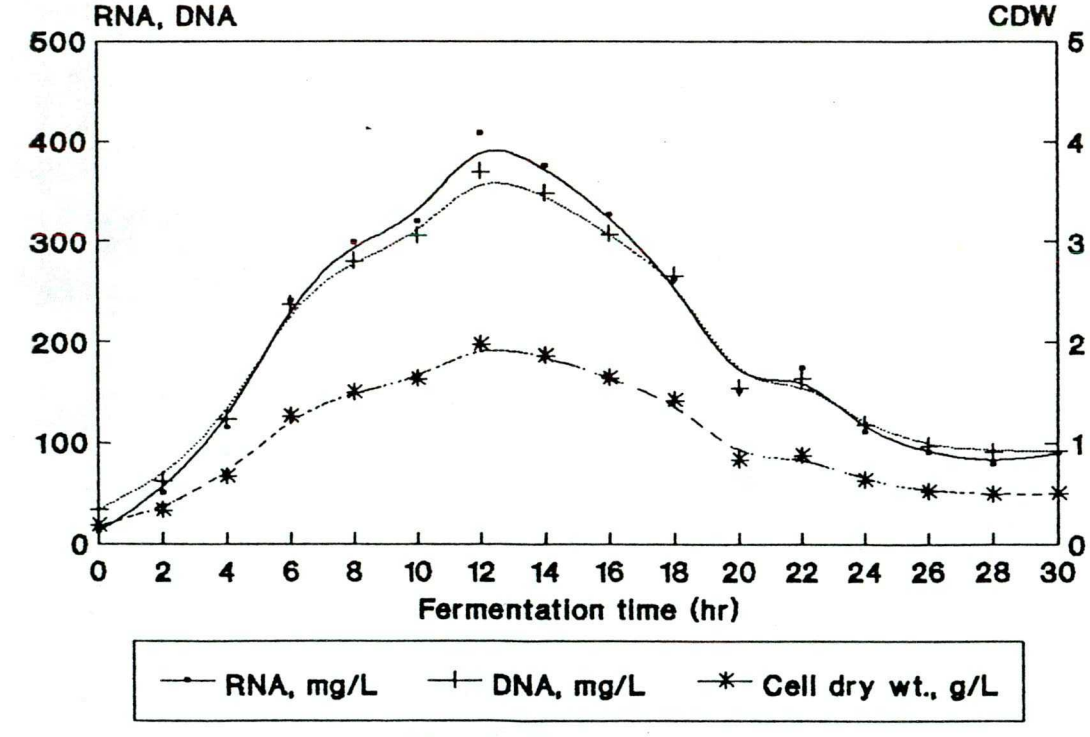


Fig. 22. Time course of batch fermentation of HC5 strain.

started in the growth phase but continued in the period of lysis and sporulation, too. Debette (1991) found in his studies using complex medium, that the production of proteinase occurred during the exponential growth and was the highest when the culture reached the stationary phase.

From the work of Sunna and Hashwa (1990) it could also be concluded that the formation of heat stable enzymes by obligate thermophiles have started in the early exponential phase, while maximal extra cellular enzyme activity was detected at the end of the decline phase. It is likely that the secretion of the proteinase belongs to the mixed-growth-associated category of fermentation pattern, noted by Wang et al. (1979) and Schugerl (1987).

From the data discussed so far, it could be concluded that enzyme fermentation likely considered as a two-step process, comprising a substrate accumulation phase, the period of which is dependent on the availability of substrate, and production phase which is substrate independent.

Due to the incomplete solubility of culture medium solid components, as well as the partial precipitation of some proteins during the sterilization process, there arose a question whether the optical density was a good measure of the cell growth. However, because of plate count or direct count methods are also laborious and have some doubt as well, a great attention was given to follow both disappearance of total carbohydrates from the medium and appearing some of the cellular materials. This idea is in accordance with the concept of Fredrickson et al. (1967) who considered that the cellular mass to be composed of two groups of substances that interact with each other and with substances in the environment to produce growth. The two groups of substances were depicted as being nucleic acids, indicative of the enzyme production capability of the cell, and protein, indicative of the enzyme concentration and also representative of the total cell mass.

From that point of view, Total nucleic acid content of the cellular materials was measured (g/l) as well as the cell dry weight (g/l) which indirectly measured to avoid the interference of medium protein with the cellular one. Data obtained are graphed in the same Fig. From the Figure, a rather good correlation was

found between the growth monitor OD, DNA, RNA, and cell dry weight either during the growth phase or during the postgrowth phase.

Typical results were obtained by Koliander et al. (1984) who found a linear relationship between net dry weight and the content of RNA measured as guanosine monophosphate. Data could also implies that former proves that optical density can be used even in this conditions of opaque media, using high dilution, while the latter shows that the decline phase in the growth curve after 12 h of fermentation was caused by the cell lysis and sporulation. These results, on the other hand, could also be confirmed by the data obtained by Millet (1970) who found more precisely correlation between spore formation and excretion of proteinase.

2.2.3.2.2. Dynamic of Population and Product Excretion

According to the model postulated by Saidel (1968) regarding the dynamic of cell population in relation to their chemical environment, if cells die because of the toxic products, the stationary phase no longer appears, however, if the cell die because of a lack of nutrient, the stationary phase is still present. Going back to the Figures (21, 22) taking into account the cell dry weight (CDW), the stationary phase will be obviously depicted. Fig. 21 shows short stationary phase begin after 8 h to 14 h with average of CDW about 2.3 g/l for ES2 strain while the long stationary phase was observed in case HC5 started after 8 h and continued to 16 h with CDW about 1.68 g/l as shown in Fig. 22.

On the other hand, the continuation of enzyme excretion after the decline of the bacterial growth curve could be explained by that there are at least two contributing factors for that phenomenon; firstly, substrate released from active cells as a results of autolytic processes; secondly, biochemical end products released from active cells as a results of partial metabolisms of the substrate. So that the contribution of exogenous substrates from metabolizing by *Bacillus* sp can not be considered negligible in development of the culture kinetics as decided by Martin et al., (1966).

2.2.3.2.3. Kinetic Parameters

The economic of a fermentation is greatly affected by the amount of substrates and dissolved oxygen needed to produce the required amount of cell and product as well. For complete view of cell growth and substrate utilization, it is necessary to know the relation between the cell growth, product formation rate and utilized substrate. This relations are expressed and summarized in Table 8.

The yield may not be as high as a typical for a mesophiles, but it has been speculated that thermophiles in general have lower growth efficiency owing to higher maintenance requirements at elevated temperature as reported by Sonnleitner et al. (1982), Sundaram (1986) and AEvarsson et al. (1991).

Table 8. Some kinetics parameters of bacterial growth.

Parameter		ES2	HC5
r_x	g/l h	1.905	1.316
r_s	g/l h	3.51	3.930
Eff.Y.	g/g	0.543	0.335
Eco.Y.	g/g	0.196	0.093
dE/dX	U/g CDW	73.100	96.365
dE/dS	U/g Glu.	29.719	26.389
μ_x	h^{-1}	0.142	0.125
μ_p	h^{-1}	9.210	8.656
μ_s	h^{-1}	0.262	0.372
t_d	h	4.880	5.540

2.2.4. GROWTH BEHAVIOR IN CONTINUOUS FERMENTATION

Growth and product formation in batch culture is a process that always terminates after some finite and relatively short time (30 h). As described in the previous chapter, all the features of the batch culture are function of time when the amount and the physiological state of the inoculum, the composition of starting medium and the physical conditions of cultivation do not change, the amount of a given product is characterized by the duration of fermentation.

In the continuous fermentation, on the other hand, the time factor is completely left out since the culture obviously develops in time but its properties are not a function of time. In a trial to apply the continuous-flow cultivation using a complex culture medium (starch & soybean meal containing medium), a single stage, non-chemostate system of continuous fermentation experiments were undertaken. In this kind of media (growth limiting unknown media) it difficult to clearly state the nature of the limiting component of the medium.

These experiments were carried out in order to examine how the microorganisms behaves, as well as, how much the end products will be comparing with what already obtained in batch system. From the fact, in continuous fermentation, that the rate of feeding (energy, carbon, and nitrogen) should be adjusted to provide for the growth requirements of organisms without maintaining any excess contents in the medium and substrate concentration is also maintained as close to zero as possible to prevent instability resulting from substrate inhibition of growth, the dilution rates were varied by step up change in the range of 0.1-0.3 hr^{-1} and the temperature was kept constant at 50°C with all the cultivation conditions previously mentioned.

2.2.4.1. DYNAMIC OF CELL POPULATION

It is well known that the growth rate has a strong influence on the macromolecular composition of bacterial cells, since the content of nucleic acids mainly RNA, depends on the growth phase and cultivation conditions. The bacterial growth rate is also greatly affected by the culture medium composition and especially

by the growth limiting substances in the dynamic cultivation.

In the Figures 26 & 30, it could be seen little weaving in the values of CDW measurements correlated side by side with the values of both RNA and DNA. This finding could bring out that the weaving in growth behavior may be owing to the different limiting growth materials containing culture medium.

Generally, the content of RNA increase greatly at increasing growth rate, whereas the DNA contents of the cells show little decline under these circumstances. The same finding was also clearly found between DNA and RNA during the course of batch fermentation in the preceding chapter (Maaloe & Kjeldgaard, 1966, and Stouthamer & Bettenhaussen, 1973).

It has commonly been assumed that, the woven graph showed in Fig. 26 & 30 may be happen and normal because of no using synchronized seed cultures, consequently asynchronously dividing should be happen. This may leads to that all the individuals in a population are not equivalent because of the growth at a certain individual rate which could be happen even in a genetically homogeneous population (Fencl et al., 1966). These weaving may also attributed to little changes in the flow rate, because of the technical dificulties of operating the system by solid components culture medium which are expected to cause the system to continue to drift from the original position to a new one.

Maruyama & Hayashi (1966) and Fencl and Novak(1969). have also explained the weaving of the growth behavior in continuous cultivation on the basis of DNA replication by the age of DNA strand between the mother and daughter cells. Furthermore, the changes in the cell wall has also a un-negligible effect in this phenomenon.

2.2.4.2. STEADY STATE EXAMINATION

The steady state which characteristic for a growth rate, demonstrates that a constant population level maintains a constant yield of product while consuming a constant amount of substrate per unit time. During these experiments, the continuous flow unit was allowed to reach steady state (not really typical) over a time period equal to at least three turnover volumes of the fermentor at a number of dilution rate used.

The steady state was attained after more than three cycles of mean liquid residence time which initially established on the basis of constant culture turbidity and the concentration of oxygen response monitor and later verified by determination of the nucleic acids of cellular materials, cell dry weight and the remaining total carbohydrates as well.

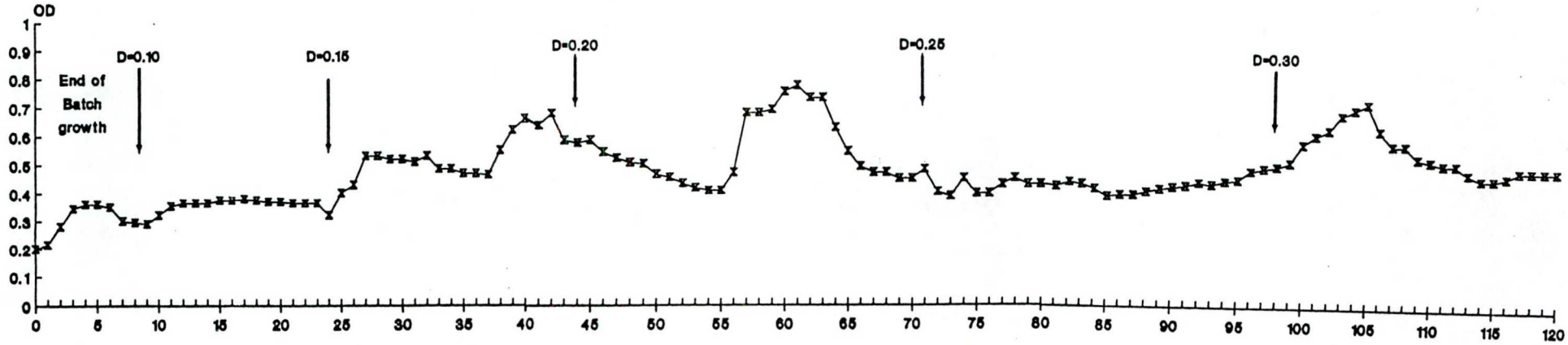
It is important to note here also that the microorganisms showed a significant response with the dilution rate change, since the biomass subsequently decrease or increase again to the previous steady state value, presented in their behavior which existing considerable lag phase for each dilution rate applied as can be seen in behavior of population density as shown in Figures 23 & 27. From these two Figures, it could be seen that HC5 strain showed little interruption in its behavior (Fig. 27) than ES2 strain (Fig. 23).

