AMYLASE PRODUCTION BY BACILLUS SPECIES ISOLATED FROM FERMENTING TEF AND KOCHO

By

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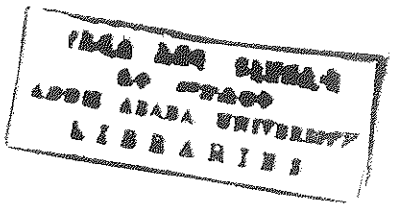

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A B S T R A C T

Amylase producing bacterial strains associated with kocho and tef fermentation were isolated. The strain from fermenting kocho was identified as Bacillus subtilis while the strain from fermenting tef was identified as Bacillus licheniformis.

Conditions for the productions of amylases were investigated. The bacterial strains secreted amylase into a liquid medium containing 1% soluble starch. When starch was replaced by disaccharide and monosaccharide, amylase production was reduced. Amylase production was higher with unfermented kocho compared to other native straches. Of the nitrogen sources tested, the greatest growth and amylase production were obtained with 0.5% peptone. The most appropriate metal salt for amylase production was found to be 0.02% $MgSO_4 . 7H_2O$. Amylase was produced over a wide pH range (4.5 to 9.5 for Bacillus subtilis and 5 to 10 for Bacillus licheniformis with the maximum activity between pH 6 and 8. Amylase produciton was greater at temperatures from 18 to 24 (room temperature) at 72 hour of cultivation.

The amylases which were found to be extracellular were optimally active at 40°C and pH 5.6. Bacillus licheniformis T 31 amylase was also active at pH 8. The amylases of both organisms retained 100% activities for 1 hour at 60°C. These enzymes were stable in the pH range of 5.5-8.5. $MgSO_4$, $CaCl_2$ and $BaCl_2$ were found to stimulate amylase activity. The amylases were stable in 0.5 M NaCl with 64% -78% of their original activities being retained after 24h. In addition to amylase, Bacillus subtilis also produced pullulanse.

Using chromatographic analysis, glucose, maltose and maltotriose were detected as products of hydrolysis.

I N T R O D U C T I O N

1.1.Amylolytic Organism

Enzymes responsible for the degradation of starch are widely distributed in nature. Among the enzymes that are concerned with the hydrolysis of carbohydrates, the most important group from the point of industrial application are the amylolytic enzymes (Rose, 1961).

Amylases from plants, animals, and micro-organisms have been studied extensively since enzymes were first discovered (Saito, 1973; Boyer and Ingle, 1972). Because of the technical and economic advantages of microbial enzymes, microorganisms are assuming predominant roles as the sources for industrial enzymes (Reed, 1975). Large quantities of plant materials are needed for the production of small amounts of enzymes. The supply of animal enzymes are limited since other uses compete for the enzyme-yielding tissues. In addition the supply of raw material for enzyme extraction from plants and animals is often seasonal and this prevents steady production (Meyrath and Volavsek, 1975).

Microorganisms offer many advantages as sources of enzymes over those obtained from higher animal or plant materials(Arima,1964).

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These advantages include the following:

a. Microbes can produce enzymes at a very rapid rate and, with few limitations, offer an unlimited source of supply.

b. Microbial enzymes can be produced at relatively low cost with respect to both cultivation of the organism and purification of the enzyme.

c. The rate of enzyme production of microbe can usually be increased by strain selection, induction of mutants, and improvements in culture conditions.

Priest (1977) put the following predictions of Humphery before the introduction of his article:

> " Enzymes almost exclusively derived from microorganisms will surely become a multibillion dollar industry in the 70's. They will be the major growth products of the fermentation industry." 1972.

At the present time, the realization of this prediction is being achieved in different industrial applications of enzymes. Culture media of bacteria and molds are rich sources of amylases (Takagi,et al. , 1971). Thus microbial enzymes are manufactured to an ever increasing extent. For example a starch liquefying amylase is one of the enzymes manufactured in large quantities. In Japan alone, upto 6,000 tons were produced annually as crude powder (Arima, 1964).

The Genus Bacillus represents one of the most important groups of bacteria that produce commercially important enzymes (Fogarty, et al., 1974; Priest, 1977). Of the 48 Bacillus species listed by Gibson and Gordon (1974) 32 hydrolyse starch. Kneen and Beckord (1946) studied the amylolytic systems produced by 41 strains of Bacillus subtilis, seven of Bacillus polymyxa and three of Bacillus macerans (cited in Fogarty and kelly, 1980). As the use of bacterial alphaamylases increases, it becomes important to isolate new high producing strains (Ingle and Boyer, 1976) and enzymes with characteristics more amenable to industrial use (Ingle and Erickson, 1978).

1.2. AMYLASES

Amylase is the name given to all enzymes that degrade starch. Amylase was one of the first enzymes discovered (Sumner and Somers, 1947) and the term has been used to designate the enzymes catalyzing the hydrolysis of alpha-1, 4- glucosidic bonds of polysaccharides such as starch, glycogen or their degradation products (Fischer and Stein, 1960). In recent years, a number of new starch-degrading enzymes have been detected in microorganisms (Suzuki and Chishiro, 1983; Tigerstrom and Steimaschuk, 1987; Buonocore et al., 1976; Lee and Whelan,1971). Increasing amounts of amylase are being produced from microorganisms and they may produce one kind of amylase or a mixture of amylases (Castro, et al., 1992).

1.2.1. Classification and Mode of Action

The amylases are classified in various ways depending upon how they act on the starch molecules (Fogarty and kelly, 1980; Frazier and Westhoff, 1978; Larsson, 1989; Lee and Whelan, 1971; Kulp, 1975; Fischer and Stein, 1960).

I. Endo-amylases: Split the bonds in the interior of substrate.

a. Alpha-amylases

(Alpha-1,4-glucan - 4-glucanhydrolase, Ec.3.2.1.1). They hydrolyse the alpha - 1,4 linkages of starch randomly and bypass the alpha-1,6 linkages by endoaction, resulting in a rapid reduction in the viscosity of starch (Farez-Vidal et al., 1992). They produce initially oligosaccharides of various lengths and, in some cases, maltose and glucose as final products (Guilbot and Mercier, 1985).

The action of alpha-amylase on amylose proceeds in two stages (kulp, 1975). In the first step amylose is changed into maltose and maltotriose rapidly. The second step (Walker and Whelan, 1960 cited in Kulp, 1975) is much slower than the first step, involving a slow hydrolysis of the oligosaccharides, with the formation of glucose and maltose as final products. The alpha - amylolysis of amylopectin yields glucose, maltose, and a series of alpha-limit dextrins, oligosaccharides of four or more glucose residues,

all containing alpha-1-6, glycosidic bonds (Kulp,1975). The work of Robyt and French (1970) shows that the action of alpha-amylase is not random but follows a regular pattern with low-molecular weight substrates. The amylase-catalyzed hydrolysis of an alpha, 1—4-linked glucose polymer is achieved by the transfer of a glucosyl residue to water (Thoma, et al., 1971). They hydrolyse polysaccharides containing three or more alpha 1,4 linked D-glucose units (Barman, 1969). Alpha-amylases were named by kuhan in 1923 (cited in Thoma, et al., 1971) because the configuration of the C_1-carbon of the new reducing ends produced is alpha.

Alpha-amylases can be classified as liquefying and saccharifying enzymes. The saccharifying amylase produces a greater yield of reducing sugars from starch. Saccharifying enzymes degrade 50-60% and liquefying enzymes degrade 30-40% of the glucosidic linkages of starch (Vihinen and Mantsala,1990). The alpha - amylases are used predominantly to liquefy starch.

Alpha-amylases are widely distributed in microorganisms, plants and animals. The mode of action, properties and degradation products differ somewhat,depending on the source of the enzyme (Robyt and Whelan, 1968). Commercially alpha-amylase is produced mainly from different strains of Bacillus species (Fogarty, 1983).

ii. Exo-amylases: hydrolyse units from the nonreducing end of the substrate.

a. Beta-amylases (Alpha-1,4-glucan maltohydrolase, Ec. 3.2. 1.2) They hydrolyse alpha-1,4 bonds and attack alternate linkages from the nonreducing end of a substrate (French, 1960).They cannot bypass alpha-1,6 linkages in amylopectin and glycogen. Beta-amylases were named by Ohlsson in 1930 (cited in Thoma, et al., 1971) because they hydrolyzed starch to yield a single product, beta-maltose. The maltose produced has the beta-configuration at the reducing end and the reaction of this enzyme proceeds with inversion of configuration about the c(1) position of the glucose from alpha - to beta (Gray,1971). The prefix "beta" or "alpha" indicates product stereochemistry not substrate specificity (Thoma,et al., 1971). Beta - amylases produce maltose from amylose, and maltose and beta-limit dextrin from amylopectin and glycogen (Fogarty and Kelly,1980).

Beta-amylase was thought to be produced by higher plants only. In recent times these enzymes have been discovered from microorganisms (Castro, et al., 1992). Most bacteria produce more alpha - amylase than beta-amylase (Frazier and Westhoff, 1977).

b. Amyloglucosidases or glucoamylases (alpha-1-4 glucan glucohydrolase, Ec 3.2.1.3.): are exo-splitting that hydrolyse alpha-1,4 and alpha 1,6 linkages in polysaccharids so as to remove successive glucose units from the non-reducing ends of the chains and the sources of these

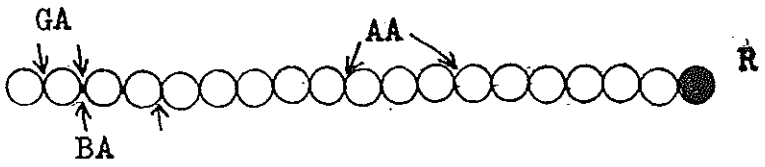
enzymes are fungi (Fogarty and Kelly, 1980). The end product of the reaction is glucose and this differentiates glucoamylase from alpha-and beta-amylases (Figure 1).

c. Other Exo-Acting Enzymes. Other amylases have been discovered that produce specific malto-oligosaccharides. These enzymes hydrolyse alpha-1,4 bonds and cannot bypass alpha-1,6 linkages and produce end products other than maltose from starch substrates (Fogarty and Kelly, 1980). Examples are the enzymes from Bacillus licheniformis (Saito, 1973) and Pseudomonas sp (Okemoto,et al., 1986) which produce maltopentaose, the enzymes from Pseudomonas stutzeri (Robyt and Ackerman,1971)and Bacillus circulans (Takaski et al., 1991) which produce maltotetraose.

iii Cyclodextrin Producing Enzymes : These enzymes degrade starch to a series of non-reducing cyclic malto-oligosaccharides or schardinger dextrins (Fogarty and Kelly, 1980). Examples are enzymes from Bacillus macerans (Depinto and Campbell, 1964; 1968).

iv . Debranching Enzymes: These enzymes hydrolyse alpha-1,6 only in amylopectin, glycogen, pullulan and related polysaccharides (Lee and Whelan, 1971). Representative examples are pullulanase (EC 3.2.1.41) and isoamylase (EC 3.2.1.68). Pullulanase converts pullulan into maltotriose and it was discovered in Aerobacter aerogenes. Pullulanases have been reported from a mesophilic Bacillus cereus (Bakashi, et al., 1992) and thermophilic Bacillus stearothermophilus (Suzuki, et al ., 1983). Isoamylase cannot degrade [illegible] and Whelan, 1971.).

Amylose



Amylopectin

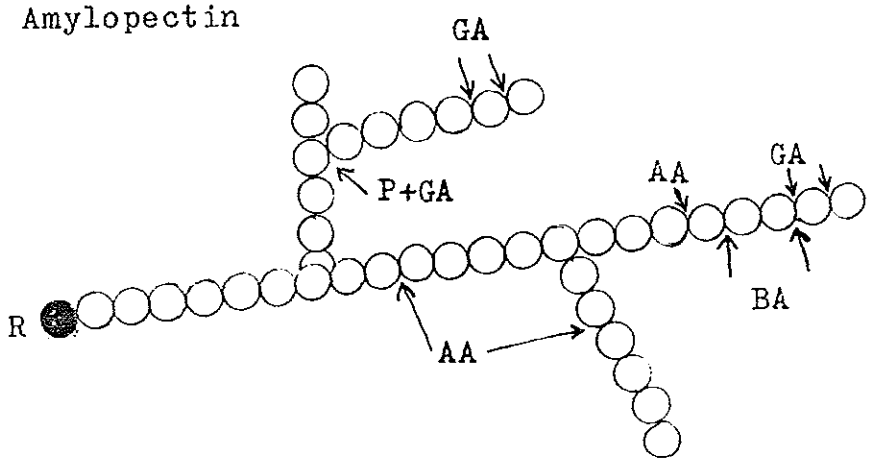


Figure 1. Enzyme action on amylose and amylopectin. (AA = alpha - amylase, BA = beta-amylase, GA= Glucoamylase, p=Pullulanase, R= reducing terminal, O = glucose units).

(Adapted from Larsson, 1989; Windish and Mhatre, 1965).

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1.3. The Substrate: Starch

Starch is an important natural substrate of amylases. It is found in all parts of the plant - the leaves, stem, shoots, and storage organs such as tubers, rhizomes, and seeds; the proportion varies from a few percent to 70 to 80% of the cereal grains (Greenwood, 1970.)

Starch granules are a mixture of two high molecular-weight polysaccharides, called amylose and amylopectin. Amylose is a linear polysaccharide whose glucose units are linked by alpha (1-4) bonds. Amylopectin is a highly branched glucose polymer. These branch points are believed to occur at intervals of about 24 to 30 glucose units (Matta and Wilbraham, 1981). The branch points are connected by alpha (1-6) linkages and the nonbranching portion of amylopectin consists of glucose units connected by alpha (1-4) linkages.

Gelatinization is an important step in starch hydrolysis using enzymes. The temperature of gelatinization process depends on the source of the starch and it ranges from 60^{o} -70^{o}C for most starches (Scott,1975). During this process the starch suspension changes into a paste and the viscosity increases with rising temperature. When heated in water, the starch absorbs water and swells, leading to the eventual rupturing of the granules and the extrusion of the water-soluble starch (Southgate, 1976).

The intact, ungelatinized starch granule is generally quite resistant to attack by enzymes. However suspensions

of starch granules in water below temperatures of gelatinization are slowly dissolved by action of amylases (MacAllister, et al., 1975).

To avoid the process of gelatinization, a search has been conducted for enzymes which could hydrolyse raw starch Ueda,1980). Examples of organisms which degrade raw starch are a bacterium, yeast and fungus (Bergmann,et al., 1988), and Aspergillus sp.(Abe, et al., 1988).

Starch has many uses in industries due to its unique properties, such as, its particulate nature, its high viscosity on gelatinization and its gelling or nongelling characteristics (Greenwood, 1970).

1.4 Factors Affecting the Production of Amylases

Because of their wide range commercial applications many investigations have been conducted on the biosynthesis of amylolytic enzymes. Some of the factors affecting the biosynthesis of amylases are growth phase, carbon source, nitrogen source, inorganic salts, the inducibility of amylase and the repression phenomena (Ramesh and Lonsane, 1991; Ingle and Erickson, 1978; Priest, 1977; Glenn,1976; Saito and Yamamoto, 1975;Coleman and Grant, 1966; Coleman and Elliott, 1962).

