

L-threonine; Ajinomoto has modified *Brevibacterium lactofermentum* for the production of L-histidine, L-phenylalanine, L-proline, L-threonine, and L-tyrosine, Tanabe has altered *Serratia marcescens* for the production of L-histidine, L-proline, and L-threonine.

Mutants producing excess amounts of amino acids have also been isolated by combining several of the above methods.

Table 3.3 lists the strains available for the microbial production of amino acids, their genetic characteristics, and the yields obtained. Processes using various carbon sources have been developed for different production strains. In this list, only the processes with the highest published yields have given and the actual industrial yields are assumed to be significantly higher.

### 3.5 L-GLUTAMIC ACID

#### Production strains

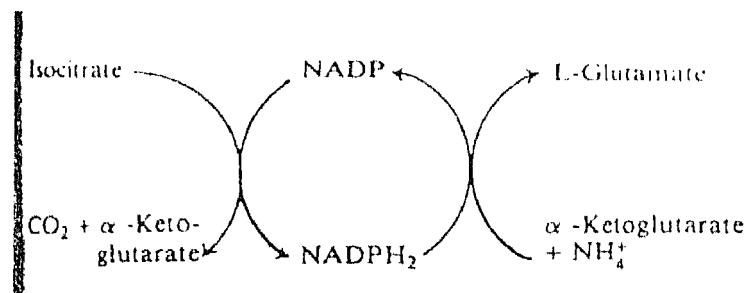
L-glutamic acid is manufactured predominantly by microbial means, although it is also manufactured chemically, Japanese researchers began developing a direct fermentation process because the D, L-glutamic acid which is formed by chemical synthesis is the racemic mixture. In screening about 2,000 microorganisms on different media, L-glutamic acid production was found to occur in a wide variety of bacteria, streptomycetes, yeast and fungi. The isolation of *Corynebacterium glutamicum* (synonym: *Micrococcus glutamicus*) was accomplished in 1957. It was immediately used industrially by Kyowa Hakko because of its high excretion of glutamic acid. Other industrially important strains with L-glutamic acid excretion of at least 30 g/L belong to the genera *Corynebacterium*, *Brevibacterium*, *Microbacterium*, or *Arthrobacter*. Morphologically and physiologically, these glutamic acid producing strains resemble *C-glutamicum*: They are usually Gram-positive, nonsporulating, nonmotile bacteria, Moreover, all glutamic acid producers require biotin, lack or show little activity of  $\alpha$ -ketoglutarate dehydrogenase, and show increased activity of glutamate dehydrogenase. In addition, some *Brevibacterium* and *Corynebacterium* mutants have lower isocitrate lyase activity. Further strain development has led to the isolation of mutants which overproduce of high concentrations of biotin. For instance, a lysozyme-sensitive mutant of

*C-glutamicum* is able to convert 40% of the added carbon source to L-glutamic acid even in the presence of 100 g/L biotin.

Successful hybrids have also been constructed by cloning *Brevibacterium* or *Corynebacterium* DNA into *Brevibacterium* or *Corynebacterium* recipients.

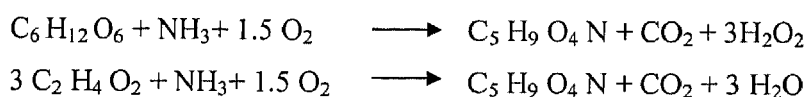
### Biosynthesis of glutamic acid

The glucose carbon source is broken down into  $C_3$  and  $C_2$  fragments by glutamic-acid-producing microorganisms through the Embden-Meyerhof- Parnas (EMP) pathway and the pentose-phosphate-cycle, and the fragments are channeled into the tricarboxylic acid (TCA) cycle. The EMP pathway is more common under conditions of glutamic acid production. The key precursor glutamic acid is  $\alpha$ -ketoglutarate, which is formed of in the TCA cycle via citrate and isocitrate and then converted into L-glutamic acid through reductive amination with free  $NH_4^+$  ions. This last step is catalyzed by the NADP- dependent glutamate dehydrogenase. The  $NADPH_2$  required at this stage of the reaction is furnished through the preceding oxidative decarboxylation of isocitrate to  $\alpha$ -ketoglutarate by the enzyme isocitrate dehydrogenase. The  $NADPH_2$  is then regenerated by the reductive amination of  $\alpha$ -ketoglutarate:



The strain used commercially for glutamic acid production has a block in  $\alpha$ -ketoglutarate dehydrogenase. In the absence of  $NH_4^+$  ions,  $\alpha$ -ketoglutarate accumulates because of the interruption of the TCA cycle. Thus efficient anaplerotic sequences are necessary to provide TCA cycle intermediates which are required for other cell reactions. Studies on the pathway of biosynthesis have been carried out using labeled compounds.

During glutamic acid formation in the presence of  $^{14}\text{CO}_2$ , the  $\alpha$ -carboxyl group of glutamate is labeled radioactively. Oxalacetate carboxylase and the NADP-dependent malate enzyme are involved with the  $\text{CO}_2$  fixation process. The malate enzyme catalyzes the carboxylation of pyruvate to malate. These anaplerotic sequences complete the TCA cycle with  $\text{C}_4$  dicarboxylic acids, Malate is then transformed via oxalacetate into citrate and isocitrate, which either serve as preliminary stages to the glutamic acid formation or are channeled into the glyoxylate cycle. Particularly with acetate as a carbon source, the energy gain and the formation of intermediates are carried out chiefly via the glyoxylate cycle for *C-glutamium*. Thus there is competition between a) the isocitrate lyase reaction, which forms succinate and glyoxylate (necessary for optimal growth) and b) the isocitrate dehydrogenase reaction which leads to the key precursor  $\alpha$ -ketoglutarate. The stoichiometry for glutamic acid formation from glucose or acetate as a carbon source would be as follows:



One mole of glutamic acid is produced from 1 mole of glucose or from 3 mole of acetate. Experiments with resting cells have shown that the actual conversion rate is between 50-70 mole%. Some part of the yield reduction is due to the reversibility of the malate enzyme reaction and the decarboxylation of oxalacetate to  $\text{CO}_2$ .