Regarding the profile of dissolved oxygen concentration, a high rate of interruption can be noticed with HC5 strain (Fig. 28) in spite of keeping constant of dilution rate, than what appeared by ES2 strain which showed very little hesitation especially in the case of low dilution rate (Fig. 24) .

The smooth transition showed by microorganisms from one steady state to another may due be to either the physiological readjustment to the new concentration of incoming medium, or to the extra time required for the synthesis of enzymes for the increased metabolic activity associated with a step up process. Data are in good line with those obtained by Matsche & Andrews (1973) using a thermophilic Bacillus sp isolated from soil, and Topiwala & Sinclair (1971) using Aerobacter aerogenes.

The complex and anomalous behavior of cell during the growth on undefined complex medium has been studied by Wang et al. (1979) who noted that it is difficult if not impossible to clearly state the nature of the limiting component of the medium which considering the main reason for this disturbances and the transition periods.

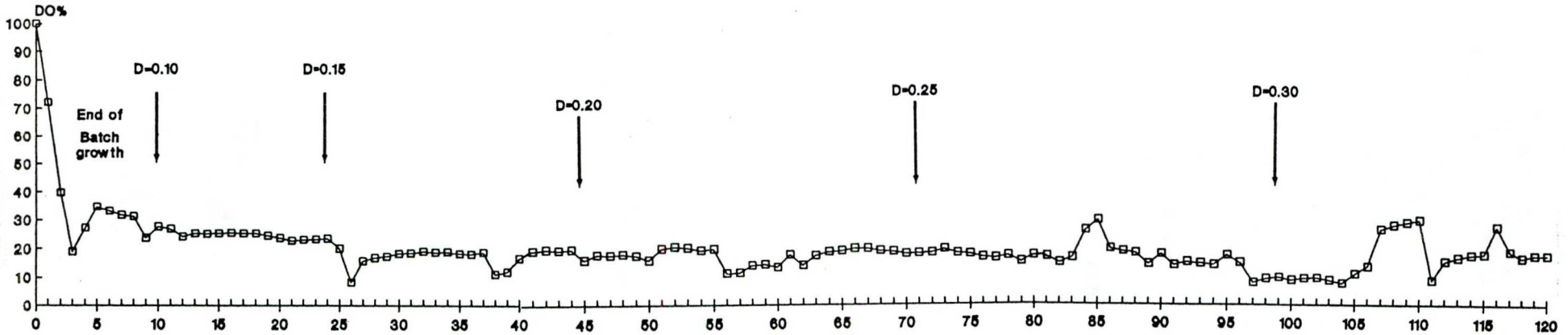
Because of the microorganisms are the most versatile form of life that they can adapt to a wide variety of environmental conditions implies that this may be correlates to RNA synthesizing capacity which explained the transition periods by the concept of Cooney et al. (1976) who pointed out that this adaptation is



Fermentation time (hr)

Fig. 23. Growth behavior of population density

in continuous fermentation of ES2 strain.



Fermentation time (hr)

Fig. 24. Dissolved oxygen tension during growth of ES2 strain in continuous cultivation.

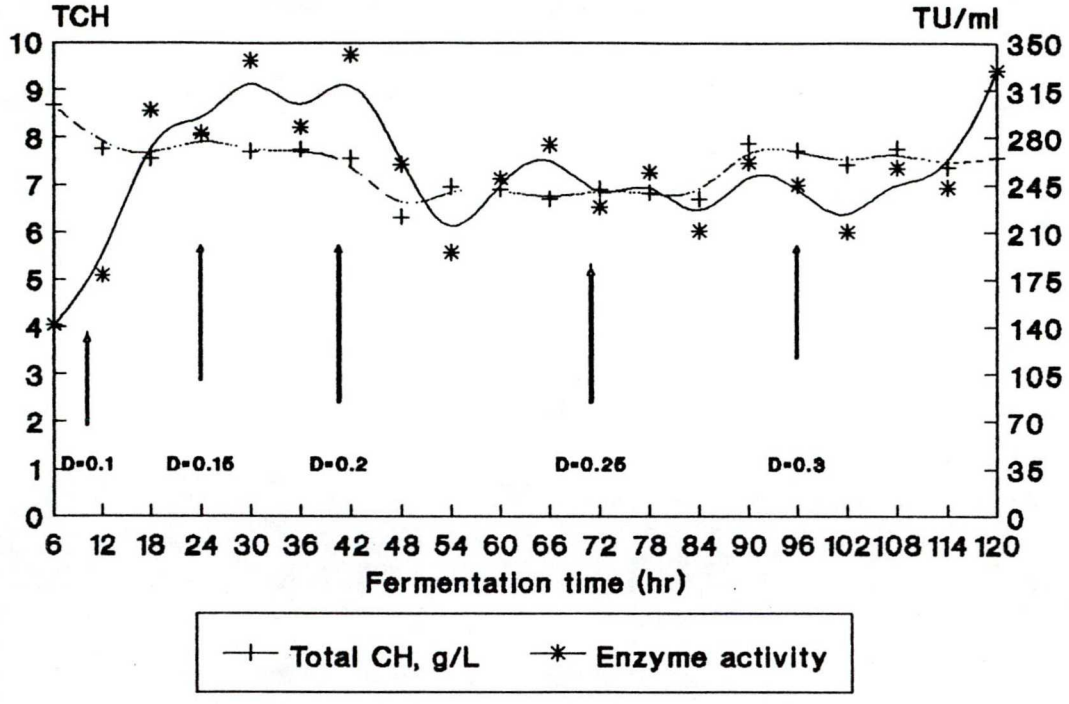


Fig. 25. Substrate concentration and enzyme activity of ES2 strain in continuous cultivation.

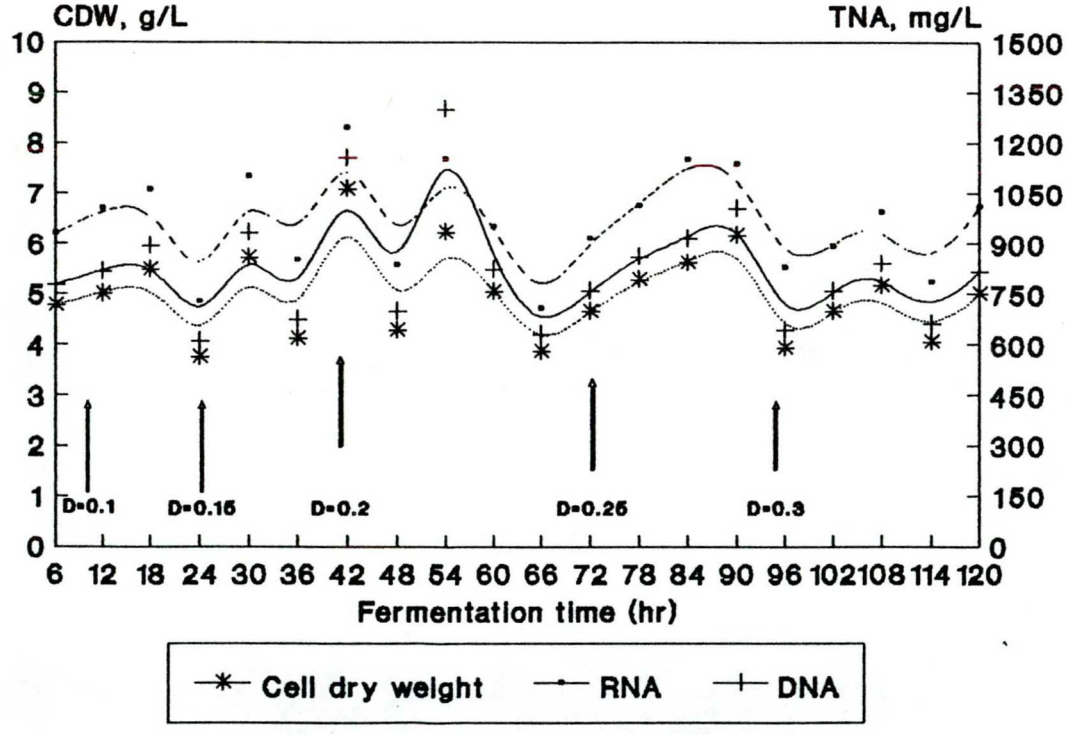


Fig. 26. Microbial growth monitoring in continuous cultivation of ES2.

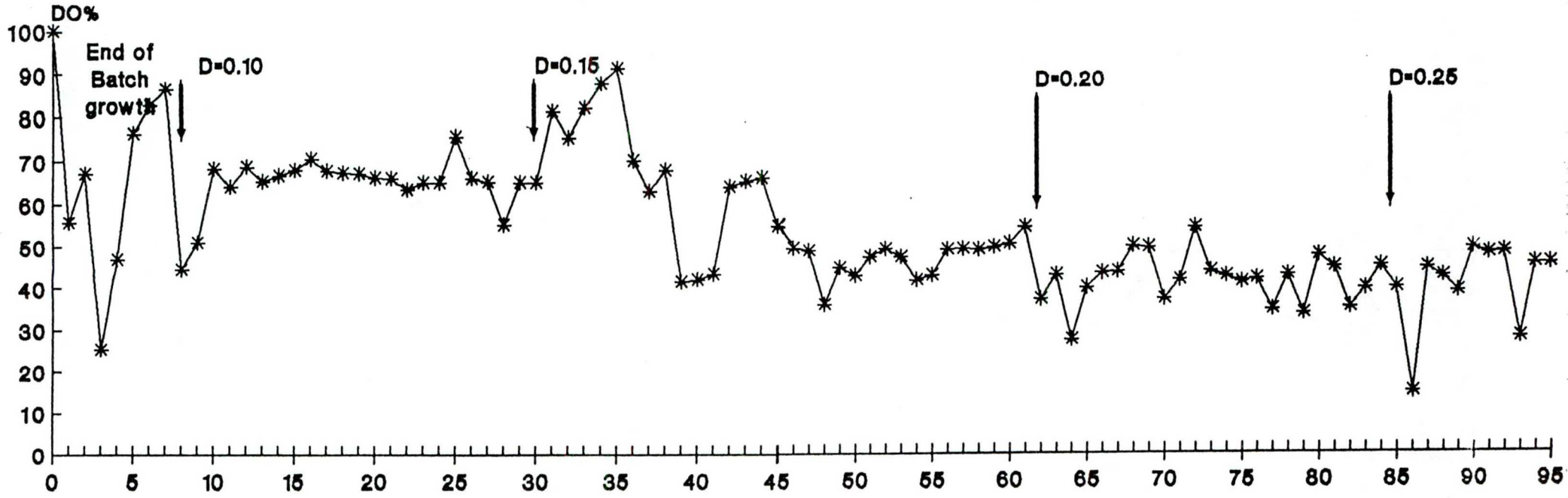


Fig. 27. Growth behavior of HC5 strain.

during continuous cultivation.

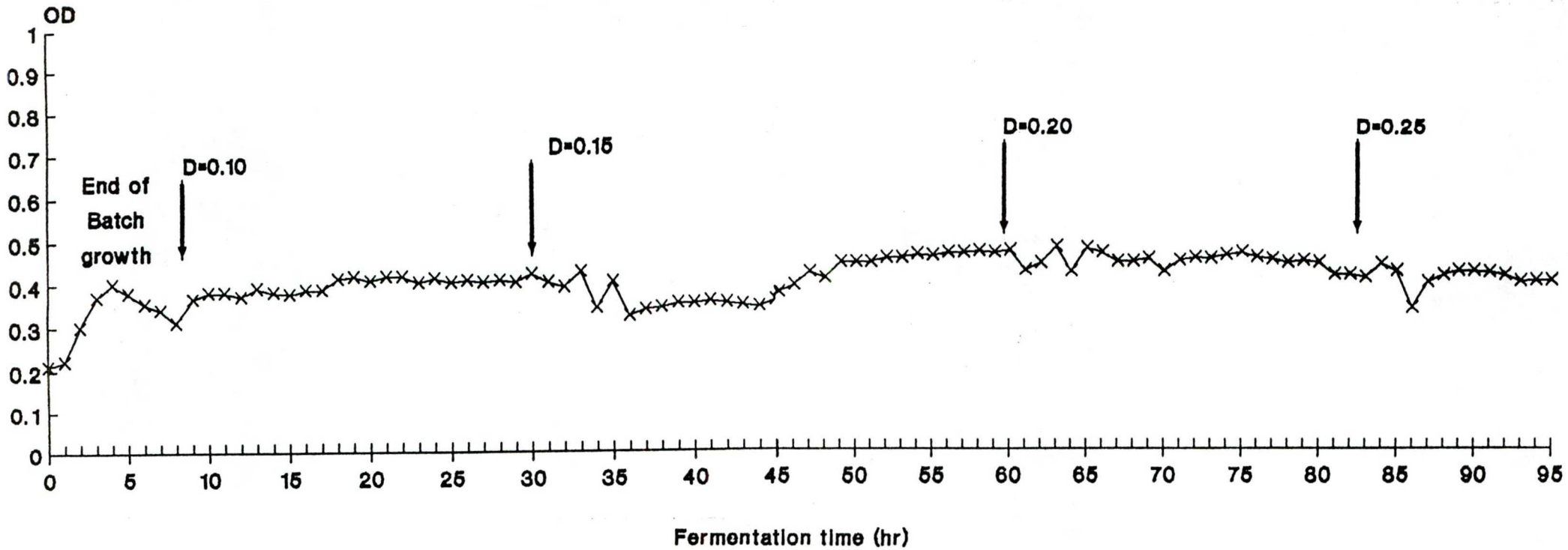


Fig. 28. Profile of dissolved oxygen concentration during continuous fermentation of HC5 strain.