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Regarding the nutrient requirement, it is important to ensure its availability to the particular organism in the correct concentration. Fukumoto, et al., (1957) showed that amylase formation by Bacillus amyloliquefaciens is greatly influenced by the type and concentration of carbon source. The presence of large concentrations of easily metabolizable sugars or aminoacids, can be detrimental to the organism. Saito and Yamamoto (1975) found that the addition of 0.5% glucose, glycerol, or acetate completely repressed alpha-amylase synthesis in Bacillus licheniformis 584.

Substrate induction and catabolite repressiona are important factors in regulating the rate of bacterial amylase synthesis. Exoenzyme synthesis may be inducible, partially constitutive, or completely constitutive and the majority of exoenzymes by the bacilli appear to be at least partially inducible (Priest, 1977).

The studies of Welker and Campbell (1963b) on Bacillus stearothermophilus and Saito and Yamamoto (1975) on Bacilus licheniformis showed that enzyme synthesis increased when maltooligosaccharides were used as substrate inducers.

Studies indicate that the biosynthetic system of Bacillus species is under catabolite repression (Ingle and Erickson, 1978). Priest (1977) defined catabolite repression as the permanent repression of inducible or constitutive

enzyme synthesis due to the presence of glucose or some other rapidly metabolized carbon source. For example studies with Bacillus amyloliquefaciens (Coleman and Grant, 1966) and Bacillus stearothermophilus (Welker and Campbell, 1963a,b) showed that carbohydrate sources or concentrations that increase the growth rate will inhibit the production of the enzyme.

1.5. Application of Amylases

Amylolytic enzymes which are produced for biotechnological applications are the second largest enzyme group in volume next to proteases (Vihinen and Mantsala, 1990). They have many uses ranging from the hydrolysis of starch to the synthesis of cyclodextrins. Amylases degrade starch to dextrins or sugars or both for different industrial applications.

The type of amylolytic enzyme depends on the raw materials used and the desired product. Thus, hydrolysis of starch can be carried out not only more easily but also more selectively than when using acid or alkaline hydrolysis (Fogarty, et. al.,1974); Windish and Mhatre,1965). Bacterial alpha-amylase is preferred for the applicaiton in starch processing and textile industries due to its action at higher temperatures (75-105^{o}c) and in neutral to alkaline pH (Shah,et al., 1991).

Some of the applications are the following:

a. Production of starch sugars: Amylolytic enzymes convert starch into different sugar solutions (Aunstup, 1979; Norman,1979) for preparations of sweet syrups. Alpha-amylases from Bacillus subtilis are found most effective for this purpose (Fogarty et al.,1974).

b. In Brewing: Amylases are used to liquefy starchy materials which serve as adjuncts in the brewing industries. The bacterial amylase is heat stable and it is therefore extermely useful in the mashing process (Bass and Cayle, et al., 1974). Bacterial alpha-amylase is also stable under cool,dry storage conditions, and may, therefore, be stocked in a brewery for either routine or emergency use (Bass and Cayle, 1975). Bacterial amylases are also known to give a more favourable taste and colour to some beers.

c. In Baking: Since ungerminated wheat has low level of alpha-amylase, it requires supplementation of the flour with this enzyme (Barrett, 1975). Because the formation of fermentable sugars by amylases is required for yeast activity and the production of a gas. The bacterial alpha-amylase is also useful to produce loaves of greater softness (Barett, 1975). Its use in the baking industry has an additional advantages in that it produces a product with greater resistance to staling (Windish and Mhatre, 1965).

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d. Desizing Textiles: In the process of weaving, the individual threads are coated with gelatinized starch to give them strength. The bacterial alpha-amylase removes the size by solubilizing it after weaving (Windish and Mhatre, 1965).

e. In Paper Industry: Coated printing papers are widely produced by the paper industry. When starch is used to prepare materials for sizing, its viscosity must be reduced with alpha-amylases of microbial origin (Mayatt, 1983).

f. Other uses: Among other industrial applications are in clarification of beer and fruit juices, in the laundary industry in a mixture with protease and lipase to launder clothes, in the preparation of glue and the incorporation of amylase into drain cleaners and septic tank rejuvenators (Windish and Mhatre, 1965). Malto-oligosaccharides such as maltotriose, maltotetraose and maltopentaose have been used as reagents for research and clinical reagents for the determination of the serum amylase activity (Saito, 1982). Cyclo-dextrins which are capable of forming complexes with other materials have many possible applications in pharmacology and industry (Fogarty and Griffin, 1974.).

1.6. Purpose of the Investigation

Amylolytic enzymes of bacterial origin have numerous uses in food, textile, paper and alcohol fermentation industries. With the increasing interest in amylase for industrial use, many investigators have given their attention to bacilli species [illegible]

Amylase producing organisms are mostly found in close association with starch or starch containing substances. In Ethiopia, fermented tef flour which is prepared from cereals of tef (Eragrostis tef) and kocho which is prepared from ensete (Ensete ventricosum) carry with them several species of bacteria some of which show amylase activity. Whether these bacteria are high amylase producers or not have not been studied in detail. Attempts are, therefore, made in this investigation to achieve the following:

1. To isolate and characterize Bacillus species which are good amylase producers from fermenting tef flour and kocho.
2. To define the proper environment (pH,temperature, ions, carbon and nitrogen sources) for growth and amylase production for the test organisms.
3. To study if the native starches in tef and kocho can serve as a carbon source.
4. To assess the effect of detergents on growth and amylase production.
5. To investigate the effect of pH, temperature affecting amylase activity.
6. To determine whether the enzymes are extracellular, cytoplasmic and/or cell bound.
7. To determine the end products of amylase activity on starch using chromatographic techniques.

2. MATERIALS AND METHODS

2.1. Isolation of Amylase Producing Bacillus Species From Tef and Kocho

The Bacillus species were isolated from fermenting tef and kocho.

Five hundred gm of tef flour (Eragrostis tef) was mixed with 900 ml of water to make a slurry (dough) in a glass jar. The jar was covered and allowed to ferment at room temperature (18-23°C) for two days. At periodic intervals 10 ml of the fermenting dough was removed and diluted appropriately with 0.1% sterile bacteriological peptone water. The dilution bottle containing the sample was heated in a water bath at 80°C for 10 min to kill all vegetative cells. (Collins and Lyne, 1976). After cooling, 1 ml of the sample was taken and poured to sterile duplicate perti dishes. Molten sterile nutrient agar (Oxoid, CM 3) supplemented with 0.5% starch (Merck), was poured into each plate and allowed to solidify. Then the dishes were inverted and incubated at 32°C for 24 hour.

Fermented kocho which was prepared from ensete (Ensete ventricosum) was bought from the market. 50 gm of fermented kocho was mixed with 90 ml water. Then 10 ml of the mixture was removed and diluted appropriately with 0.1% sterile bacteriological peptone water. This was heated in a water

bath at 80°c for lo min. to inactivate the nonspore forming members. One ml of the diluted sample was placed in a sterile petri dish. Molten sterile nutrient agar supplemented with 0.5% starch was poured into the plate, and the bacteria and agar were mixed well. When the agar was solidified, the dishes were incubated at 32°C for 24 hour.

All colonies which grew on the plates were purified by repeated restreaking on fresh plates of nutrient agar supplemented with 0.5% soluble starch. Each colony was then transferred to a nutrient agar slant as a pure culture.

Amylase activity was detected by the formation of clear zones around the colonies when flooded with a solution of 1% (w/v) I_2 in 2% (w/v) kI. Because of their high amylase activities, Bacillus species k6 from kocho and T 31 from tef were selected for further studies.

Bacillus species were maintained on slants of nutrient agar supplemented with 0.5% soluble starch and kept in a refrigerator.

2.2 Characterization of the Bacillus Species

Studies of biochemical, morphological and cultural characters were made to identify the test organisms (Claus and Berkeley,1986; Collins and Lyne, 1976). The tests made include the following:

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a) Catalase test: A suspension of the organism in distilled water was made on a slide and a few drops of 10% hydrogen peroxide was added.

b) Voges-Proskauer test: Tubes of Voges - Proskauer broth in triplicate were inoculated. After incubution for 3,5 and 7 days, acetal methyl carbinol production was tested by mixing 3 ml of 5% alcholic alpha-naphthol solution and 3 ml of 40% potassium hydroxide solution.

c) Nitrate reduction: To a 24 hour culture of the organism in nitrate broth, 0.5 ml of 0.8% sulphanilic acid in 5/N acetic acid was added. After mixing, 0.5 ml of 0.5% alphanaphthylamine in 5/N acetic acid was added.

d) Test for indole; To the broth culture a few drops of xylene was shaked gently. One ml of Ehrlich's regent was then added by running down the side of the tube.

e) Anaerobic growth: A tube of anaerobic agar was inoculated with a small loopful of nutrient broth culture by stabbing to the bottom of the culture tube.

f) Growth in sodium chloride; Tubes of nutrient broth containing 5,7,10,12% (w/v) sodium chloride with a small loopful of culture grown in nutrient broth.

g) Acid and gas production : An inverted Durham's tube was inserted in a basal medium containing diammonium hydrogen

phosphate 0.1%, potassium chloride, 0.02%, Magnesium Sulfate 0.02% and yeast extract 0.02%. The pH of the medium was adjusted to 7.0 before adding 1.5 ml of a 0.04 (w/v) solution of bromocresol purple. Sterilized glucose solution (10% (w/v)) was then added aseptically to tubes of sterile basal medium.

h) Utilization of citrate and propionate: Slants of citrate and propionate utilization medium was inoculated and incubated for 14 days.

i) Casein or gelatin hydrolysis: A nutrient agar containing casein or gelatin was inoculated and incubated for 2 days. Solutions of mercuric chloride (10%) in 20% HCl was poured over the medium.

Staining characteristics of the isolates and appearance of colonies on growth media were studied. Microscopic examinations of shape and position of spores and size of cells were conducted.

All chemicals, unless otherwise specified, were obtained from BDH.

2.3 Inoculum Preparation

Slant cultures were incubated for 24 hour before inoculation. The inoculum was prepared by suspending the cells (grown on a slant) in sterile [illegible] saline

solution (0.85%, W/v). The inoculum was transferred into sterile test tube (1.5 x 15 cm) and mixed with a vortex mixer. The absorbance was adjusted to 0.3 at 540 nm using a 21.UVD spectronic spectrophotometer (Bausch and Lamb, USA). One ml of inoculum was transferred to 250-ml Erlenmeyer flasks containing 100 ml of sterile growth medium. The flasks were incubated in a rotary shaker (Gallenkamp, UK) operating at 150 RPM. Growth was monitored at room temperature (18°C - 23°C).

2.4. Cultivation Conditions

2.4.1. Carbon Sources

The basal medium (BM) used for biomass formation and amylase production contained (g/ 100ml): KH_2PO4, 0.02; $MgSO4.\ ^{7}H_2O$, 0.02; beef extract (Oxoid L 29),0.5.

Soluble starch at different concentrations was added to the BM. The concentrations were 0.5%,1%, 2% and 3% (w/v). a medium without starch was used as a control.

The effect of other carbon sources - glucose, lactose, maltose and sucrose on growth and amylase production were also investigated.

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Regarding the native starch sources, the flour of barley, corn,wheat and tef were used. Kocho powder was prepared from the dried corms of ensete. Then the powder was washed with distilled water and dried at 60°C in an oven. The concentration used for the native starch was 1% (w/v).

2.4.2. Nitrogen Sources

Four types of nitrogen sources, namely peptone (oxoid L 37) urea, potassium nitrate and ammonium chloride were used in the BM containing optimum concentration of carbon source. The concentrations of the nitrogen sources were 0.5%, 1% and 2%. a medium without a nitrogen source was used as a control.

The media were autoclaved at 121°C 15 IbS/sq. in. for 15 min. Urea was filter sterilized and added aseptically to the sterile medium.

2.4.3 Effect of Metal Ions

The effects of different concentrations of metal ions (0.1 mM, 1mM and 5mM) on growth and amylase production were investigated. The metal ions used were $AlCl_3$ $6H_2O$, $CaCl_2$, $CuSO_4 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$, $HgCl_2$, $MgSO_4 \cdot 7H_2O$. Solutions containing the salts were separately sterilized and then

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added into the basal medium containing the optimum concentrations of carbon and nitrogen sources after heat sterilization. A medium without metal salt was used as a control.

2.4.4. Effect of Detergents

The effects of different concentrations of detergents (0.05%,0.1%,2% (v/v)) on growth and amylase production were investigated. The detergents used were Tween 20 (sigma),Tween 80 (Merck), Trition X - 100 (sigma),Tergitol 7 and sodium Lauryl sulfate (sigma). The concentration of the last detergent was in weight/volume. Before autoclaving, the detergent was added to BM containing appropriate concentrations of carbon and nitrogen sources. A medium without a detergent was used as a control.

2.4.5. Effect of Temperature and pH

The effect of temperature and medium PH on growth and amylase production were investigated. The tempertures tested were between 18-23°C, 30°C, 40°C and 50°C. The pH of the media was adjusted to 4 - 10.5 with 1N NaOH or 1 N HCl after sterilization and pH values between 4 and 10 with differences of 0.5 units were considered in this study.

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2.5. Biomass Production

Biomass was determined turbidimetrically at 540 nm when soluble starch or other soluble sugars were used as a carbon source.

Cultures containing native starches were too turbid to be measured at 540 nm. In this case biomass was estimated using a modified Lowry's method (Ghose, 1987).

The reagents used were:

A. 2%.W/V Sodium carbonate (anhydrous) in 0.1 N NaOH.

B.1 1% (w/v) copper sulfate ($CuSO_4$, $5H_2O$)

B.2 1% Sodium potassium tartarate

C. Reagents A,B.1 and B.2 were mixed in a proportion of 98:1:1:

D. Folin phenol reagent

The cell pellets were suspended in Lowry reagent A. After lysis (which was induced by heating to 60°C) the native starch and cell debris were removed by centrifugation (gallenkamp centrifuge 200 for 30 min). Five ml of reagent C was mixed with 1 ml of appropriately diluted sample. After 10 min, 0.5 ml Folin's phenol reagent was added and immediately mixed. This was left for 30 min for colour development and then absorbance was read at 750 nm using

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spectronic 21 (Bausch and Lomb). The protein concentration in the samples was determined using bovine serum albumin as a standard.

Production of amylase is expressed in terms of enzyme activity.

2.6. ENZYME ASSAYING CONDITIONS

2.6.1. Enzyme Source

At periodic intervals during growth of the bacteria, sample of culture medium (5ml) was withdrawn aseptically. Cells and suspended solids were separated by centrifugation (5,000 RPM for 15 min, IEC, clinical centrifuge, UK). The clarified supernatant was used as the source of the enzyme.