### Effect of permeability on glutamic acid production

Production and excretion of excess glutamic acid is dependent upon cell permeability. Increased permeability in glutamic-acid-producing bacteria can be attained in several different ways:

- Through biotin deficiency
- Through oleic acid deficiency in oleic acid auxotrophs

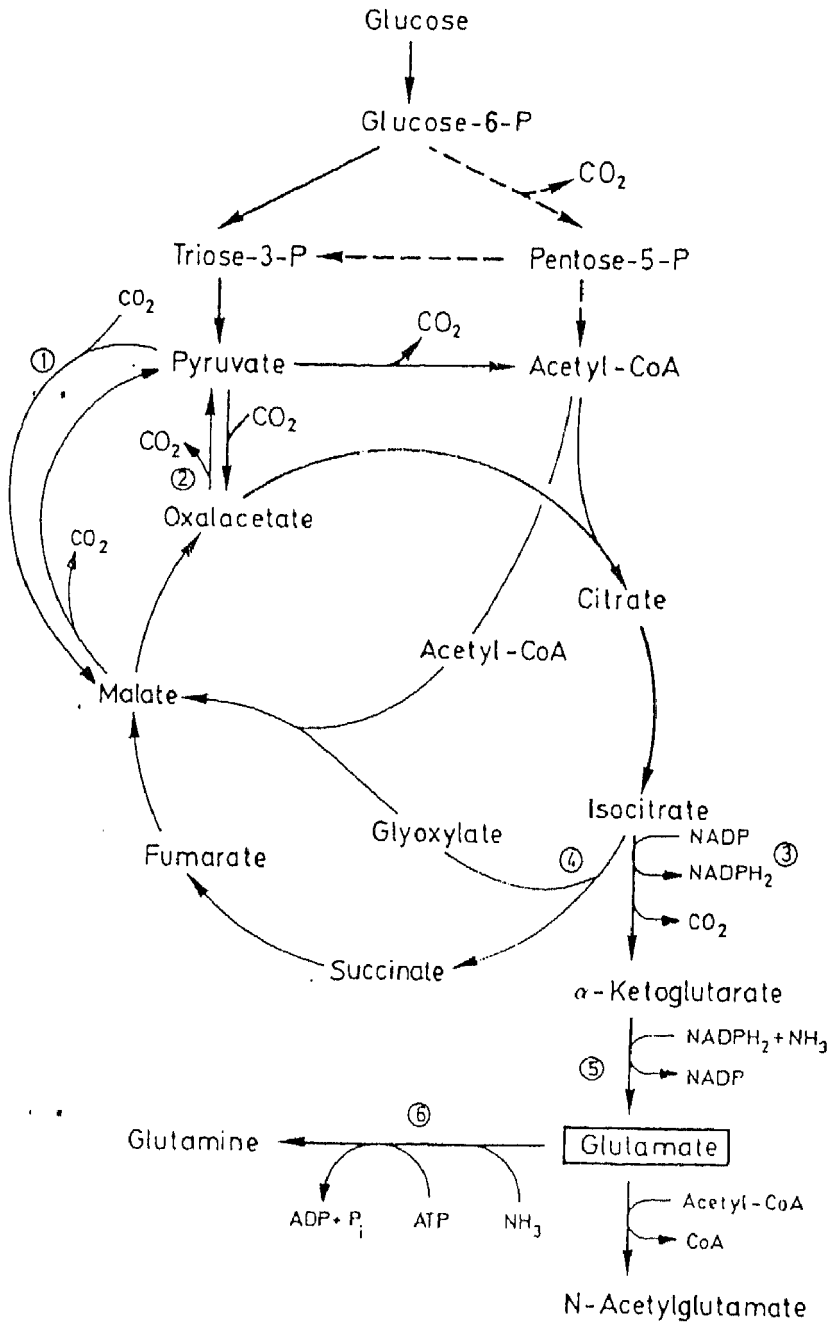


Figure 3.3 Biosynthesis of L-glutamic acid using glucose as the carbon source. Glyoxylic acid cycle, thin lines; pentose phosphate cycle, broken lines. 1, Malic enzyme; 2, Oxalacetate carboxylase; 3, Isocitrate dehydrogenase; 4, Isocitrate (Modified according to Kinoshita and Nakayama, 1978)

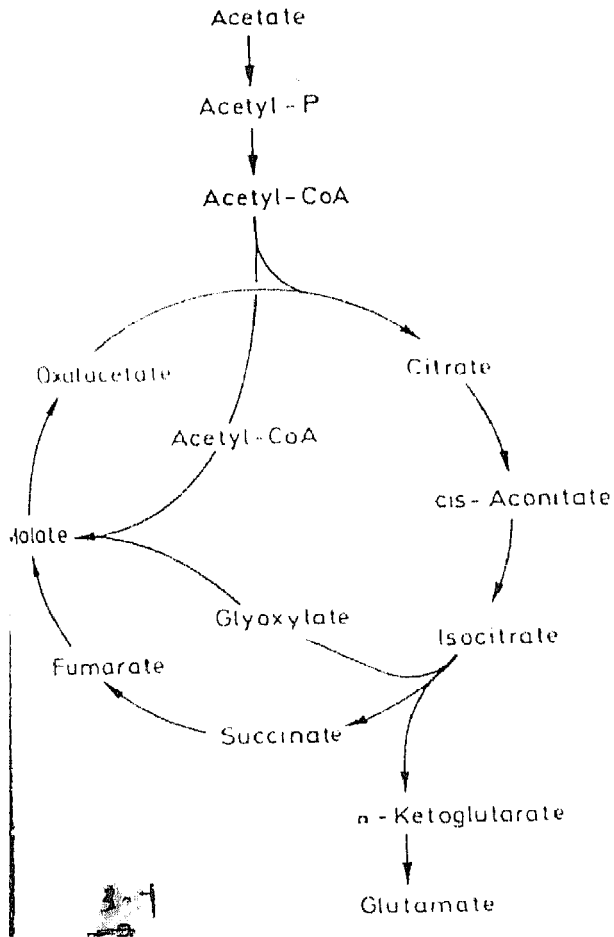


Figure 3.4 Biosynthesis of L-glutamic acid using acetate as the carbon source

- Through the addition of saturated fatty acids ( $C_{16}$ - $C_{18}$ ) or fatty acid derivatives
- Through the addition of penicillin
- Through glycerol deficiency in glycerol auxotrophs