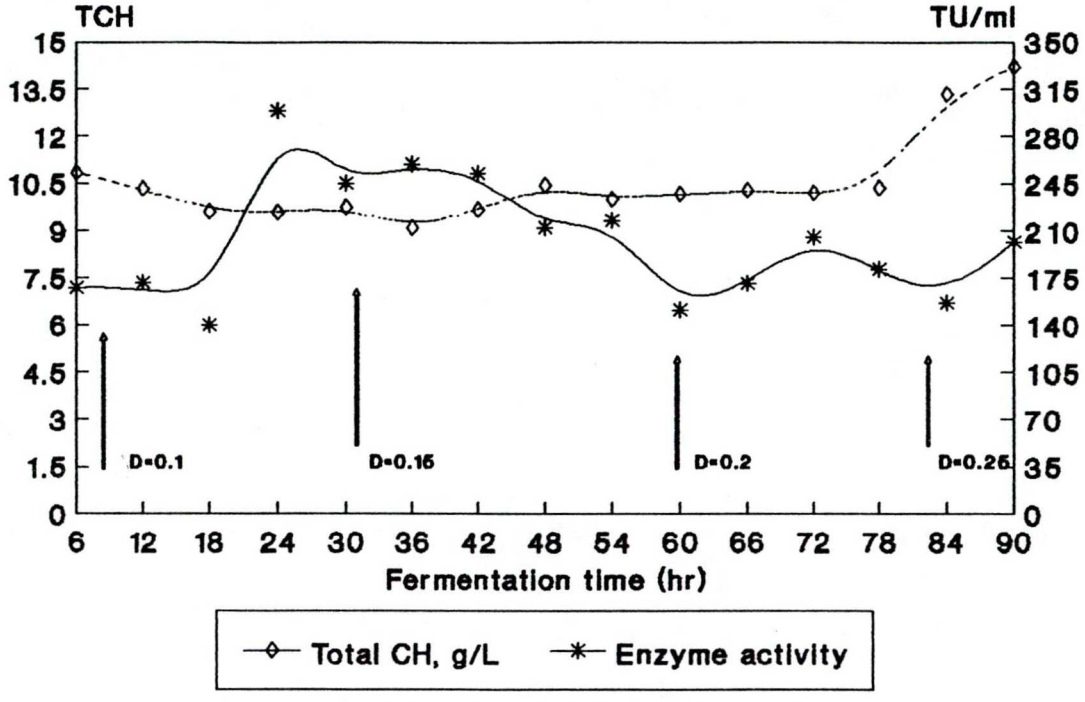


Fig. 29. Total carbohydrate and enzyme concentration in continuous fermentation of HC5.

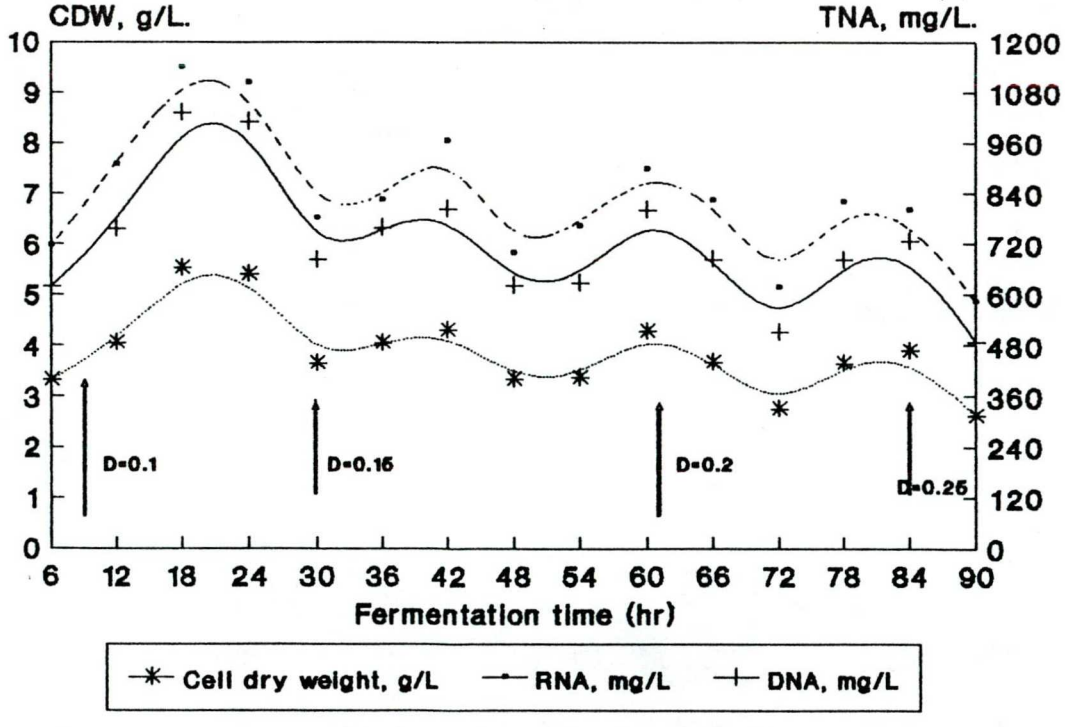


Fig. 30. Growth of HC5 strain during continuous fermentation.

accomplished by the reorganization of their macromolecular structure, induction and or repression of enzyme system, and reallocation of material in the cellular metabolic pools.

2.2.4.3. EVALUATION OF FERMENTATION

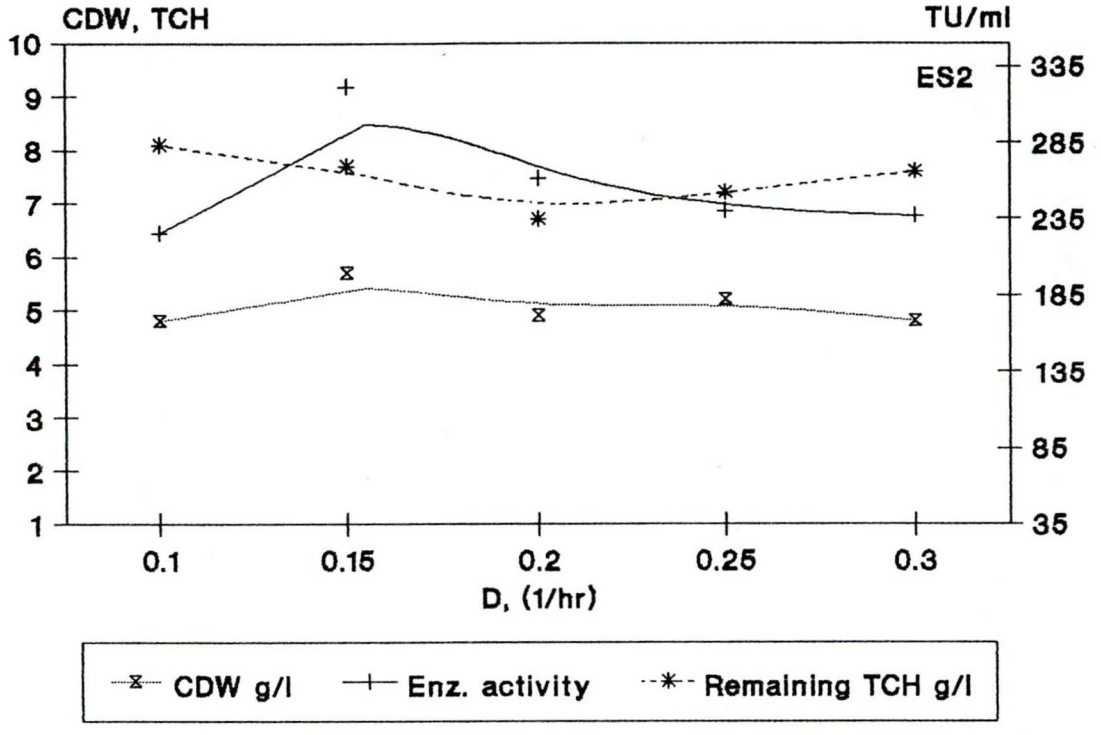
Consequently, the enzyme production was also affected owing to the above mentioned phenomena. The total carbohydrates (TCH) concentrations were plotted together with the enzyme concentration produced as can be seen in Figures 25 & 29. Data showed that the TCH seems to be constant, but woven graph regarding the enzyme activity was found in both cases. The cell dry weight was found in continuous process higher than those obtained in batch cultivation which in agreement with some of previous finding of Fencl (1966).

Fig. 31 showed the steady state under the circumstances mentioned above. The graphical plot illustrates the changes of cell dry wight, enzyme activity produced and remaining total carbohydrates in dependence on the dilution rate. Data could also proved the previous suggestion that proteinase fermentation is not growth associated pattern since significant increasing in cell mass was obtained, comparing batch fermentation with the continuous one, but the enzyme production more or less considered to be the same in both cases.

The measured values of proteinase activity are collected in Table 9 together with some growth parameters of the continuous cultivation. However, the important criteria in comparing batch and continuous cultivation can be defined and examined on an individual basis; the conversion yield, volumetric productivity, as well as the specific productivity which considered the greatest parameter in the evaluation of the fermentation process.

The volumetric productivity (DP & DX) were calculated and plotted as a function of the dilution rate as depicted in Fig. 32. The ratio of enzyme yield to increase in cell mass indicates that dilution rate 0.15 was the optimum for both strains used since higher values of (DP/DX) were calculated to be 56.12 and 62.37 U/ml/h for ES2 & HC5, respectively. A positively correlated was also obtained by plotting the specific productivity against the dilution rate as can be seen in Fig. 33.

Finally, from the results discussed so far, it could be concluded the possibility of applying the continuous-flow