2.6.2. Amylase Assay

Amylase was assayed using the method described by Bernfield (1955) with some modifications. The substrate was 1% (w/v) boiled starch. The reaction mixture contained 3ml substrate, 3 ml buffer and 3 ml appropriately diluted supernatant. It was incubated at various temperatures (between 20° C and 80°C) with shaking for 15-30 minutes. Regarding pH, the following buffer systems (0.1m) were evaluated: glycine -HCl, pH 3,3.6,acetate, PH 4,4.6,5,5.6; Phosphate,6,6.5,7,7.5; Tris-HCl, 8,8.6,9; Glycine-NaOH,9.6, 10, 10.6.

2.6.3 Estimation of reducing sugars

The estimation of reducing sugars was carried out using a modified dinitrosalicylic acid reagent (Miller, 1959).

This reagent contained the following:

- 1% 3,5-dinitrosalicylic acid
- 0.2% phenol
- 0.05% Sodium sulfite
- 1% Sodium hydroxide
- 20% Potassium sodium tartarate

From the reaction mixture, 1 ml was removed and mixed with 2 ml of dinitrosalicylic acid reagent. This was heated for 5 min in boiling water and then cooled in tap water. After addition of 7 ml of water the absorbance was measured at 540 nm against a blank which contained all the reagents except the culture filtrate. The sample values were corrected for the enzyme blanks. The optical density readings were then transformed to µg reducing sugars using glucose as a standard. One unit of enzyme activity was defined as µ mole glucose equivalent produced in one min per ml culture filtrate under the stated assay conditions.

Experiments were duplicated and results represent mean values.

2.6.4. Effect of metal ions on amylase activity

The effects of $AlCl_3$, $BaCl_2$, $CaCl_2$, $CoCl_2$, $CuSO_4$, $FeSO_4$, KCl, $MgSO_4$, $MnSO_4$, NaCl and $ZnCl_2$ on enzyme activity were investigated. The metal ions (at 5 mM) were added to the assay mixture.

2.6.5. Heat Stability

The culture filtrate was heated in a water bath at 60°C, 65°C and 70°C. The tubes were taken out after 10 minute intervals and chilled. The residual activity was checked by the assay method.

2.6.6. pH Stability

The culture filtrate at a desired pH was kept at 4°C for 24 hour and then used for activity measurements. The buffer systems were acetate, pH 4,4.5,5.5; Phosphate 6,6.5,7; Tris - Hcl, 8,8.5,9.

2.6.7 Salt tolerance

The culture filtrate was incubated in 0.1 M acetate buffer, pH 5.6, containing various NaCl concentrations (0.5,1,2,3,4,5M) for 24 hour at 4°C and residual activity was determined as usual.

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2.7 Determination of Extracellular,Cytoplasmic and Cell Bound Enzymes

Culture grown on the defined medium (100ml) was harvested on the 3rd day and centrifuged (4,000 RPM ,15 min). The supernatant was used as the source for extracellular amylase. The cell pellet was washed with distilled water and centrifuged (5,000 RPM,15 min) to remove trace amounts of extracellular enzymes. The pellet was resuspended in 30 ml o.1 M acetate buffer (pH 5.6) and the cells were broken by sonication (sonicator, MSE 150, west Germany). The disrupted cellular materials were centrifuged (50,000 x G, 15 min at 4°C) using a refrigerated centrifuge (Heraeus centrifuge, west Germany). The resulting supernatant served as the source for cytoplasmic amylase. The cell pellet was resuspended in 30 ml acetate buffer(pH 5.6) and was used to determine the cell bound amylase.

2.8. Chromatographic Analysis of Sugars

Chromatographic analysis of the supernatant from the medium containing 1% soluble starch was carried out at room temperature (19-23°C).

Paper chromatography for detection of sugars in the hydrolysates of 1% amylose and pullulan was also carried out. One ml of appropriately diluted culture filtrate was

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mixed with 1ml of 1% amylose or pullulan and 1 ml of 0.1 M acetate buffer (pH 5.6). After incubation for 15 min at 40°c in an orbital incubator shaker, the products of hydrolysis were analysed.

The solvent system was n-butanol-acetic acid-water (2:1:1 by Vol.). Spots were detected using aniline diphenylamine reagent. The standard reducing sugars such as glucose, maltose and maltotriose were obtained from sigma, USA.

3. RESULTS

3.1. Isolation and characterization of Bacillus species.

From a number of amylolytic cultures isolated from fermenting kocho and tef two bacterial strains were selected for further work. The strains were Gram-Positive, rod shaped, endospore-forming and catalase-positive. These characters suggest that they belong to the genus Bacillus.

Cultural studies indicated that Bacillus Sp. T 31 adheres strongly to agar and had hair-like outgrowth. It had white, lobate and flat colonies with mounds and was isolated from fermenting tef. Bacillus sp.k6 had cream, small, flat and round colonies and was isolated from fermenting kocho. Other properties of the two strains are listed in Table 1. Bacillus sp.k6 and Bacillus sp.T 31 were identified as Bacillus subtilis and Bacillus licheniformis, respectively (Buchanan and Gibbons, 1974; Claus and Berkeley, 1986).

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Table 1. Properties of Bacillus subtilis k6 and B. licheniformis T 31.

Properties	Bacillus subtilis k6	Bacillus licheniformis T 31
1. Cell diameter	<1.0 µm	< 1.0 µm
2. Shape and position of spore	ellipsolidal and central	ellipsoidal and central
3. Growth pH range (in nutrient broth)	4.5 - 9.5	5 - 10
4. growth (pH7)at		
a. 10°c	+ (poor)	-
b. 30°c	+	+
c. 40°c	+	+
d. 50°c	+	+
e. 55°c	-	-
f. 65°c	-	-
5. Anaenobic growth	-	+
6.Voges-Proskauer test	+	+
7. pH in v-p broth		
a) <6	+	+
b) > 7	-	-
8. Acid from		
a) D-glucose	+	+
b) D-xylose	+	+
c) L-arabinose	+	+
d) Sucrose	+	+
e) Lactose	-	+
9. Gas from glucose	-	-
10. Hydrolysis of		
a) casein	+	+
b) gelatin	+	+
c) starch	+	+
11. Nitrate reduced to nitrate	+	+
12. Utilization of		
a) citrate	+	+
b) propionate	+	+
13. Formation of		
a) indole	-	-
b) dihydroxyacetone	-	+

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14. Growth at pH		
a)6.8,nutrient broth	+	+
b) 5.7	+	+
15. Growth in NaCl		
a) 5%	+	+
b) 7%	+	+
c) 10%	+	+
d) 12%	+	-

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3.2 Effect Of Various Carbon Sources On Biomass and Enzyme Production

Different types of carbon sources were tested in order to determine their effect on growth of the test organisms and production of amylase. To determine the optimum carbon source, each carbon source was added to the basal medium (BM).

3.2.1. Soluble Starch as a Carbon Source:

The effect of different concentrations of starch on growth and enzyme yield was studied (figure 2,3). The results show the concentrations of soluble starch had differnt effects on biomass formation and enzyme production. In the absence of starch,i.e. the basal medium only,cell growth was slow and amylase production was not detected until after 72 hour of incubation in both organisms. At 72 hour incubation time, the addition of 0.5% starch resulted in increased production of biomass. The amylase produced in the basal medium was insiginificant. However,amylase production was higher when 0.5% starch was added in the medium. Increasing starch concentration from 0.5% to 1% resulted in further increase in Bacillus subtilis K6 and Bacillus licheniformis T 31 amylase activities. At higher concentrations of the substrate (2%,3%) the yield of amylase was reduced. The optimum concentration of soluble starch for both biomass and amylase production was found to be 1%.

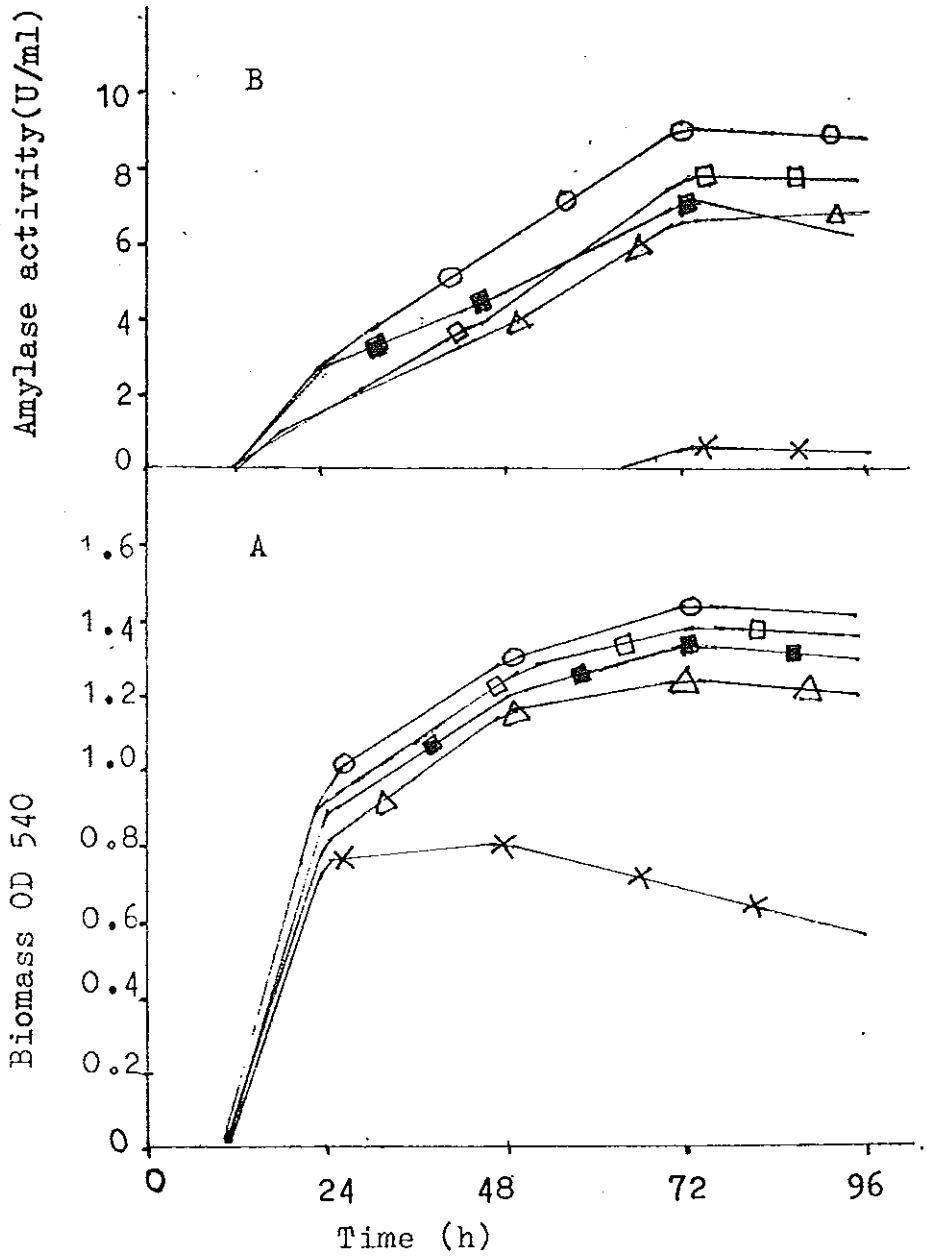

Fig.2. Effect of soluble starch on biomass formation(A) and enzyme production (B) by Bacillus subtilis k6 .

× , 0% starch
Δ , 0.5% starch
○ , 1% starch
□ , 2% starch
■ , 3% starch

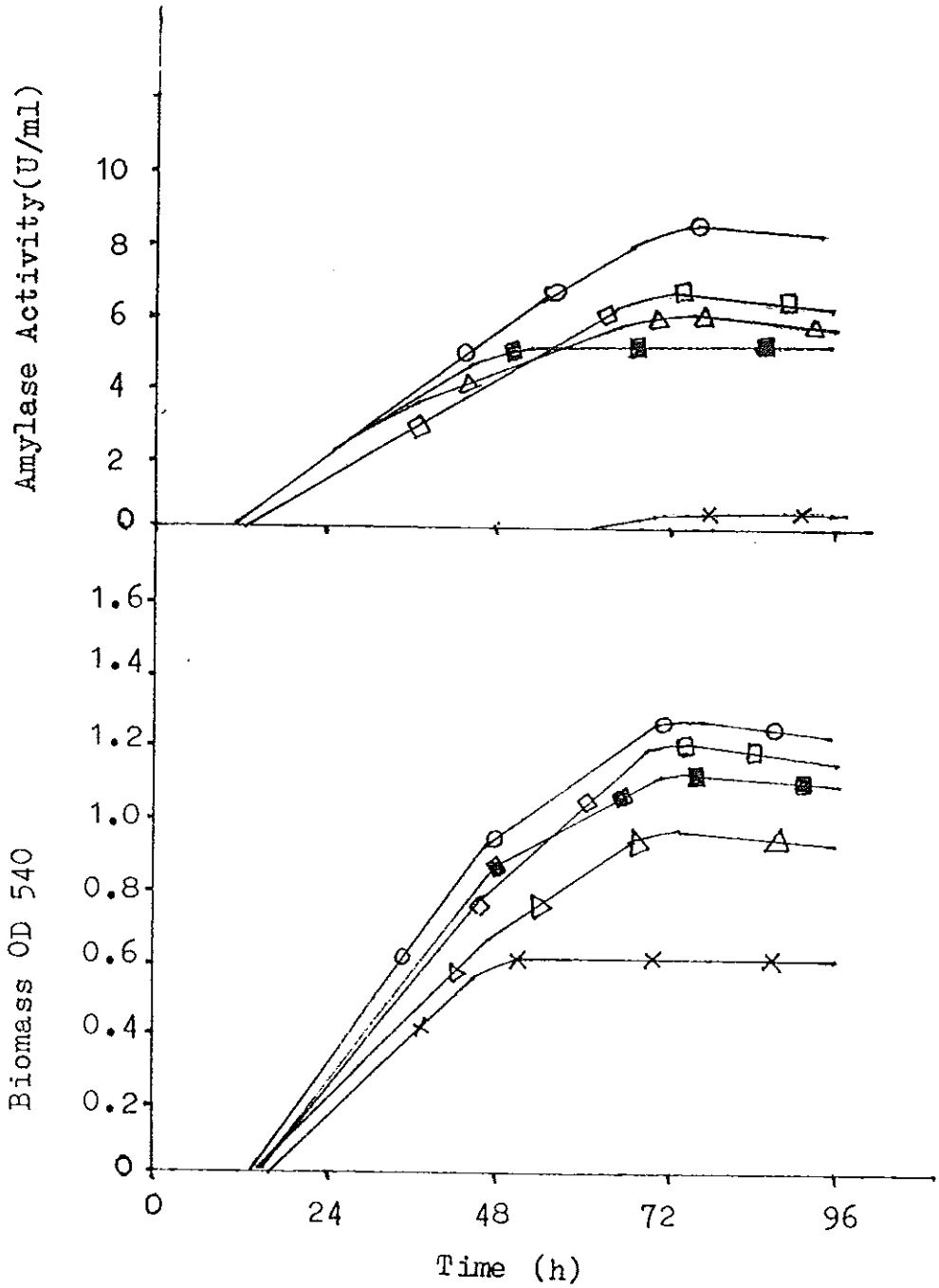

Fig. 3 Effect of soluble starch on biomass formation (A) and enzyme production (B) by Bacillus licheniformis T31.