All glutamic-acid-producing strains have a growth requirement for biotin, an essential coenzyme in fatty acid synthesis. In the presence of biotin concentrations  $>5 \mu\text{g}/\text{l}$ , increased oleic acid synthesis results in a high phospholipids content of the cell membrane. Cells with high phospholipids content are incapable of excreting glutamic acid; up to  $25\text{-}35 \mu\text{g}$  L-glutamic acid/mg dry weight is accumulated intracellularly, so that biosynthesis is stopped because of feedback inhibition. On the

other hand, growth in a biotin deficient medium causes membrane damage through reduction of phospholipids synthesis, leading to a changed ratio of saturated to unsaturated fatty acid. Under these conditions, intracellular glutamic acid can be excreted. It has been postulated that a biotin-containing acetyl-CoA-carboxylase, which catalyzes the first stage of fatty acid synthesis from acetyl-CoA, is involved in fatty acid biosynthesis. Saturated fatty acids ( $C_{16} - C_{18}$ ) repress this acetyl-CoA-carboxylase. Hence it is understandable that oleic acid auxotrophs or strains to which saturated fatty acids have been added synthesize cell membranes with lower phospholipids content; such strains also excrete L-glutamic acid in the presence of biotin.

The addition of penicillin in the logarithmic growth phase promotes significant excretion of L-glutamic acid, even in the presence of biotin. Penicillin is added to fermentations in media containing large amounts of biotin 8-12 hours after inoculation of the fermenter. A penicillin concentration is selected (between 5-300 units/ml) so that the bacterial growth rate is reduced to a level corresponding to the rate in low-biotin media.

The use of penicillin or saturated fatty acids makes possible the commercial use of inexpensive culture medium components, such as sugar cane or sugar beet molasses, which otherwise cannot be utilized due to their high biotin content. The discovery of the role of cell permeability in the production of glutamic acid has thus made possible some rational approaches to the industrial production of this important amino acid.

### **Conditions of manufacture**

Under optimal culture conditions, glutamic-acid-producing bacteria convert about 50-60% of the added carbon source to L-glutamic acid. If less favorable fermentation conditions are used so that low glutamic acid production is obtained, an increase of cell mass and an excretion of lactate, succinate,  $\alpha$ -ketoglutarate, glutamine and N-acetylglutamine have been observed. Factors which affect glutamic acid fermentation are described below.

**Carbon sources** A wide variety of carbohydrates can be used as carbon sources in the fermentation process. Among the monosaccharides, glucose and sucrose are frequently used, and fructose, maltose, ribose, and xylose find some role. Of the unrefined carbohydrate sources, sugar cane and sugar beet molasses are most important, but starch hydrolysates are also frequently employed. Since molasses has a

high biotin content (0.4-1.2mg/kg for cane molasses; 0.02-0.08mg/kg for beet molasses), penicillin or fatty acid derivatives (e.g., Tween 60) must be added to the fermentation when these inexpensive carbon sources are used. In Europe, only beet molasses is considered an inexpensive carbon source (proportion of manufacturing cost: 26%) but in other areas of the world cane molasses is chiefly used. In Japan, where acetate is inexpensive and readily available in large quantities, extensive studies have been carried out to use this carbon source but for industrial production, cane sugar molasses or starch hydrolysate are still the main carbon sources used.

Processes using methanol, ethanol, acetaldehyde, or n-alkanes have also been developed, but the cost-effectiveness of these processes depends largely on the price of petroleum. Figures 3.5 and 3.6 show the progress of typical fermentations with glucose and acetate as carbon sources.

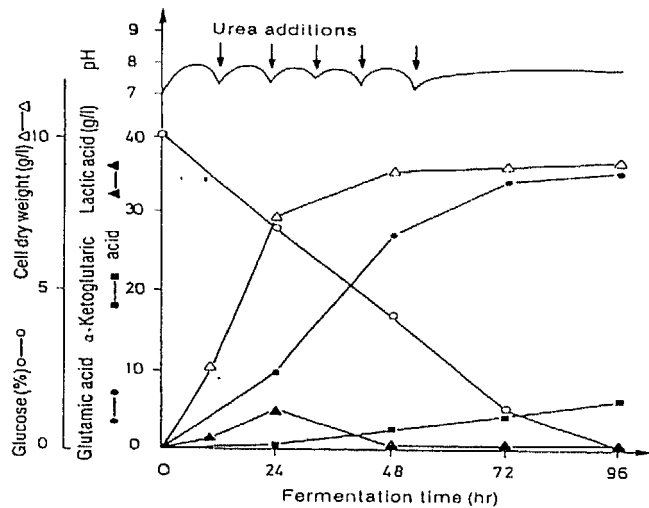


Figure 3.5 Production of L-glutamic acid with *Corynebacterium glutamicum* No.451 using glucose as the carbon source

**Nitrogen sources** In addition to ammonium salts, ammonia (gaseous or in aqueous solution) can be used as a nitrogen source. In the industrial manufacture of glutamic acid, ammonia feeding permits pH control and obviates the problem of ammonia toxicity. Most glutamic acid-producing bacteria possess urease activity, so that urea is also frequently used as a nitrogen source. In the acidic pH range with excess ammonia, glutamine is produced instead of glutamic acid.

**Growth factors** The optimal biotin concentration is dependent on the carbon source used. In media with 10% glucose, it is  $5 \mu\text{g} / \text{l}$ , in media with lower, and for acetate it is considerably lower, and for acetate it is between  $0.2\text{-}1.0 \mu\text{g} / \text{l}$ . Some strains require L-cysteine as an additional growth factor; for media based on an n-alkane, supplementation with thiamine may be necessary.