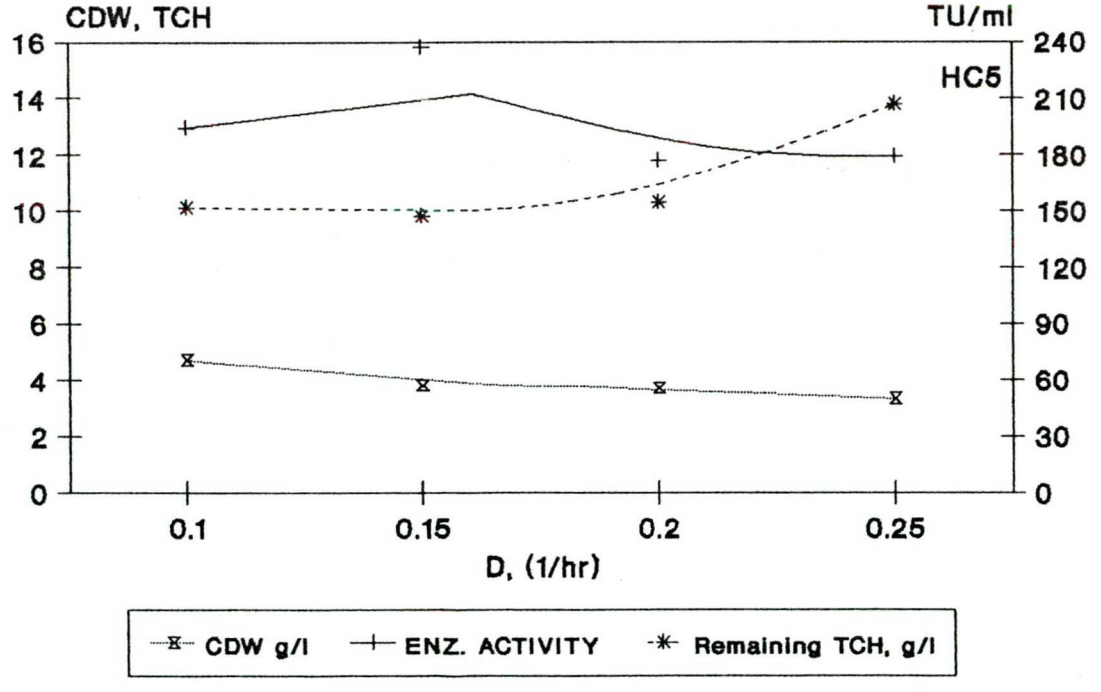
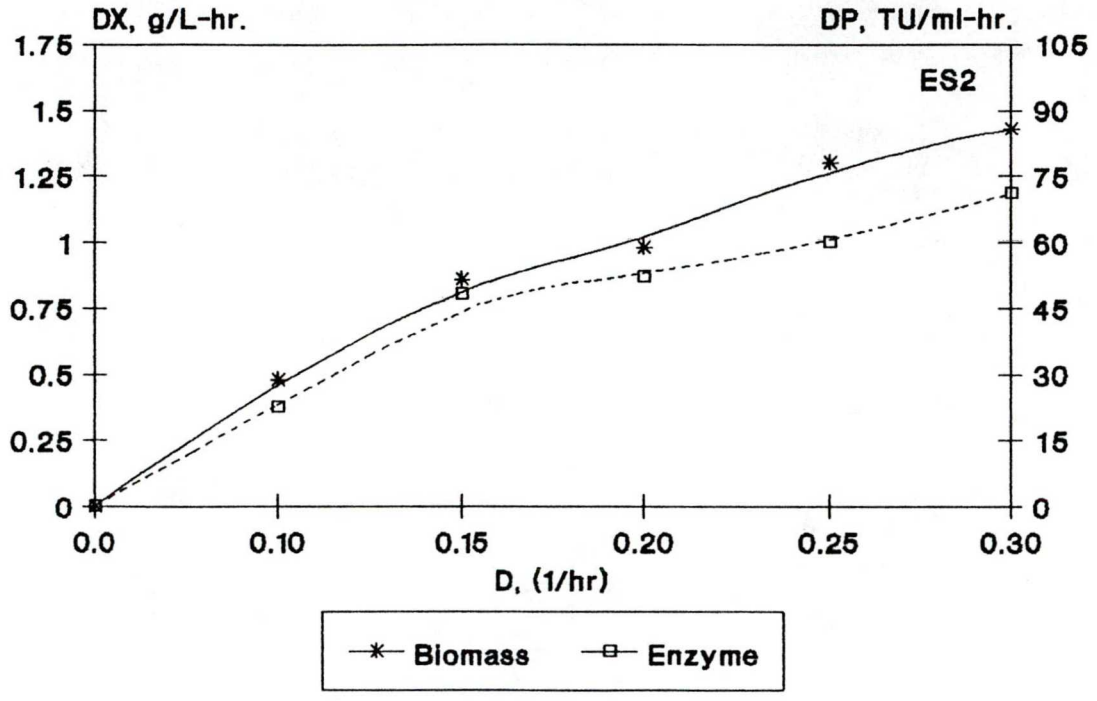


Fig. 31. Effect of D, on CDW, TCH and enzyme concentration in continuous fermentation.

Table 9. Some growth parameters of continuous fermentation.

str.	D.	X.	P.	DX.	DP.	DP/DX	DP/X
	0.10	4.78	225.6	0.48	22.56	47.00	4.72
	0.15	5.70	321.7	0.86	48.26	56.12	8.47
ES2	0.20	4.90	261.4	0.98	52.27	53.34	10.67
	0.25	5.18	239.9	1.30	59.97	46.13	11.58
	0.30	4.78	236.8	1.43	71.03	49.67	14.86
	0.10	4.65	194.15	0.47	19.42	41.32	4.176
	0.15	3.80	236.98	0.57	35.55	62.37	9.355
HC5	0.20	3.65	176.90	0.73	35.37	48.45	9.690
	0.25	3.30	178.90	0.83	44.73	53.89	13.555



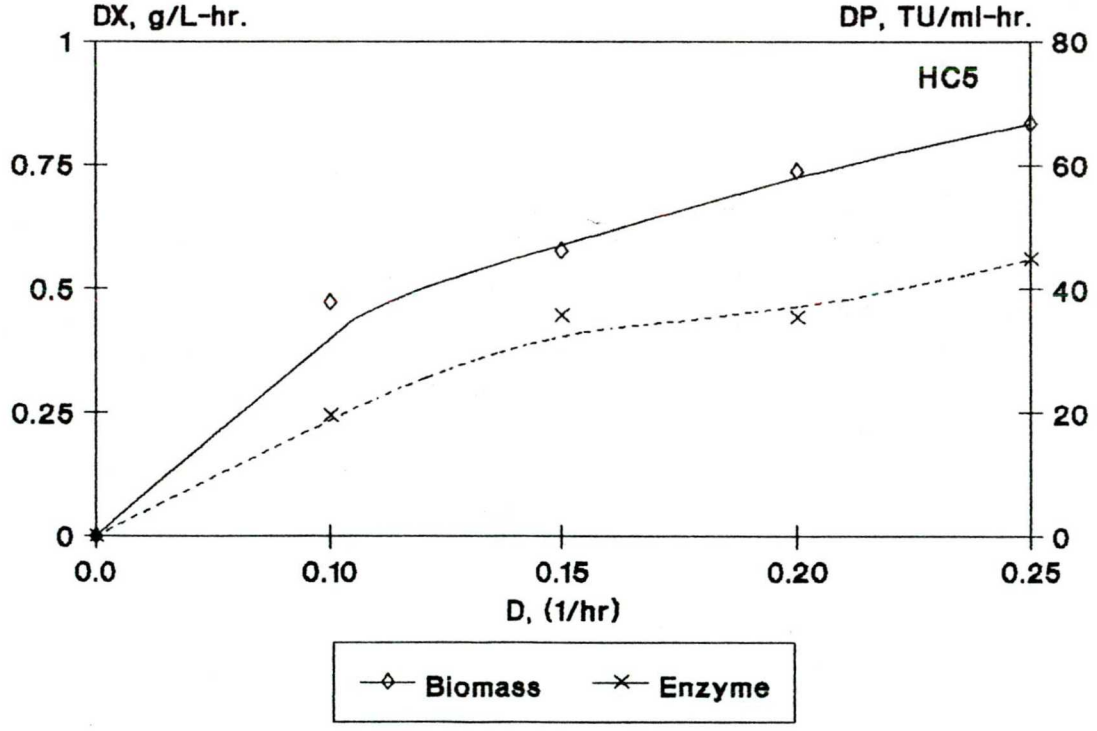
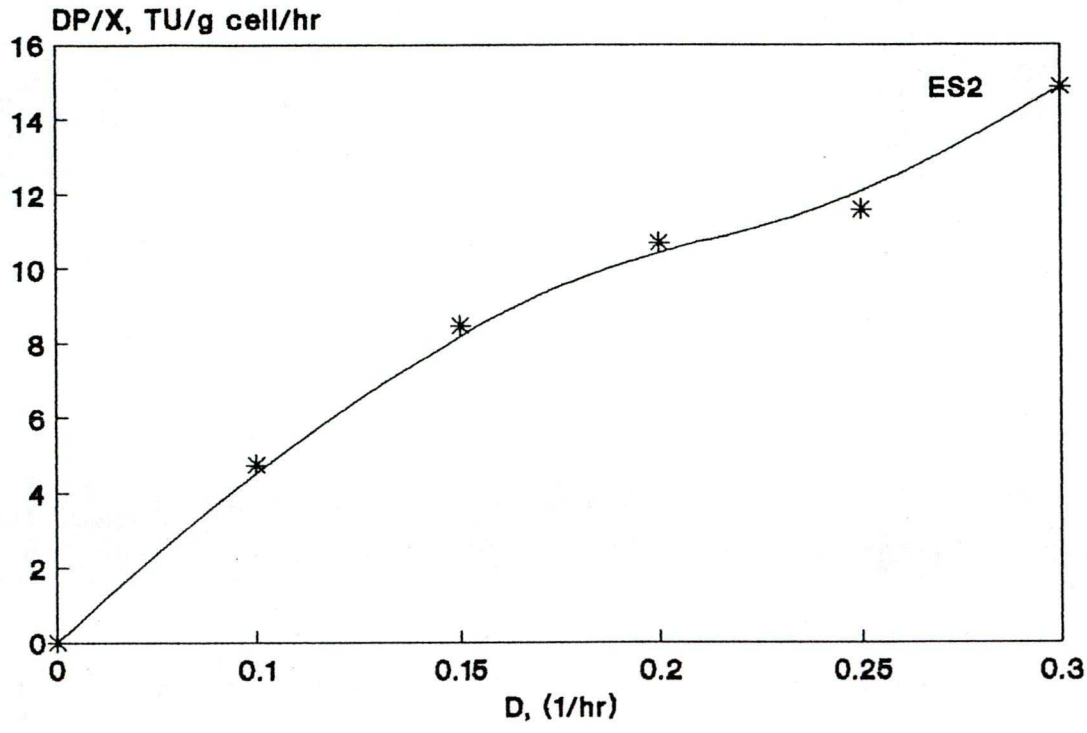


Fig. 32. Volumetric productivity as a function of the dilution rate.



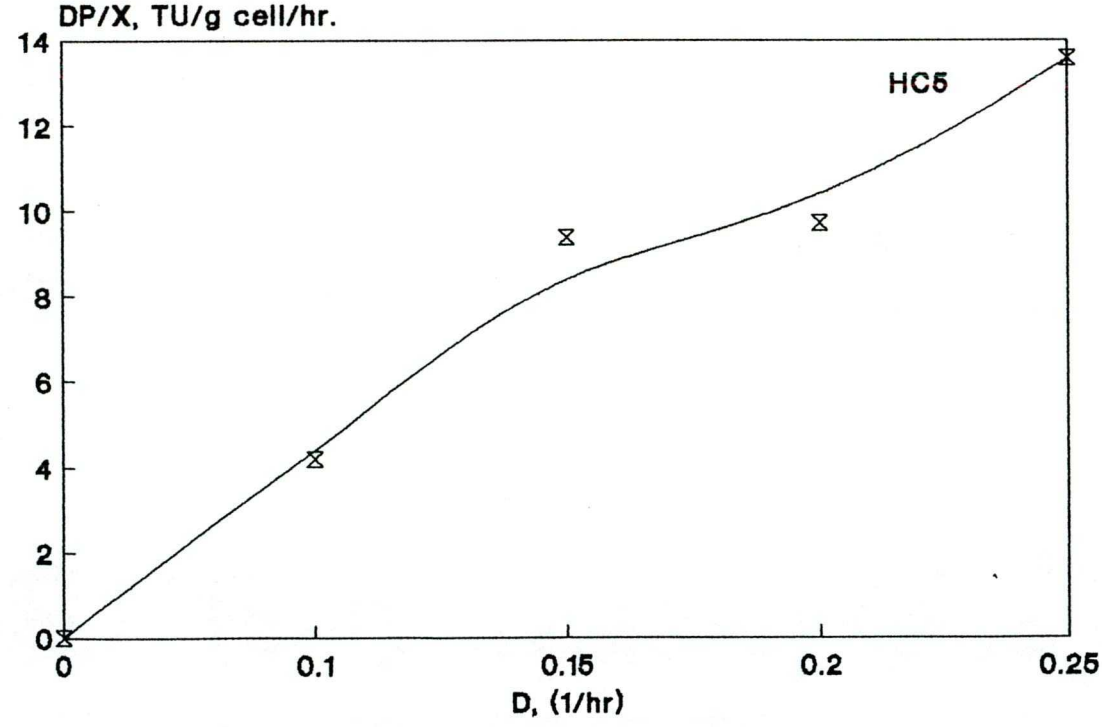


Fig. 33. Specific productivity as a function of dilution rate.

cultivation using the complex culture media under high temperature taking into account the technical difficulties during applying the process. On the other hand, however, it could bring out that, these data offered the guidelines by which further experiments and research work could be followed and carried out.

2.2.5. KINETIC OF ENZYMATIC CASEINOLYSIS

2.2.5.1. PROPERTIES OF CRUDE ENZYME

The characteristic properties and function of enzymes is the catalysis of chemical reactions. It is well known that biological system, particularly living cells, are more sensitive to changes of temperature, pH, etc., than most non biological chemical reactions, this is due largely to the properties of the enzymes on which these systems depends. Some experiments were performed to give a picture for the kinetic behavior of desired enzyme.

2.2.5.1.1. Velocity of Enzyme Reaction

The progress curves of thermostable alkaline proteinase produced in this work were measured. Data showed that the velocity falls with time which gave general form of the progress curves as shown in Fig. 34. Various causes may contribute to this falling off. For instance, the products of the reaction may inhibit the enzyme, the degree of saturation of the enzyme with substrate may fall because of the fall in substrate concentration as the reaction proceeds.