× , 0% starch
△ , 0.5% starch
○ , 1% starch
□ , 2% starch
▣ , 3% starch

3.2.2. Alternative Carbon Source

Table 2 shows that growth and amylase production were influenced by the type of carbon sources. The incubation time was 72 hour. The result showed that maximum production of amylase and biomass formation occurred with 1% (w/v) starch compared to other carbon sources.

Table 2 . Effect of some carbon sources on growth and amylase production by Bacillus subtilis k6 and Bacillus licheniformis T 31.

carbon source 1% (w/v)	B. subtilis k6		B.licheniformis T 31	
	Biomass OD 540 72 h	Amylase activity(%) 72 h	biomass OD 540 72 h	Amylase activity(%) 72 h
Soluble starch +BM	1.53	100.0	1.30	100.0
glucose + BM	0.76	4.2	0.66	3.8
Lactose + BM	0.70	68.0	0.62	71.0
maltose + BM	1.30	84.0	1.00	78.0
sucrose+BM	0.65	4.6	0.56	3.6
basal medium (BM)	0.62	5.2	0.50	3.8

Amylase activity observed in the presence of starch was taken as 100.(Its activity was 9.4 units.ml for B. subtilis ; 9 u/ml for B. licheniformis

Amylase production was not induced by glucose and sucrose. Other carbon sources did show marked change in amylase production compared to the medium without a carbon source. Next to soluble starch, maltose stimulated biomass formation and enzyme production in both test organisms. Growth was slow when lactose was used as a carbon source; however,it stimulated amylase production.

3.2.3. Native Starch as a Carbon Source:

The effects of barley, corn,kocho, tef, and wheat flour was studied on biomass formation and enzyme production (Table 3). The incubation time was 72 hour. Each carbon source was added to the basal medium.

Growth was estimated by determining cellular Protein since cultures containing native starches were too turbid to be determined using the spectrophotometer.

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Table 3. Effect of native starch on biomass formation and enzyme production.

Carbon source 1%	Protein content of cells (mg/100ml)		Amylase activity (%)	
	Bacillus subtilis k6	Bacillus licheniformis T 31	Bacillus subtilis k6	Bacillus licheniformis T 31
Barley	76	78	96.0	94.3
Corn	65	58	78.2	87.0
Kocho	85	86	100.0	100.0
Tef	68	72	78.3	81.0
Wheat	82	83	98.3	96.0
Basal medium	8	6	11.3	8.9

The 100% activity in units/ml for Bacillus subtilis k6 = 9.1; Bacillus licheniformis T 31 = 8.6.

The results showed that native starches were good carbon sources for growth and amylase production (Table 3). The enzyme yield and growth were much greater in a medium containing native starches compared to that obtained from a medium without a carbon source. The enzyme production when using kocho (Table 3), was highest. All native starches which were employed as a carbon source stimulated enzyme production in varying degrees in both test organisms.

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3.3. Effects of different nitrogen Sources on biomass formation and enzyme production

Using the basal medium and 1% (w/v) soluble starch as a carbon source, different types of nitrogen sources were tested in order to determine their effect on growth of the test organisms and production of amylase (Fig.4-7) Ammonium chloride, peptone, potassium nitrate and urea at different concentrations were employed. Both the organic and inorganic nitrogen sources enhanced biomass formation and enzyme production. But all the nitrogen sources did not stimulate amylase production equally. In the absence of the nitrogen source, growth of the organisms was slow and amylase production was very low.

The results (Fig. 4,6) show that the best growth and amylase production were obtained with peptone. Regarding the concentration, 0.5% peptone resulted in higher biomass formation and amylase production than with higher concentration (1%,2%). Hence 0.5% (w/v) peptone was employed as the nitrogen source in subsequent experiments for both test organisms.

Ammonium chloride was the next important nitrogen source for biomass formation and enzyme production.

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Growth and amylase production were lower with urea and potassium nitrate compared to peptone and ammonium chloride for both test organisms. The lowest biomass formation and amylase production were obtained with potassium nitrate (Fig.5,7)).

Hereafter, the optimized medium (OM) consisting of o.5% peptone, 1% soluble starch and the basal medium was used for biomass formation and amylase production.

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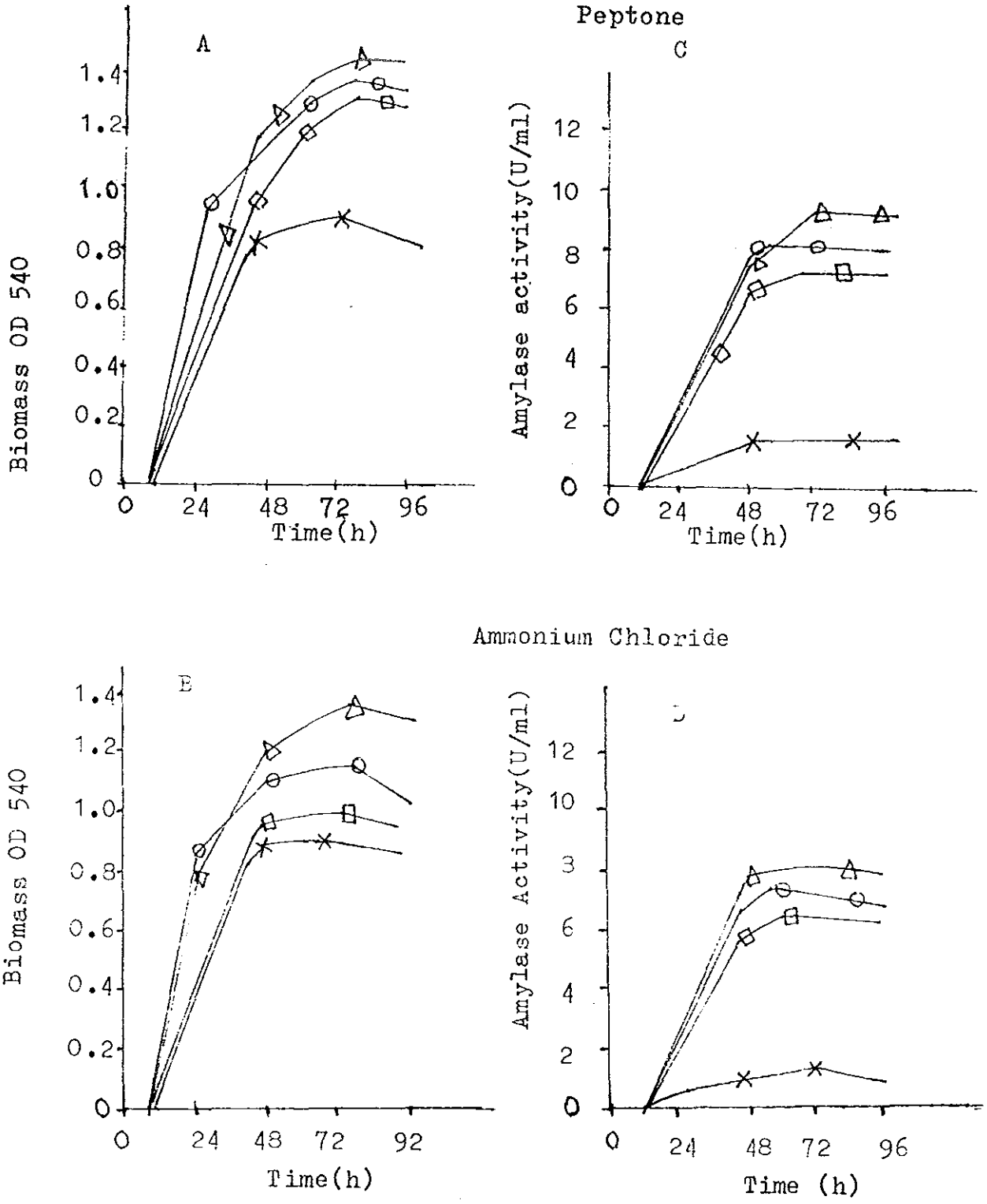

Fig.4. Effect of peptone and ammonium chloride on biomass formation (A,B) and amylase production (C,D) by Bacillus subtilis k6.

×, 0% peptone (A,C) ; NH_4Cl (B,D)
Δ, 0.5% peptone (A,C) ; NH_4Cl (B,D)
○, 1% peptone (A,C) ; NH_4Cl (B,D)
□, 2% peptone (A,C) ; NH_4Cl (B,D)

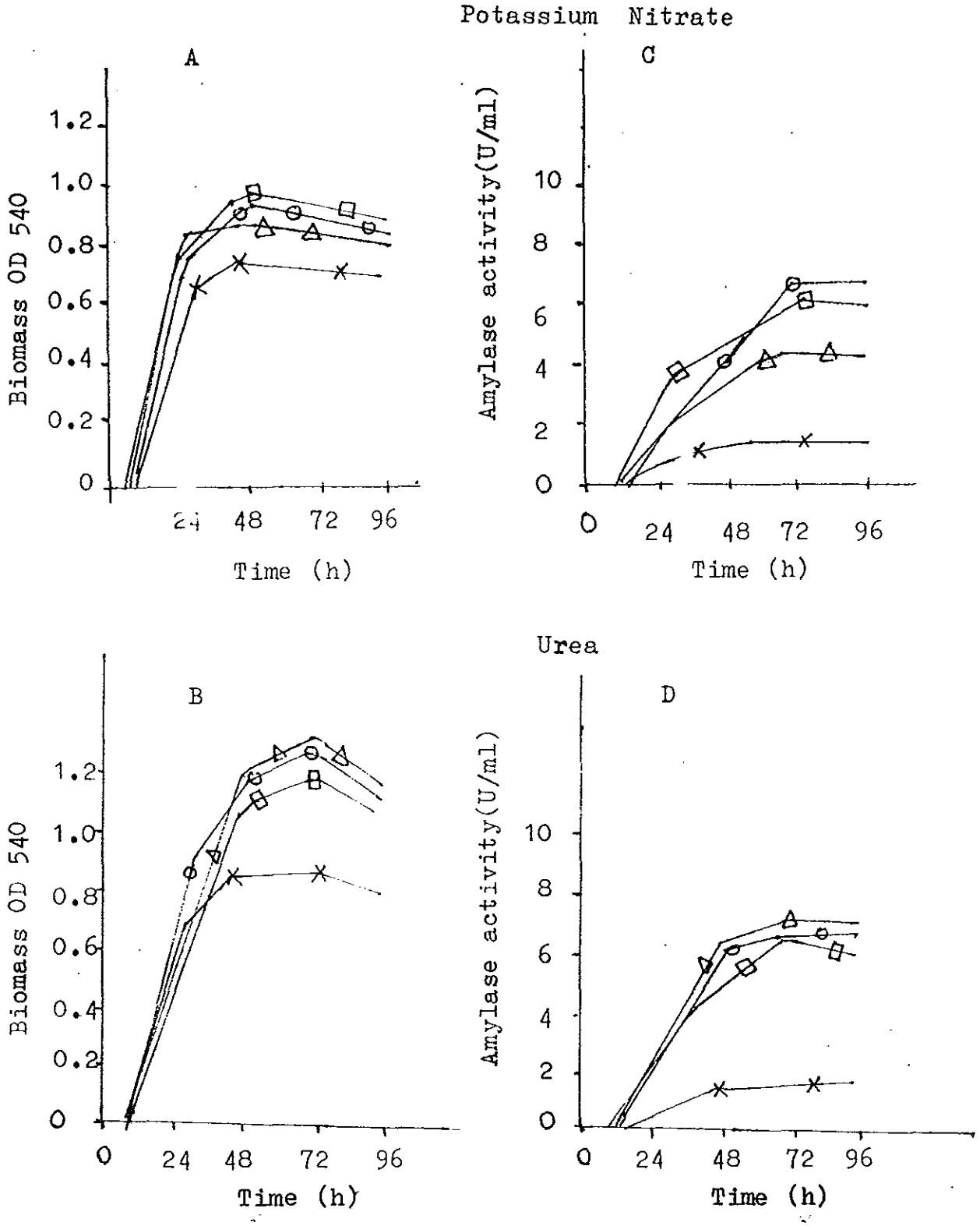

Fig 5. Effect of potassium nitrate and Urea on biomass formatio: (A,B) and enzyme production (C,D) by Bacillus subtilis k6.

0% KNO3 (A,C); Urea (B,D)
Δ, 0.5% KNO3 (A,C); Urea (B,D)

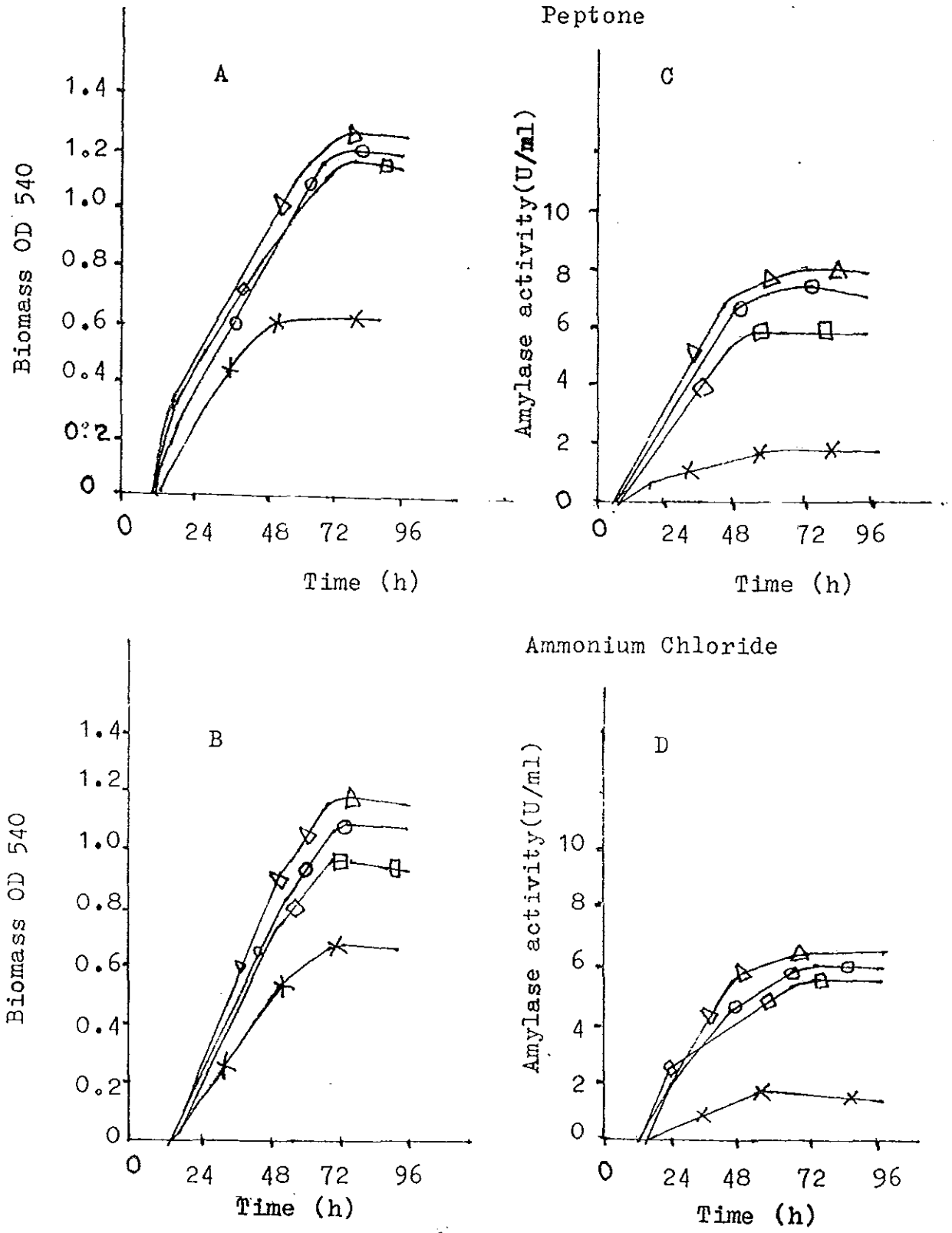

Fig.6. Effect of peptone and ammonium chloride on biomass formation (A,B) and enzyme production (C,D) by Bacillu licheniformis T31.