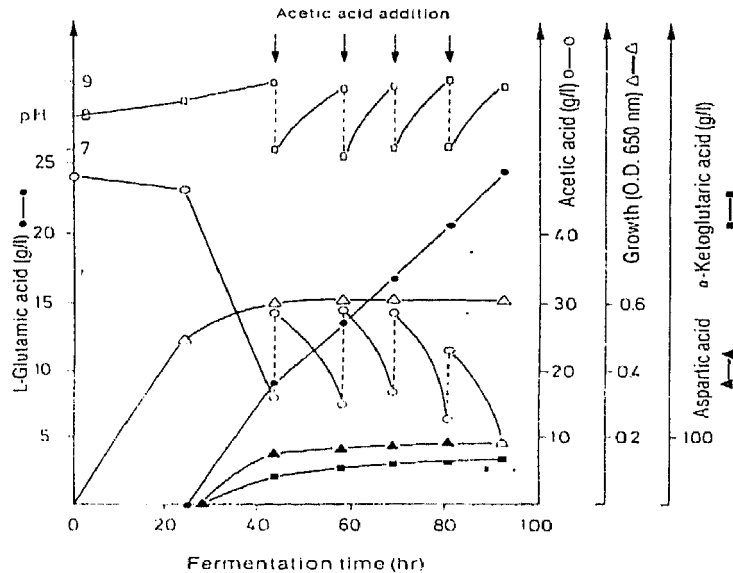


Figure 3.6 Production of L-glutamic acid with *Brevibacterium flavum* No. 2247 using acetate as the carbon source (Tsunoda et al., 1961)

**$O_2$  Supply** Optimal glutamic acid yields are obtained at a  $K_d$  value of  $3.5 \times 10^{-6}$  mole  $O_2$ /atm- min-ml. The oxygen concentration should be neither too low nor too high. Under oxygen deficiency, excretion of lactate and succinate occurs, whereas excess oxygen in the presence of an ammonium ion deficiency causes growth inhibition and production of  $\alpha$ -ketoglutarate; in both cases glutamic acid yields are low.

### Production processes

A typical fermentation from glucose with *Brevibacterium divaricatum* (NRRL B-231) runs as follows (Miescher, 1975):

- Seed culture: glucose 40g;  $K_2HPO_4$  1.0g;  $MgSO_4 \cdot 7 H_2O$  0.5g; yeast extract 1.0g; urea 8g; tap water 1L; 16h incubation at  $35^\circ\text{C}$ .

- Main culture; glucose 121g; ammonium acetate 5g; molasses from starch saccharification 6g;  $\text{KH}_2\text{PO}_4$  1.2g;  $\text{K}_2\text{SO}_4$  1.2g;  $\text{MgSO}_4$  (anhydrous) 6g;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  6ppm;  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  6ppm; antifoam agent Hodag K-87 0.1 ml; tap water 1L. Inoculum volume: 6%.

At the beginning of the fermentation, 0.65 ml/l of oleic acid is added. The pH is set at 8.5 with ammonia and is automatically maintained at 7.8 during the course of the fermentation. After beginning growth of the culture (about 14 hours), the temperature is increased from 32-33°C to 38°C. After metabolism of the glucose down to a level of 0.5-2 %, glucose feeding is done until the fermentation is completed; 160 g/l are fed on the average. Aeration is controlled so that the  $\text{CO}_2$  content in the exhaust gas not exceed 4.5 vol%. The glutamic acid content is analyzed hourly. As a rule, the fermentation is stopped after 30-35 hours with a glutamic acid yield of about 100g/l. If molasses from starch saccharification is substituted for glucose, the glutamic acid yield is 94 g/l after 36 hours. The glutamic acid yields with different carbon sources are listed in Table 3.5.

**Table 3.5 Processes for glutamic acid production with different carbon sources**

Carbon source	Organism	Yield (g/l)
Sugar beet molasses	<i>C-glutamicum</i>	> 100
Glucose + Ammonium acetate	<i>Brevibacterium divaricatum</i>	100
Acetate	<i>B-flavum</i>	98
Ethanol	<i>Brevibacterium</i> sp. 136	59
n-Alkanes	<i>Arthrobacter paraffineus</i>	62
	<i>Corynebacterium hydrocarboclastus</i>	84
	<i>C-alkanolyticum (Glycerol)</i>	72
Benzoic acid	<i>Brevibacterium</i> sp.	80
Methanol	<i>Methylomonas methylovora</i> M 12-4	7

## CHAPTER 4

### ENZYMES

#### 4.1 INTRODUCTION

The first enzyme produced industrially was the fungal amylase takediastase, employed as a pharmaceutical agent (for digestive disorders) in the United States as early as 1894. Otto Roehm's patented "laundry process for any and all clothing via tryptic enzyme additives" was announced in 1915. By 1969, 80% of all laundry detergents contained enzymes, chiefly proteases. Along with these, additional enzymes such as lipases, amylases, pectinases, and oxidoreductases were used experimentally in the detergent industry.

Due to the occurrence of allergies among production workers and consumers, the use of proteases in detergents was drastically reduced in 1971 and the world sales fell from \$ 150 million to one-third this amount. Only when special processing techniques, such as microencapsulation, were developed could dustless protease preparations be produced that were risk-free to workers and consumers. Today 95% of laundry detergents in Europe contain proteases, although such detergents are much less widely used in the United States.

The development of **amylases** and **amyloglucosidases** for the production of glucose from starch has led to a new industrial application of enzymes. Also, the use of glucose isomerase for the production of fructose has become wide spread since 1967.

**Microbial rennin** is next in order of significant enzymes. It has been used instead of calf's rennin in cheese production since 1965.

Gross world sales for enzymes in 1987 was \$ 450 million. The following enzymes are currently produced commercially:

- Enzymes used in industry, such as amylases, proteases, catalases, isomerases, and penicillin acylases;
- Enzymes used for analytical purposes, such as glucose oxidase, alcohol dehydrogenase, hexokinase, muramidase, and cholesterol oxidase;
- Enzymes used in medicine, such as asparaginase, proteases, lipases, and streptokinase.

These three areas of application require varying levels of quality and quantity (Table 4.1) On a tonnage basis, the most important are the industrial enzymes (about 1200 tons of pure protein/year) (Table 4.2). The United States and Western Europe

produce about 40-45% of the world market, and nine firms account for 90% of the total market. Industrial enzymes are produced in 120 m<sup>3</sup> fermenters, while enzymes for analytical or medical purposes can frequently be produced in pilot-plant-sized fermenters.