For that mentioned reasons, the progress curves of enzymes reaction commonly do not fit the standard chemical reactions. Positive conclusion in this respect was also came by Dixon and Webb (1964).

2.2.5.1.2. Effect of pH on Enzyme Reaction Rate

Since enzymes are proteins containing many ionizable groups, the effect of pH on enzymes is due to changes in the different states of ionization of the components of the system. The enzyme reaction mixture subsequently may undergo such changes, either the free enzyme, substrate, and the [E-S] complex, as explained by Bailey and Ollis (1986).

Generally, enzymes are active only in a limited range of pH and in these experiments a definite optimum pH is observed. In order to examine the effect of pH on the enzyme reaction, some experiments with wide range of pH (7-10.5) were carried out. The graphed data showed that pH 9.5 was the optimum for the two tested strains indicated the alkaline range required for the enzyme reaction as can be seen in Fig. 35. Experimental data showed also

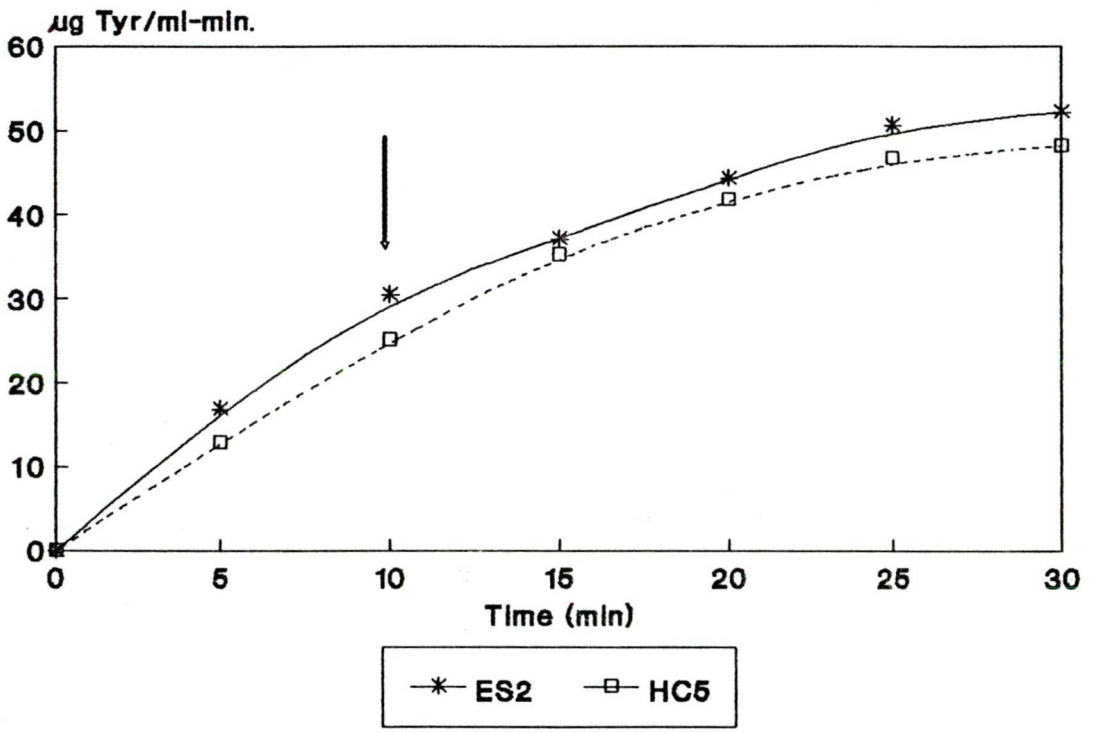


Fig. 34. Time course of enzyme reaction.

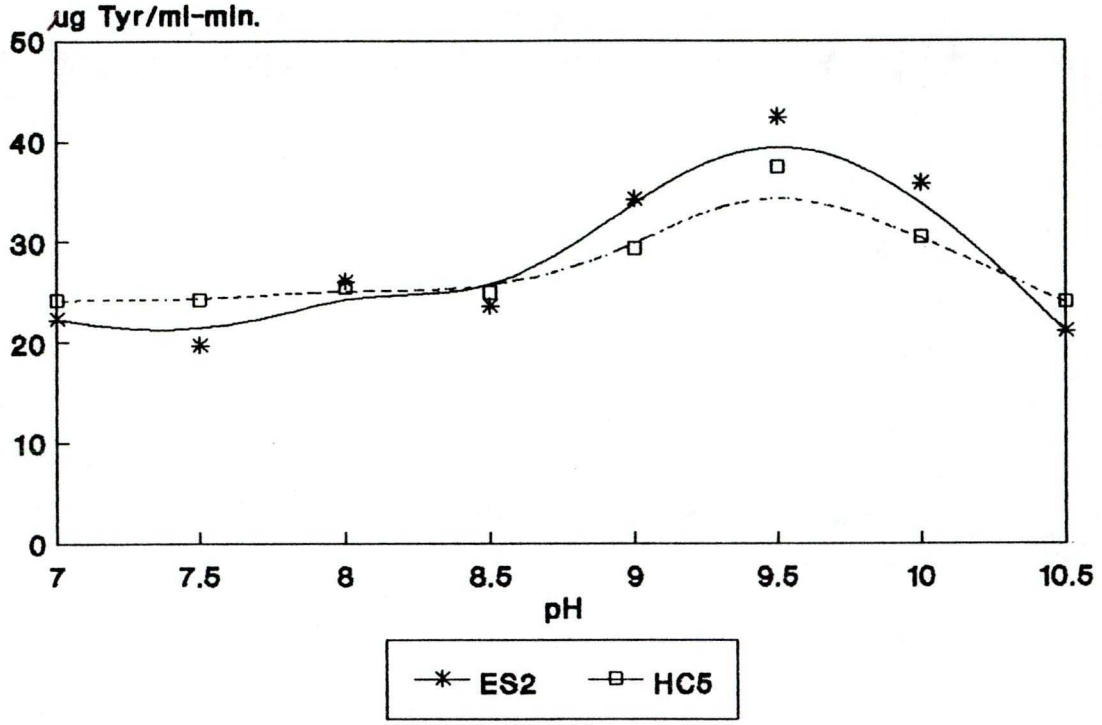


Fig. 35. Effect of pH value on enzyme reaction rate.

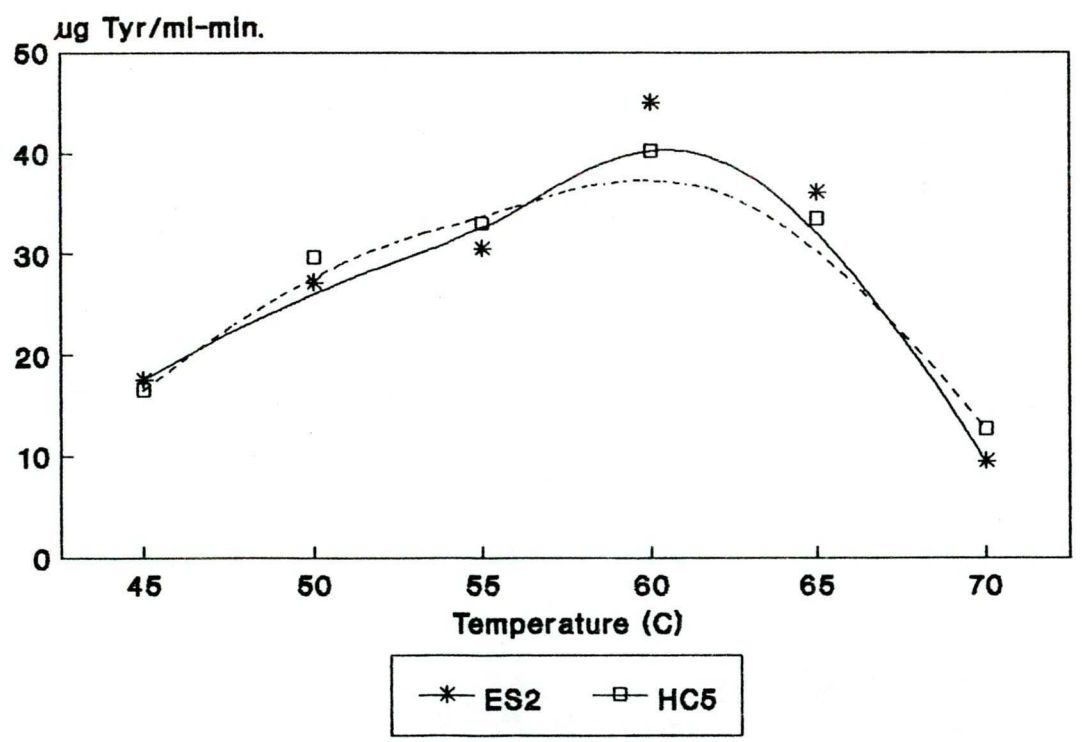


Fig. 36. Effect of temperature on enzyme reaction rate.

a moderate decrease before and a very steep decrease after the optimum point.

Therefore , the enzyme is considered to be alkaline compared with the other microbial proteinase reported so far, including that from *Bacillus*. This optimum values were again significantly different from the optimal initial pH value (7) and the final pH values (8-8.2) of cultures fluid as well.

2.2.5.1.3. Effect of Temperature on Enzyme Reaction Rate

Extracellular enzymes are biosynthetised and excreted into the surrounding environments in order to break down the high molecular weight proteins and carbohydrates to make their cellular uptake possible for the formation of new living materials in growth and reproduction.

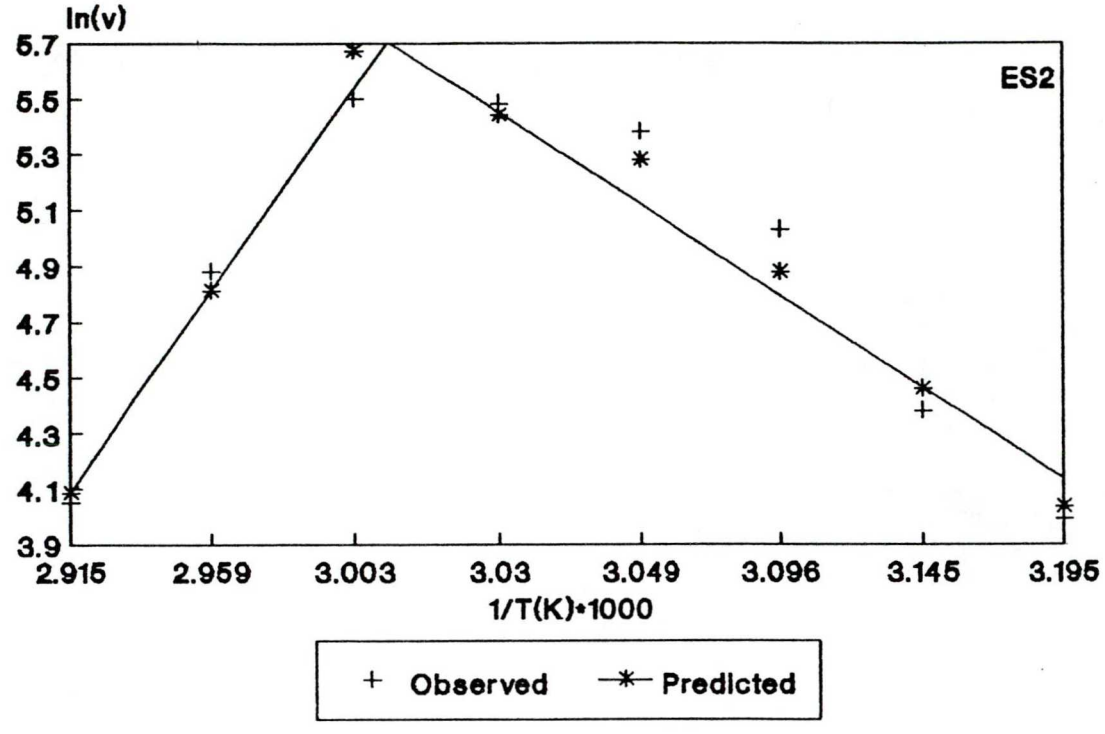
From this fact, it would be logical to suppose that the optimal pH and temperature of the action of these enzymes are likely the same as those of bacterial growth by which these enzymes were given and their production conditions too. The proteolytic activity of the cell free culture broth were examined in the range of 45-70°C.

The plotted data shows relatively flat optimum curves between 55 and 65°C and sharp decrease before and after that range as can be seen in Fig. 36. The maximal enzyme activity were found at 58°C which were significantly different from 50°C the optimum growth temperature.