× 0% peptone (A,C) ; NH_4Cl (B,D)
△ 0.5% peptone (B,D)
○ 1% peptoi (B,D)
□ 2% peptou (B,D)

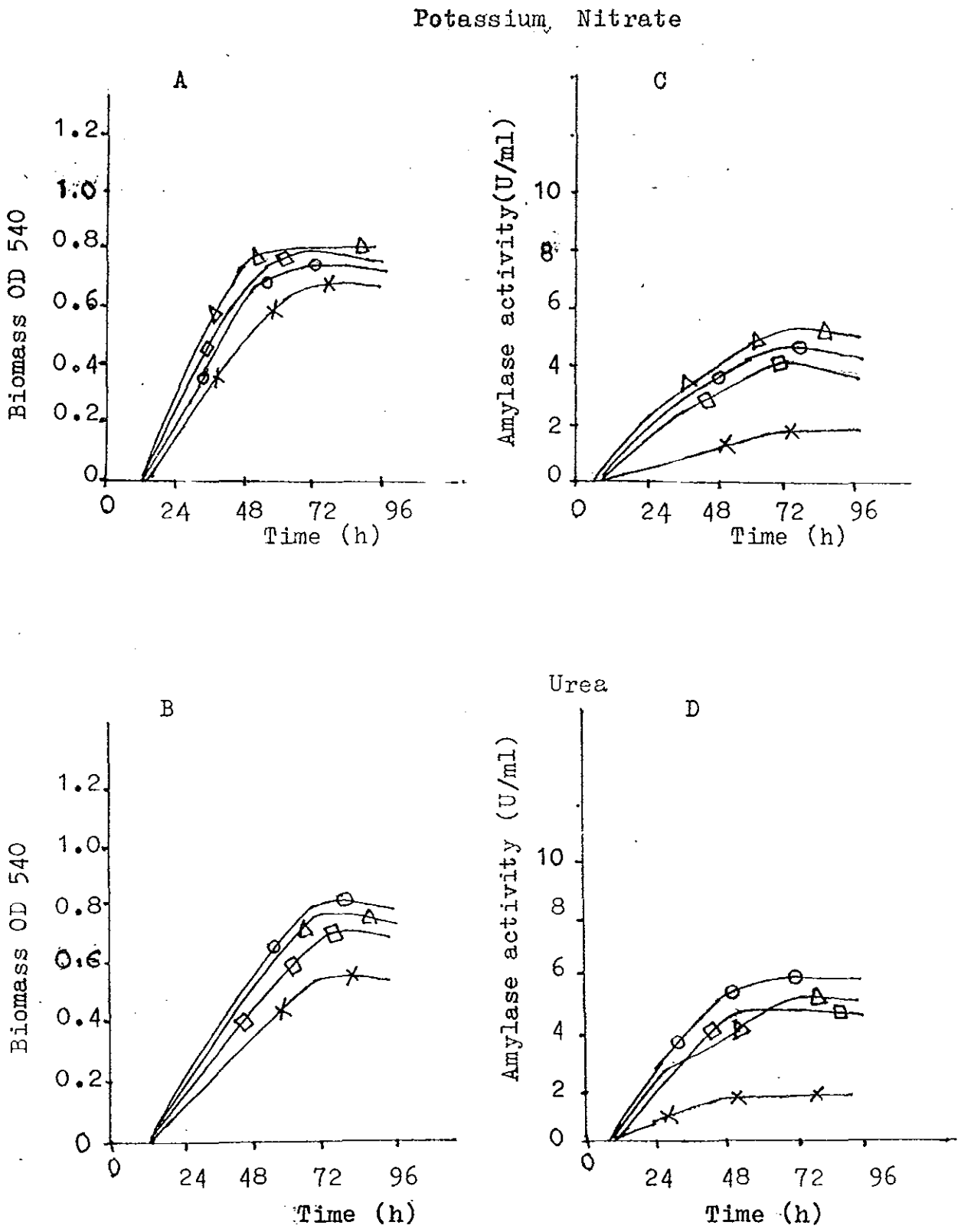

Fig. 7. Effect of potassium nitrate and urea on biomass formation (A,B) and enzyme production (C,D) by Bacillus licheniformis T31.

× 0% KNO_3 (A,C) ; Urea (B,D)
% KNO_3 (A,C) ; Urea (B,D)
KNO_3 (A,C) ; Urea (B,D)
KNO_3 (A,C) ; Urea (B,D)

3.4. Effect of temperature on biomass formation and enzyme production

Using the optimized medium, the effects of temperature (between 20°C and 50°C) on growth and enzyme production were investigated. (Table 4).

Biomass formation was high between 20°C and 40°C. However, biomass formation was low when the temperature was raised to 50°C. During the first 8 hour of cultivation, biomass was better at higher temperatures than at 20°C.

The result shows that maximum amylase production occurred at room temperature at 72 hour of cultivation. These conditions were employed in subsequent experiments.

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Table 4. Effect of termperature on biomass formation and amylase production by Bacillus subtilis k6 and Bacillus licheniformis T 31.

Temperature °C	Time h.	Biomass (OD 540) B.subtilis k6	Biomass (OD 540) B.licheniformis T 31	Amylase activity(u/ml) B.subtilis K6	Amylase activity(u/ml) B.licheniformis T 31
Room temp. 18-23	8	0.15	0	0	0
	12	0.6	1.1	1.2	0.06
	24	1.15	0.8	3.6	1.6
	48	1.3	1.2	6.0	5.2
	72	1.33	1.25	9.6	9.2
	96	1.26	1.2	9.4	8.9
30	8	0.33	0.14	0	0
	12	0.7	0.32	1.28	0.2
	24	1.2	0.8	3.8	2.2
	48	1.3	1.1	5.8	5.0
	72	1.35	1.25	9.2	8.8
	96	1.2	1.15	9	8.6
40	8	0.60	0.52	0.4	0.27
	12	0.84	0.73	3.2	1.7
	24	1.2	1.0	4.1	3.0
	48	1.25	1.1	4.8	4.6
	72	1.3	1.25	8.8	8.5
	96	1.3	1.2	8.6	7.6
50	8	0.3	0.42	0	0
	12	0.45	0.58	0.85	1.1
	24	0.5	0.6	1.2	1.5
	48	0.52	0.73	1.3	1.8
	72	0.54	0.78	1.6	2.2
	96	0.48	0.75	1.1.	1.2

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3.5 Effect of pH of the medium on biomass formation and enzyme production

Using the optimized medium (OM) and room temperature, the effects of pH of culture medium on growth and enzyme production was investigated.

Table 5. Effect of medium pH on biomass formation and amylase production after 72 hour incubation.

	Biomass (OD 540)		Amylase Activity (%)	
pH	Bacillus subtilis k6	Bacillus licheniformis T 31	B.subtilis k6	B.licheniformis T 31
4.5	0.9	0	23.0	0
5.0	1.1	0.95	58.6	38.7
5.5	1.2	1.0	78.6	58.7
6.0	1.33	1.22	87.6	76.0
6.5	1.4	1.32	98.3	88.0
7.0	1.53	1.45	100.0	96.2
7.5	1.5	1.52	97.6	100
8.0	1.45	1.5	92.0	93.7
8.5	1.43	1.45	88.3	89.3
9.0	1.4	1.43	81.4	86.0
9.5	1.3	1.32	78..0	73.2
10.0	0	0.86	0	38.0

100% activity was 9.5 units/ml for Bacillus subtilis k6; 9.1. units/ml for Bacillus licheniformis T31.

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was included

Table 5 shows that Bacillus subtilis k6 and Bacillus licheniformis T 31 had a pH range of 4.5 - 9.5 and 5-10, respectively for growth and amylase production. Bacillus subtilis k6 had a pH optimum in the range of pH 6.0 8.5,while Bacillus licheniformis T 31 had an optimum pH between 6.5 and 9. The highest peaks of activity were found to be at pH 7 and 7.5 for Bacillus subtilis k6 and Bacillus licheniformis T 31,respectively. These optimum pH for maximum amylase production were used in subsquent experiments.

3.6. Effect of metal ions on biomass formation and amylase production

Table 6 shows the effects of different concentrations of metal ions on growth and amylase production of Bacillus subtilis k6 and Bacillus licheniformis T31, respectiely. Solutions of the metal ions were autoclaved separately and added to the optimized media.

Cells of both test organisms failed to grow in the presence of $HgCl_2$, $ZnSO_4$ and $CuSO_4$ (at 1 and 5 mM concentrations). Biomass formation and amylase production were low when 5 mM Pb $(CH_3COO)_2$,$FeSO_4$ or $AlCl_3$ were added to the medium.

However, maximum production of amylase was obtained with $MgSO_4 . 7H_2O$ for both test organisims. Therefore, this comp[illegible] in the medium [illegible]r enzy[illegible] production.

Table 6. Effect of metal ions on biomass formation and amylase production by Bacillus subtilis k6 and Bacillus licheniformis T31.

Ions	Addition mM	Biomass (OD 540) B. subtilis k6	Biomass (OD 540) B. licheniformis T 31	Amylase activity (%) B. subtilis k6	Amylase activity (%) B. licheniforimis T 31
None	-	1.6	1.55	100	100
$AlCl_3$	0.1	1.4	1.35	87.3	91
	1.0	1.35	1.45	81.0	86
	5.0	0.8	0.6	23.0	28.3
$CaCl_2$	0.1	1.5	1.3	108.0	105
	1.0	1.5	1.35	97	98
	5.0	1.5	1.3	98.5	96.8
$CuSO_4$	0.1	1.25	1.2	28	31.3
	1.0	0.05	0.06	0	0
	5.0	0	0	0	0
$FeSO_4$	0.1	1.55	1.3	87.8	94
	1.0	1.6	1.2	75.3	87
	5.0	0.23	0.8	11.0	68
$HgCl_2$	0.1	0	0	0	0
	1.0	0	0	0	0
	5.0	0	0	0	0
$MgSO_4$	0.1	1.5	1.43	116	126.3
	1.0	1.5	1.55	136.2	128
	5.0	1.5	1.6	121	118
$MnSO_4$	0.1	1.55	1.5	94.2	83.6
	1.0	1.4	1.4	89.4	96.8
	5.0	1.3	1.4	82.1	88
NaCl	0.1	1.5	1.4	98.0	94
	1.0	1.5	1.5	96.1	93
	5.0	1.5	1.5	97.3	88.7
$Pb(CH_3COO)_2$	0.1	1.2	1.1	21.3	38.0
	1.0	1.1	0.9	17.0	26.3
	5.0	0.5	0.6	13.2	23.3
$ZnSO_4$	0.1	0.04	0.06	0	0
	1.0	0.03	0	0	0
	5.0	0	0	0	0

3.7. Effect of detergents on biomass formation and amylase activity

The effect of Tween 20, Tween 80, sodium lauryl sulfate, Triton x -100 and Tergitol 7 on growth and amylase production was studied using the optimized medium. The concentrations employed were 0.05% , 0.1% and 0.2%.

Tergitol 7 inhibited growth and amylase production of the test organisms. Growth was very poor when Triton x-100 was added to the medium and amylase activity was not detected when this detergent was used (Table 7).

Sodium Lauryl Sulfate at the concentration of 0.2% inhibited growth and amylase production of both test organisms. However, at 0.05% and 0.1% concentration growth and amylase activity were low.

Tween 20 and Tween 80 were better compared to other detergents mentioned above. These detergents did not inhibit or stimulate growth and amylase production at the concentrations used.

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Table 7. Effect of detergents on biomass formation and amylase production by Bacillus subtilis k6 and Bacillus licheniformis T 31

Detergent	Concentration%	B.subtilis k6		B.licheniformis T 31	
		Biomass OD 540	Activity %	Biomass OD 540	Activity %
None	-	1.40	100	1.0	100
Tween 20	0.05	1.35	98	1.1	94
	0.1	1.01	89	1.0	91
	0.2	1.1	91.3	0.9	88.3
Tween 80	0.05	1.32	97.1	1.1	100
	0.10	1.22	94	1.0	95
	0.2	1.07	87.3	1.1	91
Sodium Lauryl Sulfate	0.05	1.10	31	1.1	23
	0.10	1.0	28	0.8	19
	0.20	0	0	0.04	0
Triton X -100	0.05	0.2	0	0.25	0
	0.10	0.11	0	0.2	0
	0.20	0.10	0	0.13	0
Tergitol 7	0.05	.05	0	0	0
	0.10	0	0	0	0
	0.2	0	0	0	0

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3.8 EFFECT OF pH AND TEMPERATURE ON ENZYME ACTIVITY.

3.8.1. Assaying pH of amylase activity: The effects of pH on amylase activity was determined using buffers between pH 3 and 10. The culture filtrate for enzyme-substrate reaction was obtained after growing the test organisms in the optimized medium.

The amylases of both test organisms were inactive below pH 4 (Fig. 8A,9A). Bacillus subtilis K6 amylase activity increased when the assay pH was raised from 4.6 to 5.6. Thereafter, activity declined as the assaying pH increased. Thus the optimum assay pH for amylase activity of Bacillus subtilis K6 was found to be 5.6. However, two pH optima were ebserved for Bacillus licheniformis T 31 amylase, one at 5.6 and the other at 8.

3.8.2. Assaying temperature for amylase activity;

The effect of temperature on amylase was assayed between 20^0C and 70^0C. The pH of the assay buffer was set at 5.6. The amylases of both test organisms showed similar responses with respect to temperature (Fig.8B, 9B).The amylase activity increased when incubation temperature was raised from 20^0C to 40^0C The activity decreased with further increases in temperature. From this study, The optimum assaying temperature for maximum activity was found to be 40^oC.