The low concentration of enzymes which are normally produced by wild strains is a considerable hindrance for enzyme production. Although in 1985 about 2500 enzymes were known, only 250 were marketable, mostly in amounts around 10g, some even in mg amounts. Figure 4.1 shows the overall picture of commercial enzyme production.

The prospects for enzyme application have improved due to developments in the following areas:

- Microbial genetics. High yields can be obtained by genetic manipulation. For instance, the yeast *Hansenula polymorpha* has been genetically modified so that 35% of its total protein consists of the enzyme alcohol oxidase.
- Optimization of fermentation conditions via induction of enzyme production, use of low cost nutrients, optimal utilization of components in nutrient solution, and introduction of fed-batch fermentations.
- Release of enzymes from cells by means of new cell-breaking methods.

Table 4.1 Differences in enzyme qualities

	<b>Industrial enzyme</b>	<b>Analytical enzyme</b>	<b>Clinical enzyme</b>
Scale of application	Tons	Milligrams-grams	Milligrams-grams
Degree of purification	Crude	Pure crystalline	Pure crystalline
Secondary activities	Present	Usually none. If yes, then defined	Only isoenzymes
Origin	Microbial, usually extracellular	Microbial, animal, plant, usually intracellular	Microbial, animal, plant, usually intracellular
Multiple applications	Possible in part	Possible in part	Not yet possible
Production costs	Low	Middle-high	High

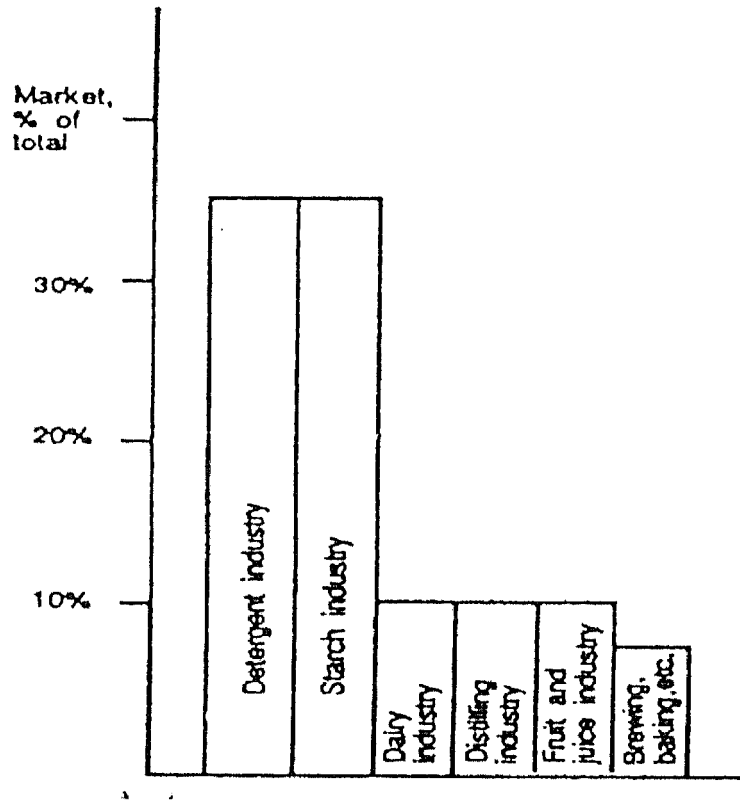


Figure 4.1 Percentage of industrial enzymes used various markets

Table 4.2 Production of industrial enzymes

Enzyme	Enzyme preparation tons/year	Sales, percent of total
Bacillus proteases	6200	45
Glucoamylases	4500	13
Bacillus amylases	4200	5
Glucose isomerases	1900	6
Rennin (microbial)	1500	10
Amylases (fungal)	800	4
Pectinases	100	3
Proteases (fungal)	100	2
Others	-	12

(Aunstrup et al., 1979)

- Modern purification processes such as countercurrent distribution, ion-exchange chromatography, molecular-sieve chromatography, affinity chromatography, and precipitation.
- Development of processes for the immobilization of enzymes and for their recycling. The amount of enzyme needed per amount of substrate converted is thus considerably decreased. The proportion of enzyme cost in some processes becomes only a few percent (Figure 4.2)
- Continuous enzyme production in special reactors. The investment cost of a new system can be minimized in a continuous operation, since smaller-sized equipment is used.

In this chapter, we will discuss those reactions which involve more or less purified enzymes (either intracellular or extracellular).

## 4.2 AMYLASES

Starch, a glucose polymer, is one of the most widely available plant polysaccharides. Amylases are enzymes which hydrolyze starch. One of the main uses of amylases is in the production of sweeteners for the food industry. The hydrolysis of starch with amylase results first in the production of short-chain polymers called dextrans, then the disaccharide maltose, and finally glucose. Maltose syrup (> 80% maltose) which is produced primarily in Japan, is of low viscosity, is weakly hygroscopic, not crystallizable, only slightly sweet, but has good heat stability and does not undergo browning reactions. Glucose is not nearly as sweet as its isomer fructose, so the next step is the conversion of glucose isomerase. Commercial sweeteners based on fructose have some economic and manufacturing advantage over the more widely used sweetener sucrose. The most important enzymes in the starch-saccharification process are  $\alpha$ -amylases,  $\beta$ -amylases, glucoamylases, glucose isomerases. Figure 4.3 shows the manner of action of these enzymes on starch.

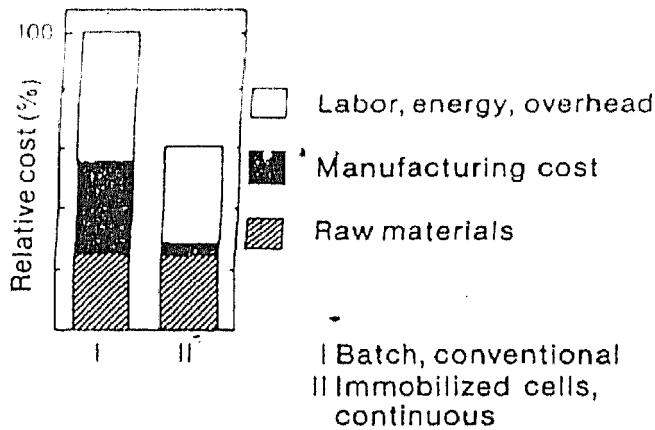


Figure 4.2 Reduction in the cost of L-aspartic acid production through use of cell immobilization techniques (From Chibata and Tosa, 1977)

### $\alpha$ -Amylases

$\alpha$ -Amylases (1,4- $\alpha$ -glucan-glucanohydrolases) are extracellular enzymes which hydrolyze 1,4- $\alpha$ -glycosidic bonds. These enzymes are endoenzymes, splitting the substrate in the interior of the molecule. Their action is not inhibited by  $\alpha$ -1-6-glucosidic bonds although such bonds are not split.