The shape of the curve reflects the temperature dependence of the reaction, a decline in the process rate with elevation of the temperature after optimum, is thought to be accounted for by the denaturing effect of heat on the enzyme operating in the rate-limiting reaction.

2.2.5.1.4. Activation Energy

There are two ways of increasing the reaction rate; one is to raise the temperature and the second is to lower the activation energy. Enzyme speed up reaction by forming transition state complex with their substrate which reduce the activation energy of the reaction (E_a). The activation energy is the amount of energy required for the transition state which represent a halfway point where the bonds of [S] are distorted sufficiently, so that



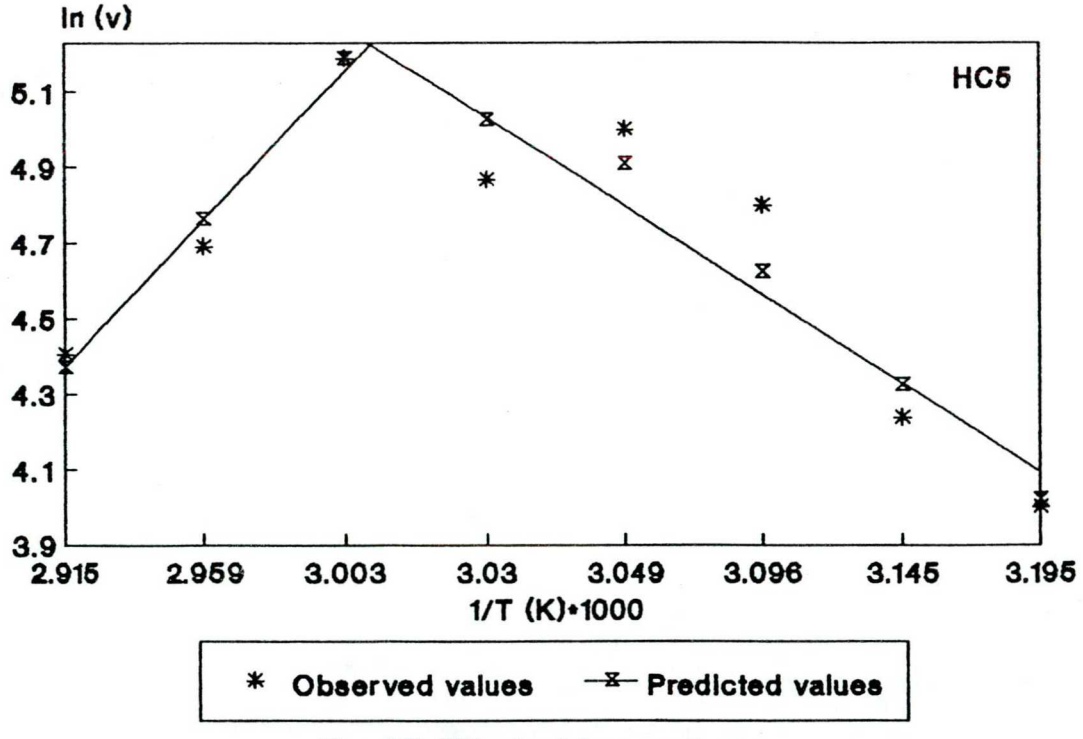


Fig. 37. Effect of temperature on enzyme stability and activity

conversion to [P] becomes possible. Living cells can exist under relatively mild environmental conditions because they possess enzymes that selectively lower the activation energy of vital chemical reactions; as noted out by Segel (1976).

Alexandrov (1977) reported that the principal reason for an enzyme to interfere in a biochemical reaction as a catalyst is to reduce the activation energy of the reaction, which sometimes resulted to increase of the reaction rate at a given temperature by up to nine order magnitude.

The relationship between (E_a) and temperature was examined using the Arrhenius equation. Data obtained are illustrated in Fig. 37. The Arrhenius plot of the casein breakdown by prepared enzyme solution based upon initial reaction rate determinations. The sharp decline of enzyme stability and the presence of break at about 60°C in the Arrhenius plot suggests the possibility that conformational changes of the enzyme protein occurred around the temperature mentioned above. The same results was found by Uchino (1982).

According to the 60°C optimum temperature, the plot consists of two linear fragments, the first belongs to the temperature limited at which the usual biological range is considered, the second concerning the significant temperature higher than the first.

From the straight line below 60°C, the activation energy was found to be 70.8 and 50.63 kJ/mole for ES2 and HC5, respectively. The values of activation energy of enzymes from thermophiles are generally the same of mesophilic enzymes; Karst et al. (1986), but it is much higher than those from psychrophiles; e.g. Jackman et al. (1983) pointed out that (E_a) for mesophilic enzyme was three times higher than in the case of psychrophiles. These results could also be confirmed by those obtained by Webster et al. (1988) who found that ATP content was lower in thermophiles than in mesophiles which may reflect a decreased activation energy required for the metabolic coupling when growth is occurring at the higher temperature.

On the other side, the straight line with positive slope over 60°C suggests a thermal deactivation of the enzyme in this range which has been attributed to conformational changes and physical changes of water. Bailey and Ollis (1986) suggested a physical mechanism for this phenomenon; as the temperature increases, the

atom in the enzyme molecule have greater energies and a greater tendency to move, then acquiring sufficient energy to overcome the weak interactions and holding the globular protein structure followed by deactivation. From this latter straight line the deactivation energy (E_d) was approximately 208 and 124.6 kJ/mole for ES2 and HC5 strain, respectively.

2.2.5.1.5. Temperature Coefficient

Another measure of activation energy is the temperature coefficient (Q_{10}) which is given by the ratio of a reaction rate at temperature (t) to that at ($t-10^{\circ}$). The measured values of (Q_{10}) were also found to be 1.6 and 1.5 for the two tested strains. Data are in general agreement with the results provided by Zeikus (1979) who pointed out that (Q_{10}) values for thermophilic enzymes lie in the range 1.4 to 1.9 which considerably depending on the molecular properties and nature of the enzyme. Alexandrov (1977) noted also that the (Q_{10}) of most chemical reactions is around 2-3. The results of Glymph and Stutzenberger (1977) showed also that the (Q_{10}) values from 45°C to 65°C varied from 1.75 to 1.82.

2.2.5.2. PARTIAL PURIFICATION OF ENZYME

Enzymes are found in nature in complex mixtures, especially in cells which perhaps contain a hundred or more different enzymes. In order to study a given enzyme properly, it must be isolated of other enzymes and purified. Because of the proteolytic enzyme is an extracellular one, it is so easy to be extracted and separated from the cell debris included in the fermentation fluid. This procedures were only realized for one enzyme which obtained by the Hungarian strain No. HC5 as follows:

2.2.5.2.1. Salting out

This is a very widely used method applying ammonium sulfate on account of its large solubility in water and absence of harmful effect on most enzymes. In the preliminary runs different concentrations of the salt were applied, 30, 40, 50, 75, and 78.5 % saturation which showed that the level 50% saturation was the most appropriate (data not shown). These experiments were carried out at temperature between 0-5°C to prevent any break down may happens to the enzyme using 50% saturation of $(NH_4)_2SO_4$. The crude

enzyme preparation was placed in an ice bath and solid ammonium sulphate (crystal) was gradually added to the enzyme solution during intensive stirring to reach 50% saturation.

After that the two fractions obtained were centrifuged separated. The precipitate was redissolved again in buffer solution, pH 9.5 and enzyme activity and total protein were measured for the crude fermentation broth and the two fractions obtained. Data obtained are collected in Table 10. It could be seen that, there are significant difference between the crude solution and the fractions obtained after applying the $(NH_4)_2SO_4$ considering the specific activity and recovering yield as well.

2.2.5.2.2. Dialysis Process

The extract containing enzyme may also contain numerous other substances both large and small molecular weight. The small molecule substances can be removed by the dialysis, leaving the large one which are predominantly protein. Of course, this step is also benefit in avoid the interference of salt with the enzyme during the gel fractionation in the next step.

In these steps, the samples were also applied at 0-5°C over night against the same buffer to allow removal the impurities. After measuring the desired parameters, data are recorded in Table 10. The dialysed samples showed highest specific activity together with the purification factor which lead to further purification steps.

2.2.5.2.3. Chromatographic Fractionation

Because having no enough information about the examined enzyme as well as from the economic point of view, a quick and small scale of the chromatographic fractionation technique was realized after which only a few minutes of centrifuging is needed to spin down the adsorbent.

The fractionation was carried out by the ion exchange chromatographic technique. The anion exchange chromatography in solid phase (fixed phase, +) using DEAE-50, and the cation exchange in solid phase (fixed phase, -) using Sp-sephadex G-50 were carried out (Plummer 1987).

Table 10. Enzyme precipitation by ammonium sulphate.

Purif. Step	Fermentation	50% $(NH_4)_2SO_4$		Dialysis
Parameters	broth	Precipitate	Supernatant	
Total volume (ml)	500	65.0	650	65.0
Total protein (mg/ml)	18.2	7.1	16.6	4.5
Enzyme activity (U/ml)	42.3	252.6	7.1	206.6
Spe. activity (U/mg)	2.3	35.7	0.55	46.1
Purification factor	1.00	15.4	0.24	19.9
Yield %	100	77.7	21.8	63.6

Table 11. DEAE-50 Anion Exchange Purification.

pH.	5.4	6.2*	8.2	8.9
Parameters				
Total protein (mg/ml)	0.70	1.09	0.97	0.79
Enz. activity (U/ml)	78.40	86.00	139.40	95.50
Spec. activity (U/mg)	111.20	79.00	143.80	120.10
Purification factor.	1.41	1.00	1.82	1.52

* Was taken as a control.

Table 12. SP-Sephadex C-50 Cation Exchange Purification.

pH.	5.8	6.2*	7.1	8.3	9.7
Parameters					
Total protein (mg/ml)	1.05	1.65	1.40	1.56	1.59
Enz. activity (U/ml)	0.00	62.00	64.50	105.00	82.80
Spec. activity (U/mg)	0.00	37.60	46.00	67.30	52.10
Purification factor.	0.00	1.00	1.22	1.72	1.38

* Was taken as a control.

In the preliminary experiments, a little volume of enzyme solution was performed in anion-cation exchanger (5ml/0.2g) in both acidic and alkaline media at 0-5°C. After 2 minutes intensive mixing (vortex type 5164, Hungarian made), the adsorbent was separated. After recovering adsorbed enzyme using buffer solution with different pH values, the Purification parameters were measured and the results obtained are summarized in Table 11 & 12.

Interestingly, no complete catching of enzyme by the resin was found during anion exchange process at pH 8.2 gave highest specific activity as well as higher purification factor (Table 11). The same finding was also came by cation exchange chromatography in the alkaline media, since the pH 8.3 showed higher specific activity and high purification factor as well (Table 12).

On the basis of these data, more amount (50 ml) of enzyme solutions were applied using DEAE-50 anion exchanger and SP-sephadex G-50 cation exchanger. To recover the enzyme molecules binded on the resin particles, buffere solutions were used in 5.5, 8.1, 9.6 pH values taking into account the dialysed sample (PH 6.2) as a control. Obtained data are recorded in Table 13. From tabulated data, it could be noticed that there is no significant difference between the two chromatographic system used, anion and cation exchange considering the specific activity and purification fold obtained.

2.2.5.3. KINETIC OF PURIFIED ENZYME

The ability to proliferation at growth temperature well above 50°C is associated with extremely thermal stable enzymes which have their unique kinetics. The study of kinetic parameters are of interest because it gives an overview about how the enzyme reaction is affected by various chemical and physical conditions and consequently how the enzyme reaction regulated in vivo. This may also gives an opportunity to produce many of industrial material under economic controlled conditions.

In order to study the effectiveness and characteristic of the desired enzyme, the examination of initial reaction rate as well as the kinetic parameters were carried out as follows:

Table 13. Batch Ion Exchange Purification.

Purif. step. Parameters.	Dialysed* sample	DEAE-50 Supernat.	SP-50 Supernat.	Sephadex Adsorbed
pH	6.2	8.1	5.5	9.6
Total volume (ml)	50.0	50.0	50.0	50.0
Total protein (mg/ml)	1.51	0.65	0.84	0.40
Enz. activity (U/ml)	6.0	14.1	0.0	9.1
Sp. activity (U/mg)	4.0	29.4	0.0	22.7
Purification factor.	1.0	7.3	0.0	5.7

* Was taken as a control.

2.2.5.3.1. Effect of Temperature on Purified Enzyme Reaction Rate

The effect of temperature on the enzyme reaction rate was examined after the purification. Obtained data was graphed in Fig. 38-39. The results suggest that the enzyme became more stable toward high temperature comparing with the crude one (Fig. 36). This may be indicated the effect of impurities on the stability which were included with the enzyme in the prepared solution.