3.9. Heat stability of amylase

To test thermal stability of the enzyme, the culture filtrate was held at different temperatures (60°C, 65°C and 70°C) from 10 to 60 minutes in acetate buffer (pH 5.6). After cooling immediately the residual activity was measured.

Fig. 10 shows that the enzymes of both test organisms were stable at 60°C. Bacillus licheniformis T 31 amylase was more stable at 65°C and 70°C than Bacillus subtilis K6 amylase. About 80% and 72% of the activity of Bacillus licheniformis T 31 amylase remained after heating at 65°C and 70°C, respectively, for 60min. However, about 60% and 45% of the activity of Bacillus subtilis K6 amylase remained after heating at 65°C and 70°C respectively, for 60 min.

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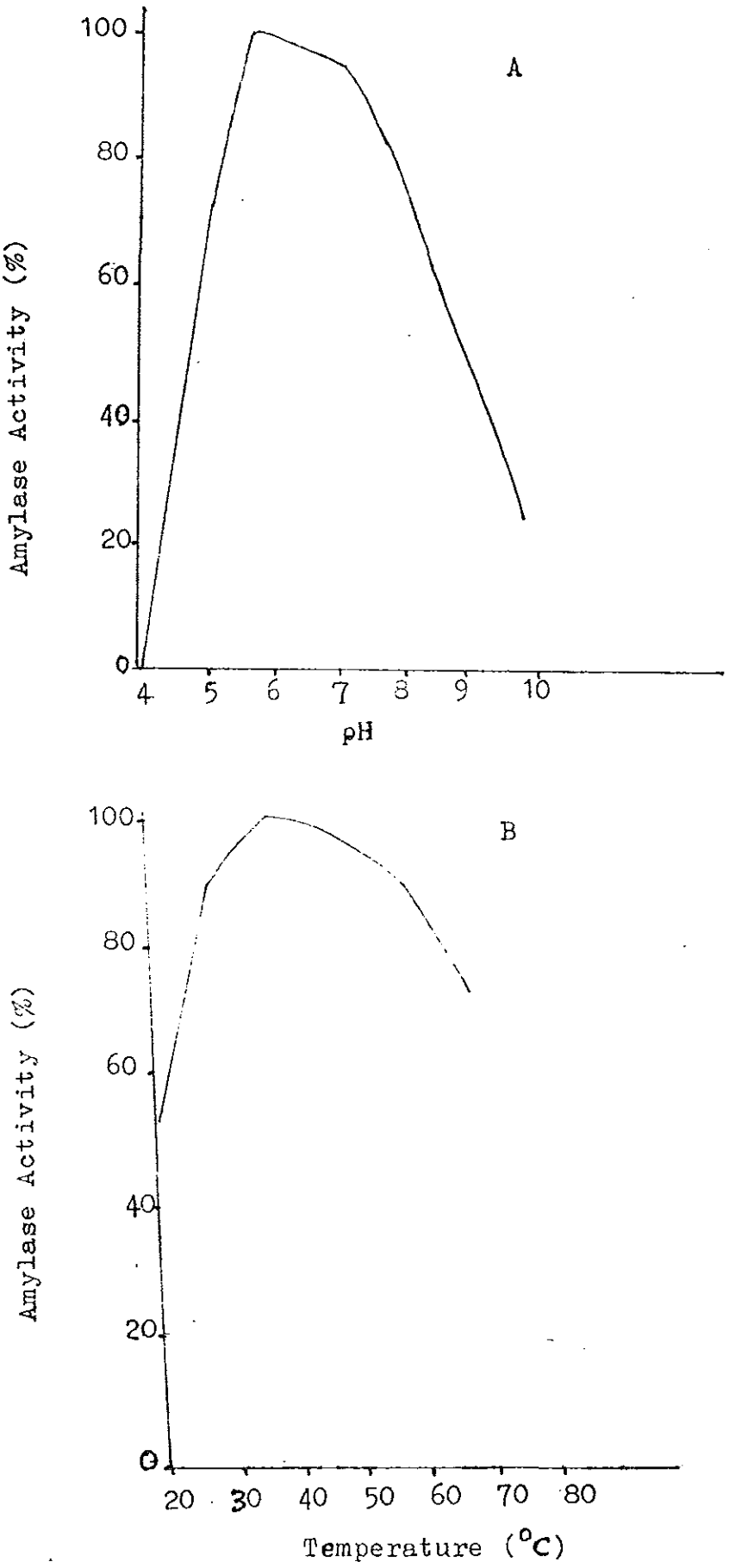

Fig. 8 Effect of pH (A) and Temperature (B) on B. subtilis

% amylase activity

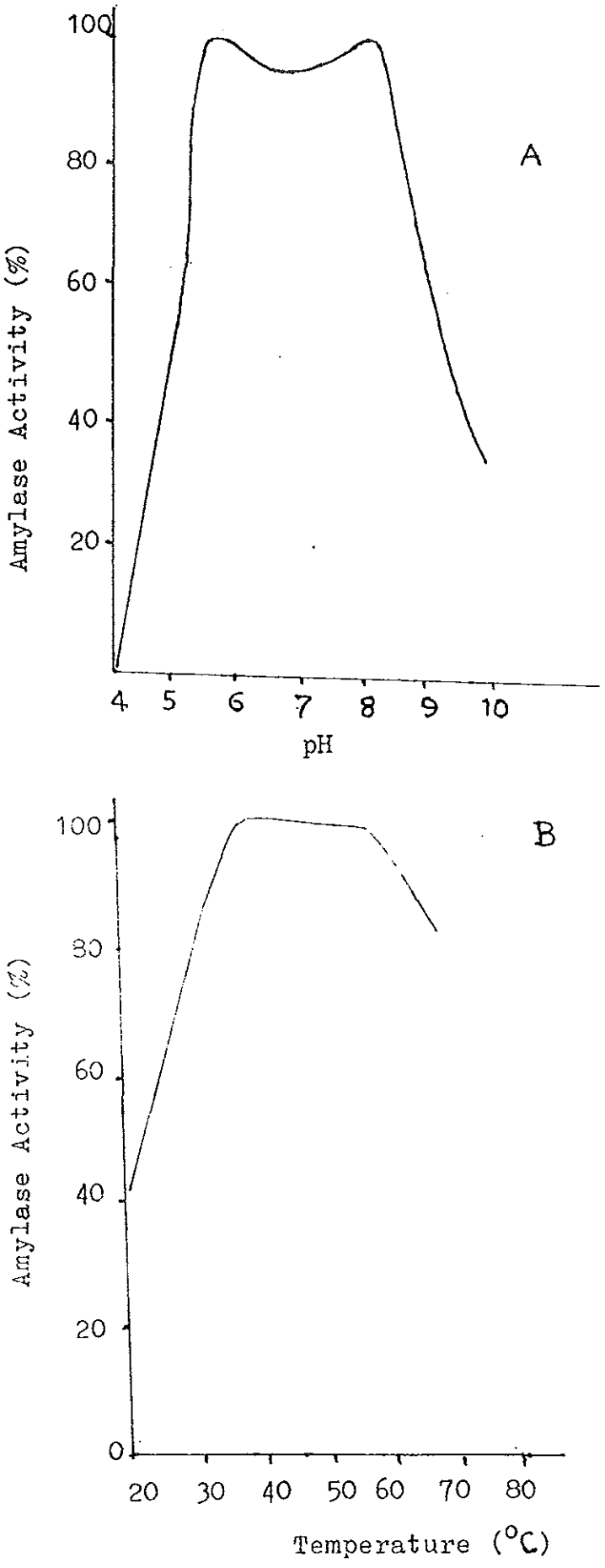

Fig.9 Effect of pH(A) and Temperature (B) on B. licheniformis T31 amylase activity.

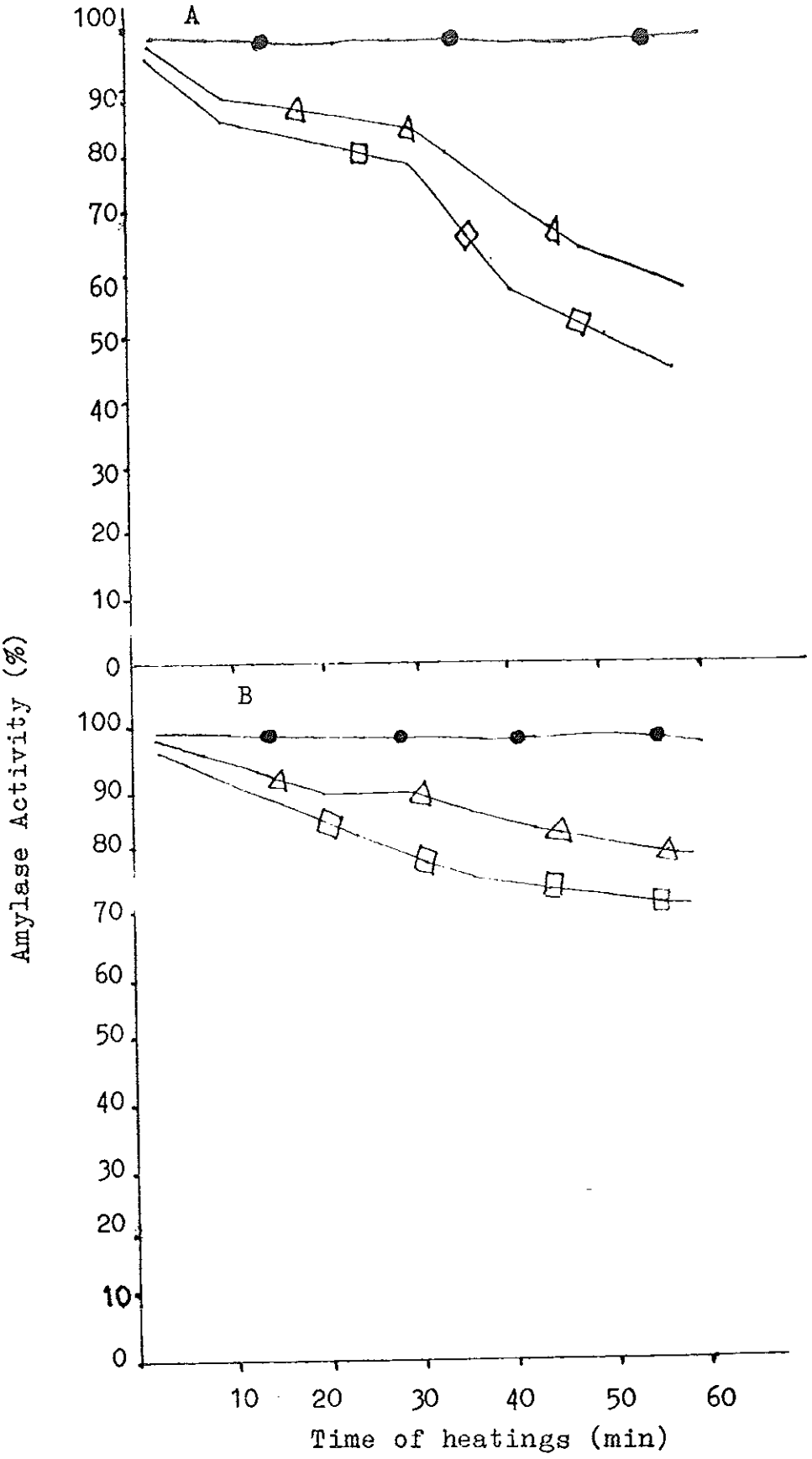

Fig.10.Thermostability of B.subtilis K6 amylase (A) and B. licheniformis T31 amylase (B) at 60°C (●), 65°C (△) and 70°C (□).

3.10. pH stability of amylases

pH stability was studied using buffers between pH 3 to 9. The culture filtrate was kept at 4°C for 24 hour. The residual amylase activity was measured.

Bacillus subtilis k6 and Bacillus licheniformis T31 showed similar responses with respect to pH stability (Table 11). The stability increased when the pH was raised from pH 4.5 to 5.5. The enzymes were stable in the pH range 5.5 - 8.5. The stability declined as the pH increased from 8.5 to 9.

Table 11. Effect of pH on stability of amylases after incubation at various pH values for 24 h.

pH	Residual Amylase Activity (%)	
	Bacillus subtilis k6	Bacillus licheniformis T 31
4	0	0
4.5	25	19
5	65	52
5.5	95	83.4
6.5	97.3	90.1
7	100.0	100.0
8	96.3	98
8.5	94.6	95
9	73.0	73

3.11 Effect of metal ions on amylase activity

Using the optimised medium, the effect of different metal ions on amylase activity was investigated (Table 9) Each metal salt (at 5 mM) was added to the assay mixture. Of the metal ions tested $CuSO_4$ and $ZnCl_2$ inhibited the enzyme activity.The addition of $MgSO_4$, $CaCl_2$, $CoCl_2$ and $BaCl_2$ stimulated enzyme activity.

Table 9. Effect of metal ions on amylase activity.

Metal ions	Amylase Activity % Bacillus subtilis k6	Bacillus licheniformis T 31
None	100	100
$AlCl_3$	96	92.3
$BaCl_2$	108	111.6
$CaCl_2$	124.2	118
$CoCl_2$	116.3	113
$CuSO_4$	23	19.6
$FeSO_4$	87.3	96
KCl	97	91.4
$MgSO_4$	119.4	126
$MnSO_4$	93	89.3
NaCl	96.4	98.1
$ZnCl_2$	23.5	[illegible]

3.12 Salt tolerance of amylases

The culture filtrate was incubated in acetate buffer (pH 5.6) containing different concentrations of NaCl (0-5M) for 24 hours at 4°C. The residual activity was measured (Table 10). The activity of the amylases of both test organisms decreased when the concentration of NaCl increased. No activity was observed at 5 M NaCl. The amylase of Bacillus subtilis k6 was relatively tolerant to salt compared to the amylase of Bacillus licheniformis T 31 . It was stable in 1M Nacl with 60% of the original activity being retained.

Table 10. Effect of NaCl on the activity of amylase

NaCl Concentration(M)	Residual Amylase Activity %	
	Bacillus subtilis k6	Bacillus licheniformis T 31
0	100	100
0.5	78	64.6
1.0	60.2	42.0
2.0	38	21.5
3.0	26	15.0
4.0	14	8.3
5.0	0	0

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3.13. Chromatographic analysis of sugars

Figures 11A and 12A show the course of hydrolysis of soluble starch in the medium by the Bacillus species.The paper chromatographic analysis of the supernatant showed presence of the products of starch hydrolysis such as glucose, maltose, maltotriose.Glucose and maltose were not detected between 0-8 h. Maltotriose was present at 8 h and its concentration decreased as the incubation time increased.

Fig.11B shows the products of the action of amylase from Bacillus licheniformis T 31 on amylose and pullulan. The action on amylose produced maltose and maltotriose as the main product. The enzyme did not act on pullulan.

On the other hand, maltotriose, maltose and glucose were produced by the action of amylase from Bacillus subtilis k6 on amylose (fig.12B).The enzyme from this species also acted on pullulan and produced maltotriose.

3.14 Determination of Extracular Cytoplasmic and cell bound enzymes

The presence of amylolytic enzymes in the culture filtrate, cytoplasm and cell debris of the test organisms were investigated. Table 11 shows the percent distribution of amylases among these cell fractions.