$\alpha$ -Amylases are formed by many bacteria and fungi. They are classified according to their starch-liquefying and/or saccharogenic effect, pH optimum, temperature ranges, and stability. Saccharogenic amylases produce free sugars, whereas starch-liquefying amylases break down the starch polymer but do not produce free sugars. Many organisms produce several  $\alpha$ -amylases.

Bacteria which produce  $\alpha$ -amylases are: *Bacillus subtilis*, *B-cereus*, *B-amyloliquefaciens*, *B-coagulans*, *B-polymyxa*, *B-stearothermophilus*, *B-caldolyticus*, *B-acidocaldarius*, *B-subtilis* var. *amylosaccharaticus*, *B-licheniformis*, *Lactobacillus*, *Micrococcus*, *Pseudomonas*, *Arthrobacter*, *Escherichia*, *Proteus*, *Thermomonospora*, and *Serratia*.

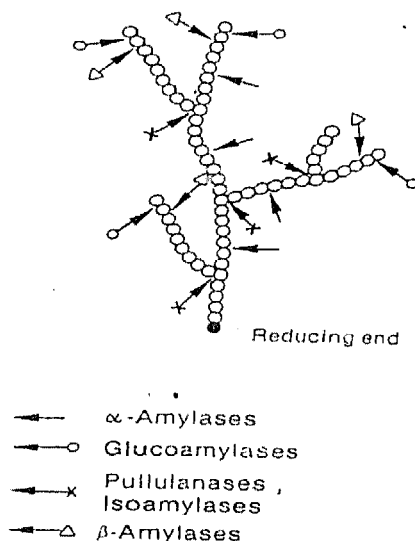


Figure 4.3 Mechanism of starch hydrolysis by amylases

Three very similar strains producing saccharogenic  $\alpha$ -amylases are *B. subtilis* Marburg, *B. subtilis* var. amylosaccharaticus, I, and *B. natto*. The strain *B. amyloliquefaciens* differs from these in that it produces a liquefying  $\alpha$ -amylases. The substrate concentration determines the extent to which the enzymes act in a liquefying or saccharogenic manner.

Some  $\alpha$ -amylases-producing fungi are from the genera *Aspergillus*, *Penicillium*, *Cephalosporium*, *Mucor*, *Candida*, *Neurospora*, and *Rhizopus*.

The molecular weights of various  $\alpha$ -amylases do not differ considerably (Table 4.3). They all contain a large proportion of tyrosine and tryptophan in the enzyme protein and most require calcium as a stabilizer.

Table 4.3 Molecular weight of some  $\alpha$ -amylases from different microorganisms

Organisms	Molecular weight X 10 <sup>3</sup>
<i>Aspergillus oryzae</i>	51-52
<i>A. niger</i>	58-61
<i>Bacillus acidocaldarius</i>	68
<i>B. amyloliquefaciens</i>	49
<i>B. subtilis</i>	24-100

(Fogarty, 1983)

The most important  $\alpha$ -amylases are produced by *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, and *Aspergillus oryzae*. *Bacillus* amylases are used much more extensively than those of *Aspergillus*. The most important areas of application for these two enzymes are shown in Table 4.4.

**Production of bacterial  $\alpha$ -amylases** Bacterial amylases production involves the function of the normal cell machinery for protein synthesis. This is shown by experiments involving the addition of antibiotics. If the specific antibiotics are used to inhibit protein synthesis in *B. subtilis* during the production of  $\alpha$ -amylases, both growth and production of amylase cease (Figure 4.4). Compared to the mRNA of intracellular enzymes (stability about 2 minutes for *Escherichia coli*), the mRNA for the production of extracellular hydrolases has an extremely long lifetime. When actinomycin D is added to the amylase-producing culture to inhibit RNA synthesis, both RNA synthesis and growth cease after 30 minutes but the production of amylase continues (Figure 5.4)

For industrial production,  $\alpha$ -amylases are produced either in batch or in fed-batch fermentation. The enzyme formation rate is very low during exponential growth in many production strains, but just before the growth rate decreases and spore formation begins, amylase production increases (Figure 4.6)

The production of  $\alpha$ -amylases is regulated by several genes, which have been only partially characterized.

**Table 4.4 Important applications of  $\alpha$ -amylases**

Industry	Source		Application
	<i>Bacillus</i>	<i>Aspergillus</i>	
Starch industry	+		Liquefaction of starch for production of glucose, fructose, maltose
Milling		+	Modification of $\alpha$ -amylase-deficient flour
Alcohol	+	+	Liquefaction of starch before the

			addition of malt for saccharification
Baked goods		+	Increase in the proportion of fermentable carbohydrates
Brewing	+		Barley preparation, liquefaction of additives
		+	Improved fermentability of grains, modification of beer characteristics
Paper	+		Liquefaction of starch without sugar production for sizing of paper
Textiles	+		Continuous desizing at high temperatures
Feed industry	+		Improvement of utilization of enzymatically treated barley in poultry and calf raising
Sugar	+		Improvement of filterability of cane sugar juice via breakdown of starch in juice
Laundry and detergent	+		Increase in cleansing power for laundry soiled with starch, additive in dishwasher detergents