On the other hand, oposite results were found regarding the calculated values of (E_a) and (E_d). The values of activation and deactivation energy of purified enzyme are 27.3 and -45.9 kJ/mole which are less than those given by the crude one. These results suggested the effect and the contribution of the other impurities proteins involved in the solution of crude enzyme.

2.2.5.3.2. Effect of Substrate Concentration

The initial reaction rate of the enzyme was measured at different substrate concentrations. Data showed a curve similar to the rectangular hyperbola which proved first order kinetic of enzyme reaction rate at low substrate concentration while at high substrate concentration, v is independent of [S] giving zero order kinetic as can be seen in Fig. 40. The decreasing of values as a result of the inhibition effect of substrate concentration is also clearly illusrated in the Figure.

2.2.5.3.3. Kinetic Constant Examination

In order to calculate the values of the kinetic parameters, K_m and V_{max} , three different methods were followed as described by Lee (1992). The data are arranged in Table 14 by which the calculation of these constant by the three different linear plots were realized.

The numerical value of K_m was 0.047, 0.045, and 0.024 mg/ml by the method of Lineweaver- Burk ($1/v = K_m . 1/s + 1/V$), Eadi-Hofstee ($v = V - K_m . v/s$) and Hanes ($s/v = K_m/V + 1/V.s$), respectively. These results of K_m (low numerical value) proved the high affinity of enzyme-substrate. On the other hand, the value of V_{max} was 0.881, 0.874 and 0.867 ug tyrosine/min by the three methods used, respectively. This value could also showed what is the level of substrate at which the enzyme is saturated by the substrate.

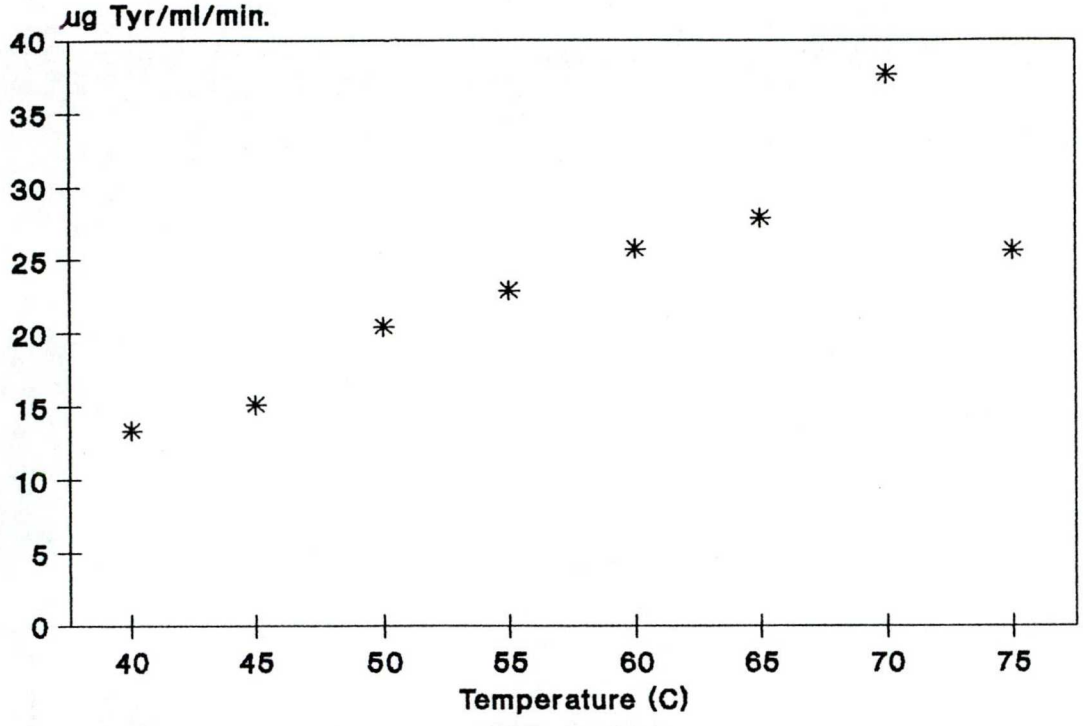


Fig. 38. Effect of temperature on purified enzyme reaction rate, HC5.

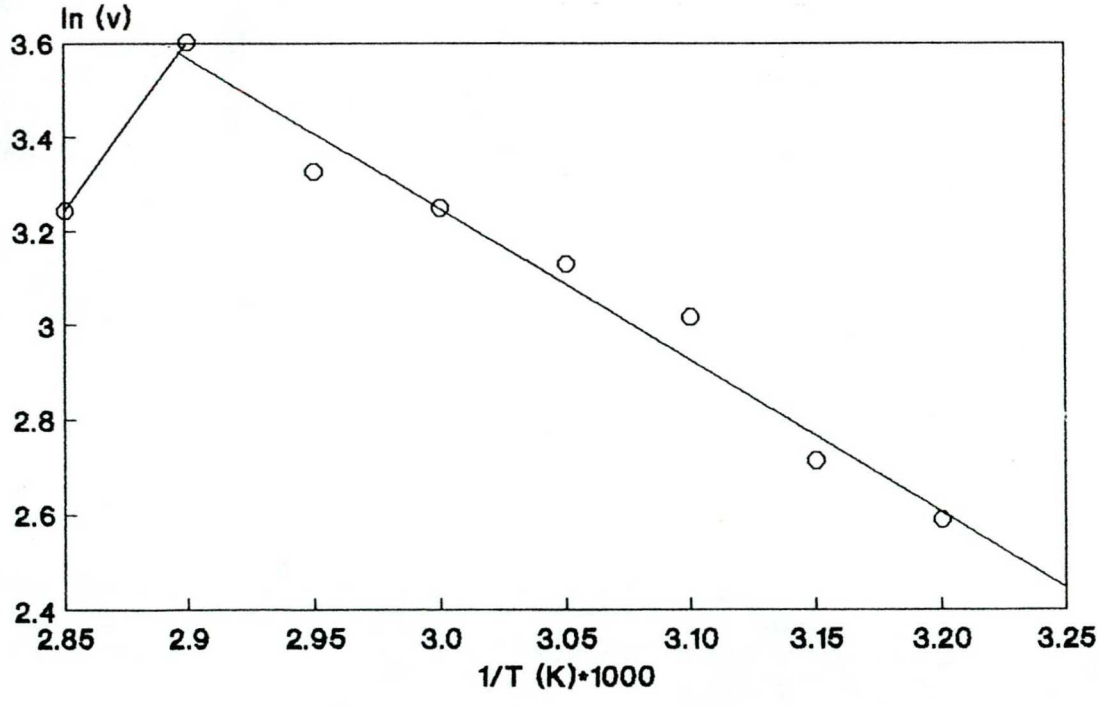


Fig. 39. Effect of temperature on stability and activity of purified enzyme (HC5).

Table 14. Parameters of kinetic constant measurement.

[S] g/l	v µg tyro/min	1/[S]	1/v	[S]/v	v/[S]
0.1	0.602	10.0	1.660	0.166	6.02
0.25	0.76	4.0	1.316	0.329	3.04
0.35	0.721	2.857	1.387	0.485	2.06
0.5	0.855	2.0	1.170	0.585	1.71
1.0	0.827	1.0	1.209	1.209	0.827

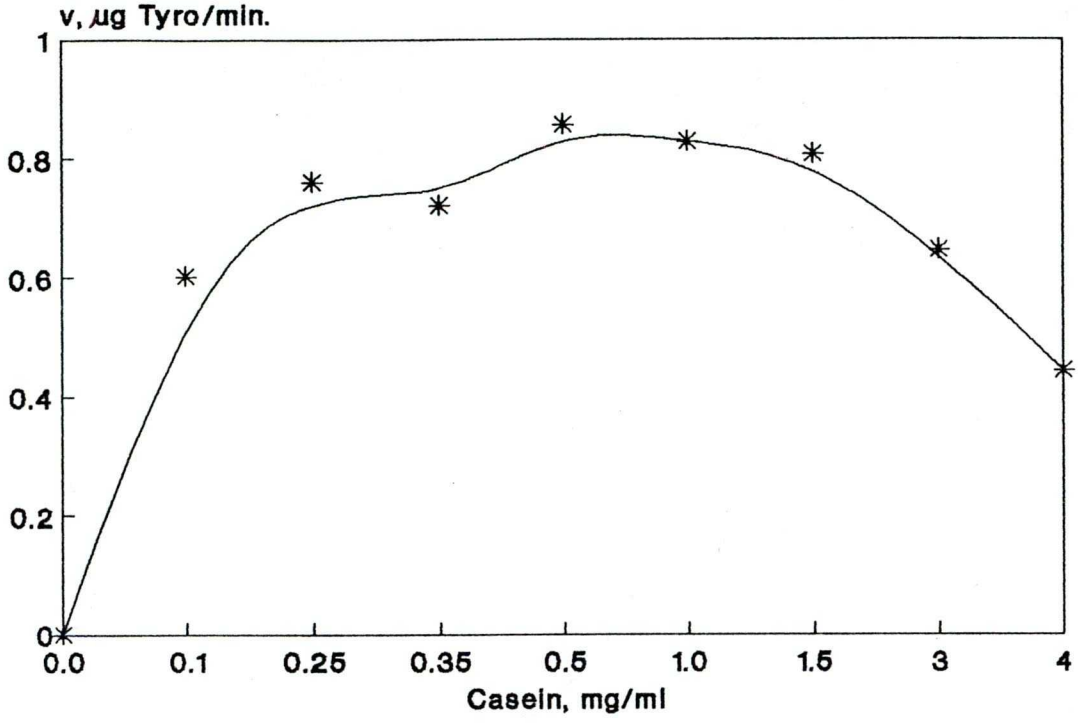


Fig. 40. Effect of [S] concentration on enzyme reaction rate

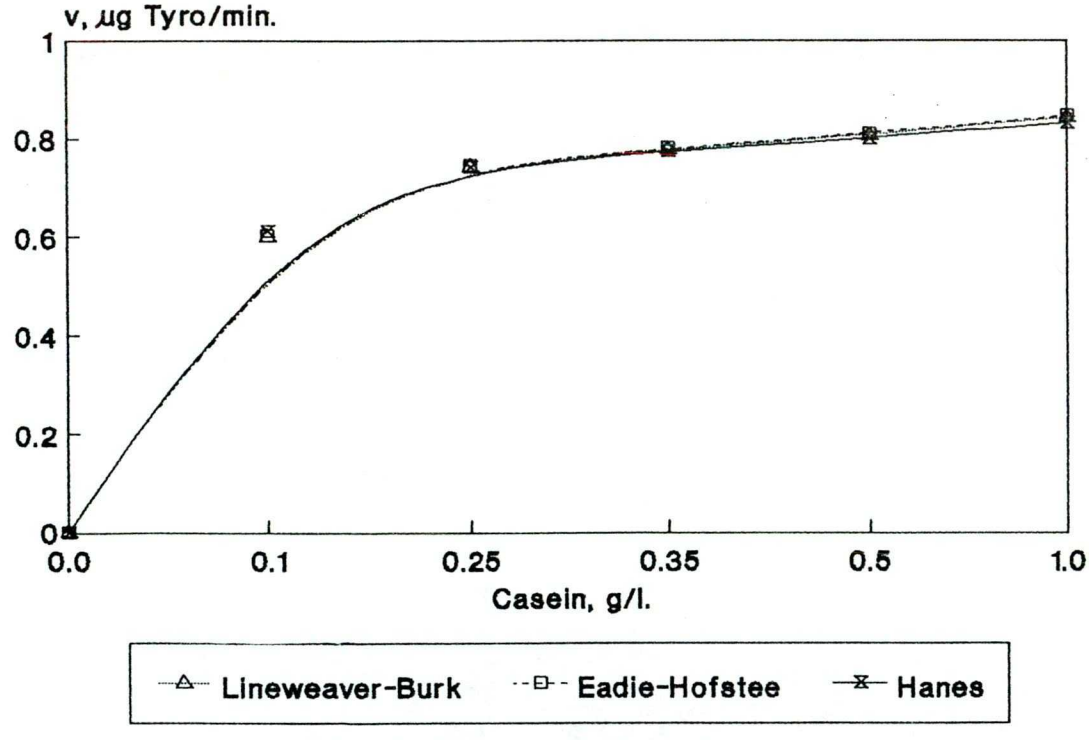


Fig. 41. Enzyme reaction rate curves derived by Michaelis-Menten equation

In order to examine the accuracy of the previously obtained data, the application of Michaelis-Menten equation $v = V / [1 + K_m/s]$ was realized. The different curves were plotted in Fig. 41. The graphed values proved the validity of the three kinetic models used.