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Table 11. The distribution of amylases among extracellular, intracellular and cell bound amylases.

Enzyme Source	Enzyme activity %	
	Bacillus subtilis k6	Bacillus licheniformis T 31
Culture filtrate	80.4	69
Cytoplasm	4.6	3.6
Cell debris	15	27.4

The result shows that the activities of the amylolytic enzymes released in the medium (i.e, extracellular) were considerably higher than the cytoplasmic or cell bound amylases.

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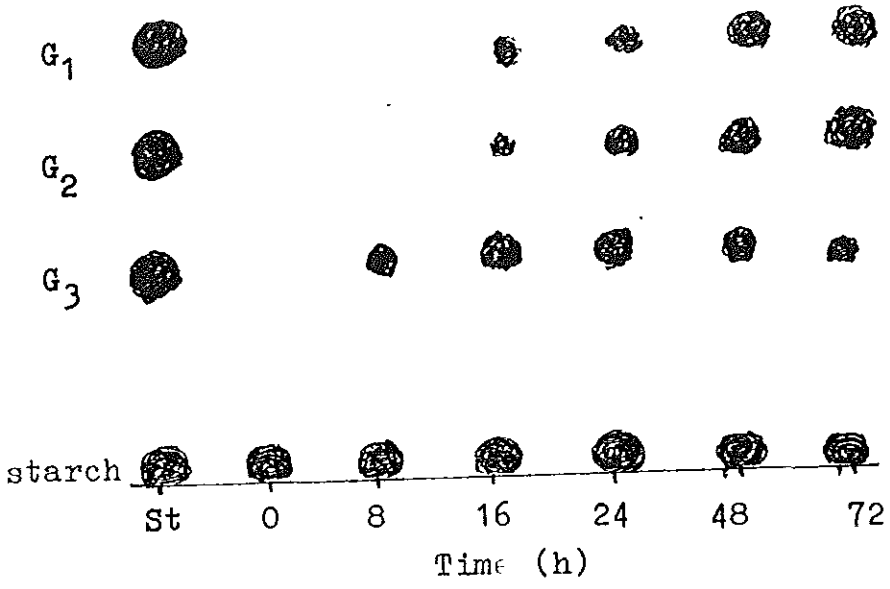

Fig. 11A. Chromatographic analysis of supernatant in the medium containing 1% soluble starch from B.licheniformis T31 Culture, Symbols: St, standard sugars (G_1, glucose; G_2, Maltose; G_3 maltotriose). The sizes of the spots show relative amounts of products

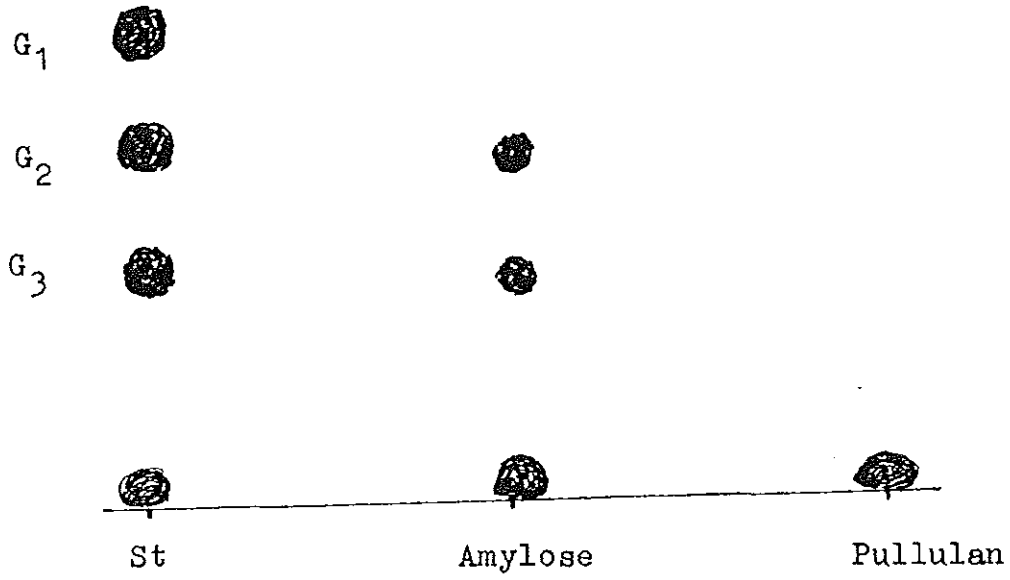

Fig.11B. Chromatographic analysis of the products produced by the action of the enzyme of B.licheniformis T31 on amylose and pullulan.

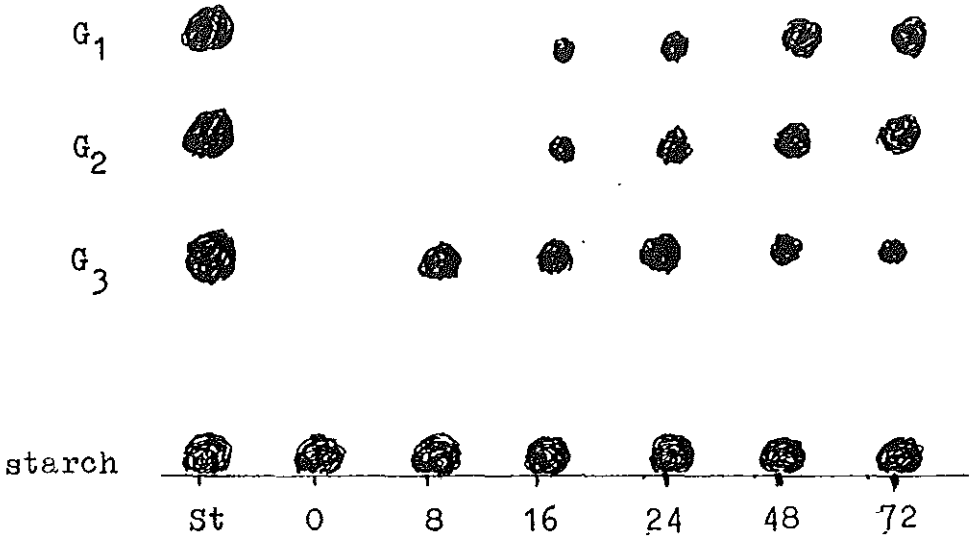

Fig.12A. Chromatographic analysis of supernatant in the medium containing 1% soluble starch from B.subtilis K6 Culture. symbols: St, standard Sugars (G_1, glucose; G_2 maltose; G_3, Maltotriose). The sizes of the spots show relative amounts of products.

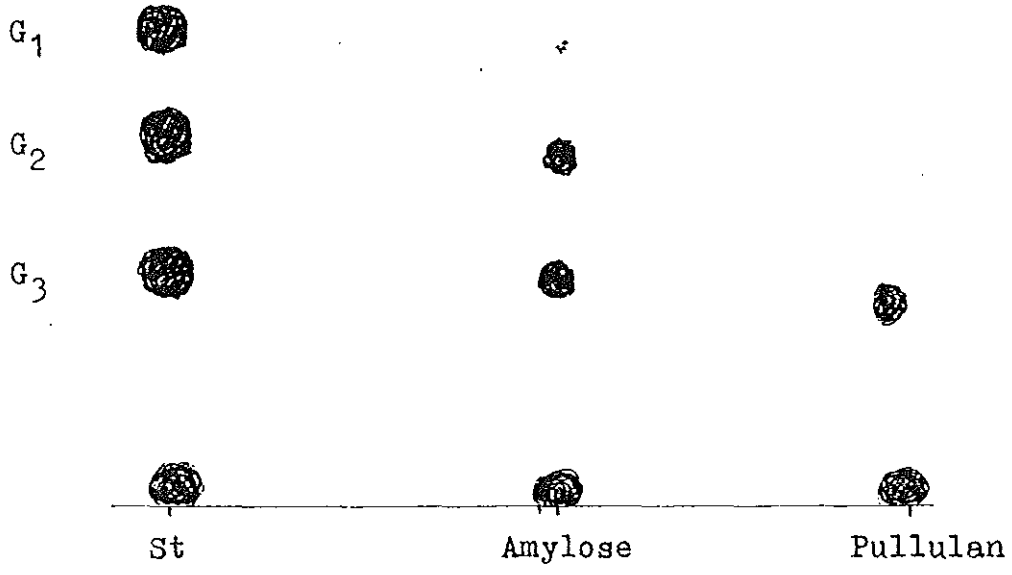

Fig.12B. Chromatographic analysis of the products produced by the action of the enzyme of B.subtilis K6 on amylose and pullulan.

DISCUSSION

Two amylase producing Bacillus species were isolated from fermenting tef flour and kocho (one from each source). Obtaining a suitable organism is of little value unless the proper physico-chemical characteristics for growth and enzyme production are defined. The composition and concentration of chemicals in media greately affect the growth and synthesis of amylases (Chandra, et al., 1980; Fukumoto, et al., 1957).

In this study, both the type and concentration of the carbon and nitrogen sources in the culture medium affected biomass and amylase production. The highest amount of amylase production was obtained with 1% soluble starch. Earlier work shows that starch prevents catabolic repression (Castro, et al, 1992). As the substrate concentration was increased from 1 to 3%, the enzyme activity decreased. Amylase production was low when glucose and sucrose were used as a carbon source. When glucose was used as carbon source amylase activities were 4.2% and 3.8% for Bacillus subtilis k6 and Bacillus licheniformis T 31, respectively. On the other hand, when surose was used the activities were 4.6% and 3.6 for Bacillus subtilis k6 and Bacillus licheniformis T 31, respectively. Glucose and sucrose have been known to repress production of amylase (Fukumoto,et al., 1957: Ingle and Erickson, 1978).

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Amylase production was stimulated when native starches such as kocho, tef, wheat, etc. were used as carbon source. This has also been confirmed by others using media containing starch, such as maize, barley or wheat (saito and Yamamoto, 1975). In this study amylase production was high when kocho was used as a carbon source. This suggests that it might be used as cheaper carbon substrate instead of commercially available soluble starch for the production of amylase for industrial purposes.

In addition to carbon sources, amylase production was also found to be affected by the type of nitrogen sources used in the growth medium. Peptone at 0.5% resulted in higher amylase production when compared to other types of nitrogen sources used in this study. Inorganic nitrogen sources may also be used as major sources of nitrogen in media for amylase production (Meyrath and Volavsek, 1975). In this study ammonium chloride was found as an important nitrogen source next to peptone.

The presence of zinc, mercury, copper and lead in the culture medium were found to inhibit amylase production. Thus, heavy metals should not be included in the medium. However, Magnesium salt which stimulated amylase production should be added in the growth medium.

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The effects of different detergents at various concentrations in the medium on amylase production were compared Triton x -100, Tergitol 7 and Sodium lauryl sulfate inhibited growth and amylase production at 0.05,0.1 and 0.2% concentrations. Detergents cause loss of membrane phospholipids in microbial cells (Demain, and Birnbaum, 1968).The detergents used in this study might have caused the disintegration of the organisms at the concentrations present in the medium. The result shows that Tween 80 and Tween 20 didn't stimulate or inhibit amylase production at the concentrations used.

Physical factors such as medium pH and growth temperature had important influences on both biomass formation and amylase production. Both test organisms had a broad pH optimum (6-8.5) for growth and amylase production. This could be advantageous to cultivate the organisms for amylase production in acidic, neutral or alkaline conditions. Bacillus subtilis k6 was more tolerant to acidic pH than Bacillus licheniformis T 31. On the other hand, Bacillus, licheniformis T 31 was more tolerant to alkaline PH than Bacillus subtilis k6. During isolation of the test organisms, the pH of fermenting kocho and tef were 4.5 and 5, respectively.

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The temperature at which the organisms were cultured affected both growth and amylase production. Maximum growth was observed between 20°C (room temperature) and 40°C. However, maximum amylase production was obtained at room temperature. This could be advantageous to cultivate the organisms at mesophlic conditions for amylase production.

The activity of amylase on starch is dependent on pH of the assay buffer and assay temperature.

The optimum pH of amylase from Bacillus subtilis k6 was 5.6; however it showed activity between pH 4.6 to 9. Two pH optima were observed for amylase from Bacillus,licheniformis T 31, one at 5.6 and the other at 8. The observation of two pH optima in Bacillus licheniformis T 31 could be due to the presence of two different amylases. Saito (1973) observed that Bacillus licheniformis amylase had optimum pH value between 5 and 8. Amylase activity was not detected below pH 4. The effect of pH on the stability and activity of amylases has an important industrial application. For example, the destruction of cereal alpha-amylase at pH value of 3.4 4.0 has been known to be important in the baking industry. This property has been used to produce a bread at low pH in order to prevent excessive dextrinization and the production of stickiness in the bread (Kulp, 1975). In this study, the amylases of both test organisms were stable under refrigeration conditions when kept in buffers where

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pH is in the range of 5.5 - 8.5 . This property is important to stock the amylolytic enzymes for future use in industry (Bass and Cayle, 1975).

Regarding the assay temperature, the amylase activity increased when incubation temperature was raised from room temperature to 40°C. The amylases produced by the test organisms were less thermostable than those of other bacterial species reported in the litrature (Kochhar and Dua, 1990; Scott, 1975). In this study, the amylases were stable at 60°C for 1 hour, Bacillus licheniformis T 31 amylase was more stable than Bacillus subtilis k6 amylase at 65° C and 70°C. Ingle and Erickson (1978)and Saito (1973) isolated thermostable amylase from a mesophilic Bacillus licheniformis. Thus, a continuous search for increasingly thermostable amylases should be carried out.

This study showed that amylase activity increased when solutions of divalent metals, such as barium, calcium, cobalt and magnesium were added to the assay mixture Amylases are stabilized by calcium (Vihinen and Mantsala, 1990; Fischer and Stein, 1960). Thus, barium, cobalt and magnesium could be used to replace calcium for stabilizing amylases.

Salt tolerance test showed that Bacillus subtilis k6 amylase was stable in 0.5 M Nacl with 78% of the original activity being retained after 24 hours. On the other hand,

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64% of the original activity of Bacillus licheniformis T 31 amylase was retained in the same concentration of Nacl. Moseley and keay (1970) reported that Bacillus subtilis NRRL B 3411 amylase activity was lost at 0.5 M NaCl. However, a salt tolerant amylase which was stable in 5M Nacl was isolated from Bacillus sp. 64 (khire and Pant, 1992).

Chromatographic analysis of the supernatant from medium containing 1% soluble starch, showed the presence of the products such as maltotriose, maltose and glucose. Maltose and glucose were found to increase in their concentration as the incubation time increased. Thus the isolated amyloytic organisms could be useful in the process of sugar productions.

The action of amylases of both test organisms on amylose produced maltose and maltotriose. Thus it can be concluded that the enzymes are most probably alpha-amylases. Alpha-amylases are known to produce initially oligosaccharides of various lengths followed by maltose and glucose as final products (Guilbot and Mercier, 1985). The culture filtrate from Bacillus subtilis k6 was active on pullulan and produced maltotriose. This means that Bacillus subtilis k6 produces pullulanase in addition to alpha-amylase. More recently, the simultaneous production of different enzymes by Bacillus subtilis Mir-5 has been reported (Castro, et al.,1992).