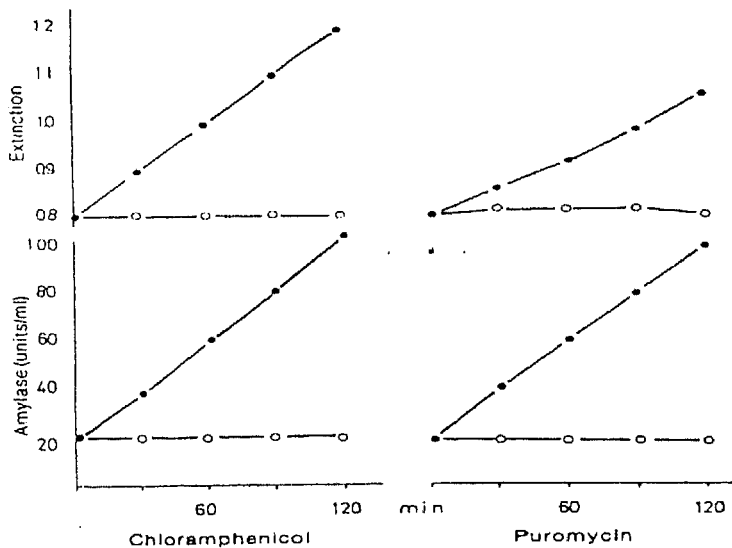


Figure 4.4 Effect of chloramphenicol and puromycin  $\alpha$ -amylase production in *B. subtilis*. O—O antibiotic added (10  $\mu$ g/ml chloramphenicol, 100  $\mu$ g/ml purmycin); •—• control. (From Terui, 1973)

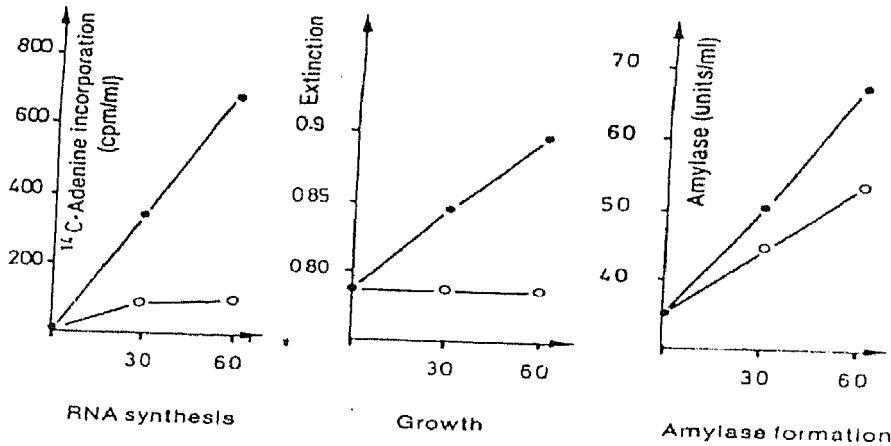


Figure 4.5 Effect of actinomycin D on growth, RNA synthesis, and  $\alpha$ -amylase production. O—O actinomycin D (0.6  $\mu$ g/ml); •—• control. (From Terui, 1973)

characterized. Whereas single-step mutations increase yields by a factor of 2-7, mutants have been selected after 5 steps which produce yields 250 times greater than the wild strain.

Since starch is a macromolecule, it cannot be taken up by the cells and hence cannot act as an inducer of  $\alpha$ -amylase synthesis. It is assumed that a small amount of enzyme is produced constitutively and excreted into the medium where its action on starch leads to the production of low molecular-weight inducers. **Catabolite repression** is significant in the production of most extracellular enzymes. Glucose promotes the best growth compared to other substrates, but is the least favorable in terms of amylase production. In continuous fermentation with carbohydrates as a limiting factor, growth is inversely proportional to the  $\alpha$ -amylase production. When nitrogen is used as a limiting factor with excess glucose, only traces of amylase are produced.

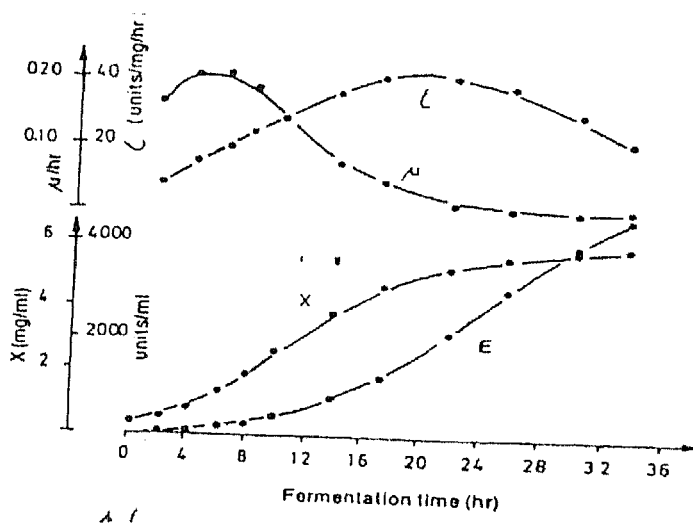


Figure 4.6 Amylase production in *Bacillus amylosolvens*.  $\mu$  specific growth rate,  $\epsilon$  specific enzyme production rate, X cell concentration, E enzyme concentration (From Terui, 1973)

It seems unlikely that amylase is excreted by pure diffusion, since the molecule is quite large and is hydrophilic. There is good evidence that the biosynthesis as well as the steps leading to excretion of extracellular proteins such as amylases and proteases takes place at the outer membrane of the cell. As it is formed, the protein contains an additional peptide, the *signal peptide*, which participates in the transfer of the nascent protein from the ribosome through the membrane. Only after the protein has passed through the membrane does it fold into its tertiary configuration and the signal peptide is removed.

A **medium** for the production of  $\alpha$ -amylases in a 100 m<sup>3</sup> fermenter with *B. subtilis* consists of: 5% starch, 0.56% NH<sub>4</sub>NO<sub>3</sub>, 0.28% sodium citrate, 0.13% KH<sub>2</sub>PO<sub>4</sub>, 0.05% MgSO<sub>4</sub>.7 H<sub>2</sub>O, 0.01% CaCl<sub>2</sub>.2 H<sub>2</sub>O, 0.5% peptone, 0.2% yeast extract; pH 6.8.

The effect of temperature on enzyme production and growth are is given in Figure 4.7.