SUMMARY

This investigation could be summarized as follows:

1. Screening for proteolytic enzymes

1.1. First Screening

After the enrichment, isolation, and purification of thermophiles, the first screening was carried out on milk agar plates on the basis of qualitative enzyme activity measurement. Of 14 isolates, only ten were selected by this step.

1.2. Second Screening

The isolates showed higher ratios (Z-G = 7-12 mm) were applied for second screening on shake flasks technique using different recommended culture media. On the basis of the quantitative measurement of enzyme activity, the results showed that the isolates No. ES2 and HC5 showed higher enzyme production with medium No. 3. being 195 and 162.5 TU/ml culture filterate for the two selected strains, respectively.

These two isolates which originally comes from the Egyptian soil and Hungarian compost samples were then selected for further investigations after examining some of their characteristics. The plan of the research work will be comparative studies between two strains comes from different sources and different environmental condition as well.

2. Optimization for enzyme production

2.1. Effect of environmental conditions on enzyme production

2.1.1. Effect of Temperature

The growth temperature as an important factor was examined. Data shows that 50°C was the most favorable in respect of growth and enzyme activity for ES2 strain, while 55°C was the best for HC5. On the basis of definiation of thermophily, These results proved that these strains are considered to be thermophilic bacteria.

2.1.2. Effect of pH

In spite of the organisms produced the alkaline proteinase in appreciate amount than neutral one, the experiments showed that

the optimum value was at pH 7.0. as initial level for culture medium. The results obtained suggest that those strains are thermoneutrophile bacteria.

2.2. Culture Medium Composition

In optimizing the culture medium composition, two different designs were applied taking into account three factors, C, N, and P, as follows:

2.2.1. The First Design

The first design was linear term with interaction between carbon and nitrogen source (CN). A three factor, two level first order orthogonal composite factorial design was realized in respect of alkaline proteinase activity as an objective function after 24 & 36 h cultivation time. The experimental data were calculated by multiple regression and formulated in equation 1 & 2 (24 h) and 3 & 4 (36 h) for ES2 & HC5, respectively.

$$Y = -183.1 + 115.3\,C + 105.9\,N - 24.5\,P - 17.3\,CN \quad \text{----------[1]}$$

$$Y = -842.9 + 219.9\,C + 106.7\,N - 3.3\,P - 21.3\,CN \quad \text{----------[2]}$$

$$Y = 168.1 + 3.3\,C + 55.2\,N - 14.8\,P - 4.4\,CN \quad \text{----------[3]}$$

$$Y = -1017.5 + 284.5\,C + 134.7\,N - 6.4\,P - 29.6\,CN \quad \text{----------[4]}$$

2.2.2. Second Design

In order to achieve higher production rate of desired enzyme, further optimize with two factor, three level, second order orthogonal composite experimental design was followed. After the calculation, data were statistically analyzed and formulated in quadratic equations, 5 & 6 (24 h) and 7 & 8 (36 h) as follows:

$$Y = 388.9 + 52.4\,(N-10.8) + 27.6\,(C-3) - 23.6\,(N-10.8)(C-3) + 20.2\,(N-10.8)^2 - 47.9\,(C-3)^2 \quad \text{----------[5]}$$

$$Y = 523.5 + 18.9\,(N-7.6) + 32.9\,(C-7) + 15.8\,(N-7.6)(C-7) + 8.6\,(N-7.6)^2 - 4.3\,(C-7)^2 \quad \text{----------[6]}$$

$$Y = 546.4 + 32.7\,(N-10.8) + 20.4\,(C-3) - 11.3\,(N-10.8)(C-3)$$

$$-16.5\ (N-10.8)^2 - 149.8\ (C-3)^2 \quad \text{----------[7]}$$

$$Y = 312.8 - 12.2\ (N-7.6) + 30.9\ (C-7) - 7.1\ (N-7.6)(C-7)$$

$$+ 42.2\ (N-7.6)^2 + 10.7\ (C-7)^2 \quad \text{----------[8]}$$

Data showed also that strain ES2 was reached its maximum production after 24 hr while HC5 strain continues to 36 hr. The results were also fitted the model by developing the residual analysis since the values of residual analysis lies in the acceptable range (+-3).

3. Kinetics of batch fermentation

3.1. Growth Monitor

During the time course of batch fermentation, the growth of population was indirectly monitored by OD, DO, substrate decreasing and product formation. The direct monitoring of the population was also achieve by measuring the cell dry weight and total nucleic acid (DNA & RNA) as well.

3.2. Parameters of Growth Kinetic

The economic of the fermentation is greatly affected by the substrate consumed and dissolved oxygen, as a function of time, needed to produce the required amount of biomass and desired product formation as well. Thus, in order to examine these features, the kinetic parameters were calculated.

4. Growth behavior in continuous cultivation

4.1. Dynamic of Cell Population

The dynamic of cell population was followed during the course of continuous fermentation by measuring OD, DO, substrate concentration, RNA, DNA, and cell dry weight as well.

4.2. Evaluation of Fermentation

In order to evaluate the fermentation process, the overall productivity, the conversion yield of substrate and the specific productivity were calculated.

5. Kinetic of enzymatic caseinolysis

5.1. Properties of Crude Enzyme

5.1.1. The progress curve was measured by plotting the product formation against the time of enzyme reaction. Then the proper time of enzyme reaction (10 min) was taken from the linear part of the curve for the two tested strains.

5.1.2. The effect of temperature on enzyme reaction rate was examined. The 60°C was the optimal at which the product formation was at the maximal for the two strains.

5.1.3. Effect of pH of the enzyme reaction mixture on the enzyme reaction rate was also studied. The value of 9.5 was noticed to be the best for ES2 and HC5.

5.1.4. Because the enzyme speed up the reaction by forming transition state complex with their substrate, the activation energy (E_a) of enzyme required for that role was measured from Arrhenius plot being 70.8 kJ/mole (ES2) and 50.6 kJ/mole (HC5).

5.1.5. The temperature coefficient (Q_{10}) was also calculated to be 1.6 and 1.5 for ES2 and HC5, respectively.

5.2. Partial Purification

The enzyme was partially purified by salting out using ammonium sulphate and chromatographic fractionation using ion exchange in matrix with ionized group and solvent buffer (anion exchange, - mobile phase and cation exchange, - fixed phase). High specific activity of enzyme were obtained after anion exchange as well as the factor of purity than those found with cation exchange.

5.3. Properties of Purified Enzyme

5.3.1. Temperature examination

Some properties of purified enzyme were examined. The purified enzyme showed more stability toward the temperature (up to 70°C) than the crude one (up to 60°C), but at the same time lower value of activation energy was found for the purified enzyme (27.3 kJ/mole) than the crude one (50.63 kJ/mole).

5.3.2. Kinetic constant

Regarding the kinetic constant, the enzyme showed high affinity with the substrate used (Hammerstein casein) represented in the low numerical value of K_m (0.045 mg/ml) and the high V_{max} (0.88 ug tyrosine/min) calculated by three different methods, Lineweaver-Burk plot, Eadi-Hofstee and Hanes plot. These values were fitted by applying Michaelis-Menten equation.

CONCLUSION

From these results it could be concluded as follows :

1- The possibility to isolate thermophilic microorganisms from different sources in spite of the difference of environmental conditions, either from cold weather areas (Hungary) or from tropical countries (Egypt) as well.

2- Using the organic materials as nutritional sources for the microorganisms are of interest in biotechnology from the economic view point. At the meantime, it is so great to perform that by using cheap raw materials either from the crop residues or industrial by-product form which are available anywhere and at any time.

3- The optimization of culture media required for producing appreciate yield of enzyme was successfully carried out leading to use only the amount of growth substances consumed by the organisms to avoid the inhibition effect of substrate in high concentration level.

4- The batch fermentation are clearly showed the importance of the phosphate source as a main component in culture media used in enzyme production. The experiments of batch run also showed that the enzyme production belongs to mixed growth associated category of fermentation pattern.

5- Because of the difficulty to clearly state the nature of the growth limiting component of the complex culture medium, it should be concluded that it is so difficult, to get a real steady state if the culture media used in undefined chemical form, when applying the continuous flow-cultivation system. At the same time, the high productivity both the volumetric and specific one were given during the experimental run.

6- Regarding the difficulties of applying the classical system to express the microbial growth in the undefined culture media, the use of total nucleic acids of cellular material comes applicable and accurate.

7- The differences between the crude enzyme and purified one could also came in the conclusion, especially the high stability to temperature. The importance of enzyme purification should also be noted when the study of kinetic is required.

8- The kinetic constant of the purified enzyme showed the high affinity of enzyme-substrate as well as it gives an accurate view about the concentration of substrate and product in the cell. Consequently, the regulation of enzyme reaction in vivo can be easily done under control.

9- The characteristic properties of produced enzyme suggested that it is a qualified candidate for many application in biotechnology such as the laundry and biodetergent formulation as well as in the tanning and leather industries.

10- The appreciate yield of biomass produced as a by-product of these processes are also considered unforgettable or neglected factor in this conclusion because it can be used as nutritional additives in the poultry and animal feeding. These fermentation by-products play an important role in the field of animal nutritional from the economic point of view.

11- It should be also concluded that, although the two tested strains are completely different from each other in the source of isolation and environmental conditions as well, they are showed identical results in some tests, and different characteristic in the others are also got.

12- Data suggest the genetic stability of the organisms under study even at high population level which could be depicted by comparing the three systems used for microbial cultivation; shake flask, batch cultivation and continuous fermentation. In all these

systems the organisms showed the same pattern of fermentation since the enzyme production was of mixed-growth associated mode.

NEW SCIENTIFIC RESULTS AND THEIR APPLICABILITY

From the study of this dissertation, it could be noted some of the new scientific results which could be concluded together with their applicability as follows:

1- New thermophilic bacterial strains (14 isolates) which have the potency of proteolysis at 55°C were isolated from different sources and different environmental circumstances represented in Hungarian compost and Egyptian soil, proved the versatility of this kind of organisms in spite of the significant effect of the environmental factors (2.2.1.).

2- The physical parameters of optimum microbial growth were examined (temperature & pH) and the chemical composition of culture medium were also quantificated (carbon & nitrogen), which has to be considered in batch technology according to the requirement of microorganisms, (2.2.2.).

3- A proposed mathematical model was used (two design were used) for optimizing the composition of culture medium and some modification (the ratio of the components) of the growth medium components was performed as well which could be used for industrial design purposes, (2.2.2.).

4- By following the profiles of batch fermentation in two different pattern of cultivation (pH-controlled and uncontrolled one), after performing the shake flasks technique, has given a real view of enzyme production mode by both to be possible in large scale production, (2.2.3.).

5- High productivity, both volumetric and specific have given by applying continuous cultivation using undefined culture medium which could lead to more study of kinetics parameters regarding the enzyme production in continuous pattern which will be very important in biotechnology especially from the economic view point, (2.2.4.).

6- Accurate determination of biomass has been lacking in complex solid containing culture medium became practically without any confusion by using the nucleic acids (DNA) of cellular materials as an growth monitoring side by side with cell density and dissolved oxygen as well as the disappearance of total carbohydrates during the time course of fermentation both batch and continuous, (2.2.4.).

7- Most important parameters of enzyme kinetics were examined (temperature and pH) and obtained results proved the validity of using this enzyme in detergent preparations and leather industry, (2.2.5.).

8- Studying the kinetic constant of purified enzyme showed High affinity of enzyme-substrate represented in the low numerical value of K_m (0.045mg/ml) and the high V_{max} value (0.88 ug tyrosine/min), (2.2.5.).

9- Significant difference between the conditions of microbial growth and their enzymatic reaction parameters were demonstrated ($55^{\circ}C$ for the growth and production, $60^{\circ}C$ for enzyme reaction; pH 7.0 for the growth and production, 9.5 for the reaction) an interesting point of view of understanding the kinetics behavior both microbial growth, enzyme production and enzymatic action as well, (2.2.5.).

10- Almost two-fold increase in enzyme yield was achieved as a result of the screening programme followed; 195 & 162.5 TU/ml were given by the original medium composition while 330 & 296 TU/ml has obtained by applying the new composition of culture medium.

11. The overall productivity of batch system are considerably higher, 73.1 TU/ml (ES2) & 96.4 TU/ml (HC5) TU/ml than the counterpart obtained by continuous fermentation 56.12 TU/ml (ES2) & 62.4 TU/ml (HC5) because of higher biomass produced by the second system while the enzyme yield was the same in both cases.

12- Interestingly, the most significant results found when continuous fermentation was performed on the basis of the data derived by batch run (saturation level of dissolved oxygen and consumed ratio of substrate) is that the physiological behavior of the microbe in continuous cultivation is identical in the metabolic activities of the organisms corresponding batch data

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