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A study of the distribution of amylases in the test organisms revealed that the highest percentage activities were detected in the culture filtrate. This showed that the amylases were truly extracellular.

The Bacillus species isolated in this study are involved in tef and kocho fermentation since they produce amylolytic enzymes. Earlier work showed that species belonging to the genus Bacillus were present in fermenting kocho and tef (Meaza and Berhanu, 1985; Berhanu, et al., 1982).

Tef and kocho are the most important source of food in Ethiopia. The duration of kocho fermentation, which could take up to several months, might be reduced using pure cultures of amylolytic bacteria. Such bacteria could also be used to prevent occassional failures of the fermentation of tef dough. Thus, they could be useful to develop starter cultures for the fermentation.

It is hoped that the amylolytic bacteria isolated in this study could also be used as sources of amylases for fermentation industries. It has been found that the low level of alpha amylase in ungerminated wheat requires supplementation of the flour with this enzyme for proper functioning in the production of baked materials (Barrett, 1975).

R E F E R E N C E S

Abe, J., Bergamann, F.W. , Obata, K, and Hizukuri, S., (1988). Production of the raw-starch digesting amylase of Aspergillus sp. K-27. Appl. Microbiol. Biotechnol. 27: 447-450.

Arima, k (1964) Microbial enzyme production. In: global impacts of applied microbiology (Starr, M.P.,ed.), PP.277 - 294. Almqvist and Wiksells, Sweden.

Aunstrup, K. (1979). Production, isolation and economics of extracellular enzymes. App.Biochem. Bioeng.2:27-69.

Bakshi, A., Patnaik, P.R. and Gupta, J.K (1992). Thermo-stable pullulanase from a mesophilic Bacillus cereus isolate and its mutant UV. 7.4. Biotechnol. Lett. 14: 689-694.

Barman,T.E. (1969). Enzyme Hand Book Vol II. springer- verlag Berlin Heidelberg. New York.

Barrett, F.F. (1975). Enzyme uses in the milling and baking industries. In: Enzymes in food processing (Reed,G.ed.), 2nd ed. PP. 301-330, Academic press, New York.

Bass, E.J. and Cayle, T. (1975). Beer. In: Enzymes in food processing (Reed, G. ed.), 2nd PP 455-471. Academic

[illegible] Biotechnol Lett.

Bergmann, F.W., Abe, J. and Hizukuri,S.(1988). Selection of microorganisms which produce raw-starch degrading enzymes. Appl. Microbiol. Biolechnol. 27; 443 - 446.

Berhanu A. Gashe, Meaza Grima and Abraham Besrat (1982) Tef fermentation I. The role of microorganisms in fermentation and their effects on the nitrogen content of tef. SINET. Ethiop. J. Sci. 5(2): 69-76.

Bernfield, P (1955). Amylases alpha and beta. In: Methods in enzymology (Colowick, S.P. and kaplan, N.O.,eds.), Vol. 1.PP 149 - 158 Academic press,New York.

Boyer, E.W. and Ingle, M.B (1972). Extracellular alkaline amylase from Bacillus species. J. Bacteriol. 110:992 -1000.

Buchanan, R.E and Gibbons,N.A. (1974). Bergey's manual of determinative bacteriology, 8th ed.PP. 529-550, The Williams and wilkins Company, Baltimore.

Buonocore, V., Carporale, C, De Rosa. M., and Gambacorta,A. (1976). Stable,inducible thermoacidophlic alpha-amylase from Bacillus acidocaldarius. J. Bacteriol. 128: 515-521.

Castro, G.R. Ferrero, M.A. , Abate,C.M. Mendez, B.S., and Sireriz, F.(1992). Simultaneous production of alpha and beta amylases by Bacillus subtilis Mir-5 in batch and continuous culture [illegible] 14:49-54.

Chandra, A.K., Medda, 5. and Bhadra, A.K. (1980). Production of extracellular thermostable alpha-amylase by Bacillus licheniformis. J. Ferment. technol. 58:1 10.

Claus,D., Berkeley, R.C.W. (1984) In: Bergy's Manual for Systematic Bacteriology (Holt J.E., ed.) Vol. 2, PP. 1122-1135. Williams and Wilkins, London.

Coleman, G., and Elliott, W.H. (1962). Studies on alpha-amylase formation by Bacillus subtilis. Biochem.J. 83:256-263.

Coleman, G. and Grant, M.A. (1966).Characteristics of alpha-amylase formation by Bacillus subtilis. Nature (London) 211: 306-307.

Collins, C.H. and Lyne, P.M. (1976). Microbiological methods. 4th ed. Butterworths, London, Boston.

Demain, A.L and Birnbaum, M.J. (1968). Alteration of membrane permability for the release of metabolites from microbial cells. Curr.Top.Microbiol.4:1-25.

Depinto, J.A. & Campbell,L.L.(1964). Formation and degradation of cyclic dextrins by intracellular enzymes of Bacillus macerans. Science. 146: 1064-1066.

../

Depinto J.A. and Campbell,L.L. (1968). Pattern of action of the amylase and the cyclodextrinase of Bacillus macerans. Arch. Biochem, biophys. 125: 253 - 258.

Farez-vidal M.E., Fernandez-Vivas, A. and Arias,J.M. (1992) production of alpha-amylase by Myxococcus coralloides D. J. Appl. Bacteriol. 73:148-156.

Fischer, E.H., and Stein, E.A. (1960). Alpha - amylases. In : The enzymes (Boyer, P.D., Lard. H., and Myrback, eds.). 2nd ed., Vol. 4, PP. 313-343 Academic Press, New York.

Fogarty, W.M., Griffin, P.J. and Joyce. A.M. (1974). Enzymes of Bacillus species. Part 1. Process Biochem. 9: 11-24.

Fogarty, W.M.and Kelly, C.T. (1980). Amylases, amylogluco sidases and related glucanases. In: Microbial enzymes and bioconversions. Vol. 5. PP. 115-170.

Fogarty, W.M. (1983). Microbial amylases. In: Microbial enzymes and biotechnology (Fogarty, W.M., ed.) PP. 1-92. Applied science publishers, London and New York.

Frazier, W.C. and Westhoff, D.C. (1978). Food Microbiology. McGraw - Hill Book Co., USA.

../

French,D. (1960). Beta-amylases. In: The enzymes (Boyer,P.D., Lard, H. and Myrback, K., eds.). 2nd ed., Vol.4, PP. 345 -368. Academic Press, New York.

Fukumoto, J. Yamamoto, T. and Tsuru,D., (1957). Effects of Carbon sources and base analogues of nucleic acid on the formation of bacterial amylase. Nature (London) 180: 438-439.

Ghose, T.K. (1987) Recommendations on the measurement of cellulase activities.Pure appl. Chem. 59: 257 -268.

Gibson, T. , and Gordon ,R.E, (1975). Endospore forming rods and cocci. In: Bergey's Manual of Determinative Bacteriology (Buchanan, R.E. and Gibbons, N.E., eds.), 8th ed. The Williams and wilkins co., Baltimore.

Glenn, A.R. (1976). Production of extracellular proteins by bacteria. Ann. Rev.microbiol. 30: 41 - 62.

Gray, C.J. (1971). Enzyme -catalysed reactions - Ban Nostrand Reinhold co., London.

Greenwood, C.T. (1970). Starch and glycogen. In: The Carbohydrates (Pigman,W. and Horton,D., eds.). 2nd ed.Vol. IIB.PP 471 - 513. Academic press. New York and London.

../

Guilbot, A. and Mercier C. (1985). Starch. In: the polysaccharides 3(Aspinall, G.O., ed.), pp. 209 -282, Academic Press, New York.

Ingle, M.M. , and Boyer, E.W. (1976). In: Microbiology (Schlessinger, D. ed.),pp.420-426. Am. Soc. Microbiol., Washington, D.C.

Ingle, M.B. and Erickson,R.J. (1978). Bacterial alpha-amylases. Advances in Appl. Microbiol, 24:257 278.

Khire, J.M. and Pant,A.(1992).Thermostable, salt-tolerant amylase from Bacillus sp.64 world J.Microbiol. 8:167-170.

Kochhar, S. and Dua. R.D. (1990). Thermostable liquefying alpha - amylase form Bacillus amyloliquefaciens. Biotechnol. Lett. 12: 393-396.

Kulp, K. (1975). Carbohydrases. In: Enzymes in food processing (Reed,ed.). 2nd. PP. 53 - 120. Academic Press, New York.

Larsson, M. (1989). Applications of aqueous two-phase systems in biotechnology. Department of biotechnology. University of Lund. Lund, Sweden.

../

Lee.Y.C. and Whelan, W.J. (1971). Glycogen and starch debranching enzymes. In: The enzymes (Boyer,P.D. ,ed.). 3rd ed. pp. 191-234. Academi Press, New York.

MacAllister, R.V., Wardrip, E.K., and schnyder, B.J. (1975). Modified starches, corn syrups containing glucose and fructose, and crystalline dextrose. In: Enzymes in food processing (Reed, G,ed.) 2nd ed. pp. 331-359. Academic Press, New York.

Matta, M.S. and Wilbraham, A.C. (1981) Atoms,molecules and life. the Benjamin/Cummings publishing Co. Inc., California PP.721.

Mayatt, J.H. (1983).Paper. In:Industrial enzymology.(Godfrey, T. and Reichelt, J.R., eds.).Macmillan Pub.Ltd.,London.

Meaza Girma and Berhanu A. Gashe (1985). Studies on the microbial flora of kocho and bulla purchased from markets in Addis Ababa. SINET: Ethiop. J.Sci. 8(1&2):29-36.

Meyrath,J. and Volavsek G. (1975) Production of microbial enzymes. In: Enzymes in food processing (Reed,G.,ed) 2nd ed.pp.255-300. Academic Press New York.

Miller, G.L. (1959). Use of Dintrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31: 426-428.

Moseley, M.H. and Keay,L. (1970).Purification and characterization of the amylase of B.subtilis NRRL B. 3411. Biotechnol. Bioeng. 12:251-271.

Norman, B.E (1979). The application of polysaccharide degrading enzymes in the starch industry. In: Microbial polysaccharides and polysaccharases.(Berkeley,R.C.W. Goodday, G.W.and Ellwood,D.C. eds.) PP. 339-376. Academic press, New York.

Okemoto,H., Kobayashi., S. Momma,M., Hashimoto, H., Hara, K., and kainuma, K., (1986). Isolation and cultivation of a novel microogranism producing a maltopentaose forming enzyme. Appl. Microbiol. Biotechnol. 25: 137-142.

Priest, F.G. (1977). Extracellular enzyme synthesis in the genus Bacillus. Bacteriol.Rev. 41: 711-753.

Ramesh, M.V. and Lonsane, B.K. (1991). Regulation of alpha-amylase production in Bacillus licheniformis M 27 by enzyme end - products in submerged fermentation and its overcoming in solid state fermentation system. Biotechnol. Lett. Vol. 13:355-360.

Reed, G. (1975). General characteristics of enzymes. In: Enzymes in food processing (Reed, G,ed.), 2nd pp. 15-19, Academic press, New York.

Robyt, J.F. and Whelan, W.J. (1968). In: Starch and its derivatives. (Radely, J.A.,ed.), 4th ed, pp. 430 476 Chapman and Hall, London.

Robyt, J.F., and French,D. (1970). The action pattern of procine pancreatic alpha-amylase in relationship to the substrata binding site of the enzyme.J.Biol.Chem. 245: 3917-3927.

Robyt, J. F. and Ackerman. R.J. (1971). Isolation, purification and characterization of a maltoteraose producing amylase from Pseudomonas stutzeri. Arch. Biochem. Biophys.145: 105 -114.

Rose,A.H, (1961). Industrial Microbiology. Butterworths London.

Saito, N. and Yamamoto, K. (1975). Regulatory factors affecting alpha - amylase production in Bacillus licheniformis. J. Bacterol. 121:848-856

Saito,N. (1973). The thermophilic extracellular alpha-amylase from Bacillus licheniformis. Arch. Biochem. Biophys. 155:220-298.

Saito,N.(1982). Evaluation of meltopentaose as a substrate for alpha-amylase determination in serum. J.Jap.Soc. Starch Sci. 29:153-160.

../

Scott,D (1975). Miscellaneous applications of enzymes. In: Enzymes in food processing (Reed,G.,ed.), 2nd.pp.445 -471.Academic press, New York.

Shah, N.K., Ramamurthy, V. and Kothari, R.M (1991). comparative profiles of fungal alpha-amylase production by submerged and surface fermentation. Biotechnol.Lett. 13:361-364.

Southgate, D.A.T. (1976). Determination of Food Carbohydrates. Applied science Pub. Ltd.,London.

Sumner,J.B. and Somers,G.F. (1947). Chemistry and methods of enzymes. 2nd ed. Academic Press, Inc., Pub., New York.

Suzuki, Y. and Chishiro, M. (1983). Production of extracellular thermostable pullulanase by an amylolytic obligately thermophilic soil bacterium, Bacillus stearothermophilus KP 1064.European J. appl. microbiol.Biotechnol. 17:2429.

Takagi,T., Toda,H.and Isemura,T.(1971).Bacterial and mold amylases. PP.233-271 In: The enzymes (Boyer,P.d.,ed.), Vol.5.3rd ed.Academic press, New York and London.

Takasaki, Y, Shinohara, H., Tsuruhisa,M., Hayashi, S. and Imada, K. (1991). Maltotetraose-producing amylase from Bacillus Sp. MG-4. Agric. Biol.Chem. 55(7): 1715-1720.

Tigerstrom, G.V. and Steimaschuk,S. (1987).Purification and characterization of an amylase from Lysobacter brunescens. J.Gen.Microbiol. 133:3437-43.

Thoma, J.A., Spradin, J.E. and Dygert, S.(1971).Plant and animal amylase. In: The Enzymes (Boyer,P.D.,ed.),Vol.5 3rd ed. PP. 115-189. Academic press, New York and London.

Ueda, S. (1980). Raw starch digestion by mold glucoamylases and debranching enzymes. In: Mechanisms of saccharide polymerization and deploymerization.(Marshall, J.S.ed.). PP 55-72. Academic Press, New York.

Vihinen, M. and P. Mantsala (1990). Characterization of a thermostable Bacillus stearothermophilus alpha-amylase. Biotechnol.Appl. Biochem. 12:427435.

Welker, N.E. and Campbell, L.L. (1963a). Effect of carbon sources on formation of alpha-amylase by Bacillus stearothermophilus. J. Bacteriol. 86:681-686.

welker,N.E. and Campbell,L.L. (1963b). Induction of alpha-amylase of Bacillus stearothermophilus by maltodextrins. J. Bacteriol. 86 : 687-691.

Windish, W.W. and Mhatre, N.S. (1965). Microbial amylases. In: Advances in applied microbiology (umbreit,W.W., ed.), Vol 7. pp. [illegible] New York.