At 45°C the maximal specific enzyme formation rate is reached with the test strain after 18 hours; however, the maximal amount of enzyme produced (up to 3000 units/ml) is obtained at considerably lower temperatures (27 to 30°C). Thermophilic strains are used in newer processes. *Thermomonospora*, isolated from compost, has a

temperature optimum for growth and amylase production at 53°C. Figure 4.8 shows the narrow pH optimum for amylase production by this strain.

***α*-Amylase production from fungi** The production of fungal amylases is constitutive, but as with other enzymes, it is repressed by regulators. For amylase production using *Aspergillus oryzae*, the following nutrient solution can be used: 8% starch, 1.2 % NaNO<sub>3</sub>, 0.1% K<sub>2</sub>HPO<sub>4</sub>, 0.1% MgSO<sub>4</sub>, 0.05% KCl, 0.003% FeSO<sub>4</sub>, 0.08% Mg (NO<sub>3</sub>)<sub>2</sub>, 0.05% Mg (H<sub>2</sub>PO<sub>4</sub>)<sub>2</sub>, 2.0% malt extract. The medium composition must be optimized, because as seen in Table 4.5, a marked shift in enzymatic activities is observed when different carbohydrates are used.

The optimal temperature lies in a narrow range between 28-30°C, and the duration of the fermentation process is 3-4 days.

### ***β*-Amylase**

*β*-Amylase (*α*-1-4-glucan-maltohydrolases) are usually of plant origin, but some microbial producers are also known: *Bacillus polymyxa*, *B. cereus*, *B. megaterium*, *Streptomyces* sp., *Pseudomonas* sp., and *Rhizopus japonicus*. Although yields in wild strains are usually low, mutants have been discovered which produce 200 times more enzyme than the wild type.

Bacterial *β*-amylases have greater heat resistance (> 70°C) than plant *β*-amylases and the pH optimum is also higher (about pH 7.0). In contrast to *α*-amylase, calcium is not necessary for stabilization and activation of bacterial *β*-amylase.

In the future it is likely that *β*-amylases will be used for the production of maltose syrup.

### **GLucoamylases**

GLucoamylases (*α*-1-4-glucan-glucohydrolases) act on starch by splitting glucose units from the nonreducing end. Maltose is broken down only slowly, while 1,6-bonds in the branched polysaccharides are hardly attacked. Thus glucose, maltose and limit dextrins are the end products of glucoamylase action.

Microorganisms used to produce glucoamylases are *Aspergillus niger*, *A. oryzae*, *A. awamori*, *Rhizopus niveus*, *R. delemar*, *R. formosaensis*, and *R. javanicus*. Production strains in western Europe and in the United States are mutants of *A. niger*

and *R. niveus*. Today, industrial fermentation of glucoamylases is carried out almost exclusively in submerged fermenters up to 150 m<sup>3</sup> in volume. Due to the increasing demand for glucoamylase in fructose-syrup production, smaller systems which involved growth on the surface of liquid or solids are no longer economical. However, in genetic studies, strains are frequently isolated which produce higher yields in surface culture than in submerged culture (Table 4.6) Experiments with batch-fed fermentations have been successfully carried out over periods as long as 320 hours.

Frequently, several glucoamylase isoenzymes are produced by one strain; in addition, the enzymes may be modified during the fermentation process. *A. awamori* var. *Kawachi* produces three *glucoamylases*, only one of which hydrolyzes crude corn starch. Due to the action of proteases and glycosidases, which are also present under various experimental conditions, one of the other two enzymes may be formed instead of the glucoamylase which hydrolyzes crude corn starch. These other enzymes can only act on corn starch which has been swollen by heat or chemical treatment. Therefore the changes in glucoamylase activity must be carefully examined. When high-yielding strains are isolated and when fermentation conditions are being optimized.

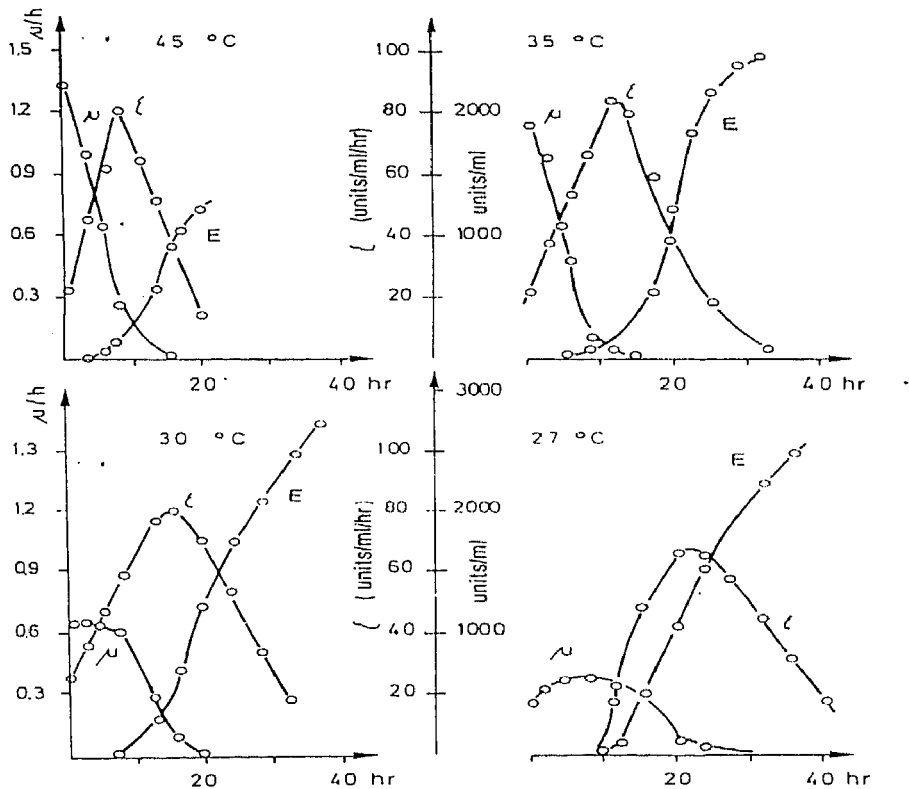


Figure 4.7 Relationship of growth and  $\alpha$ -amylase production to temperature. See Figure 11.6 for symbols. (Modified from Terui, 1973)

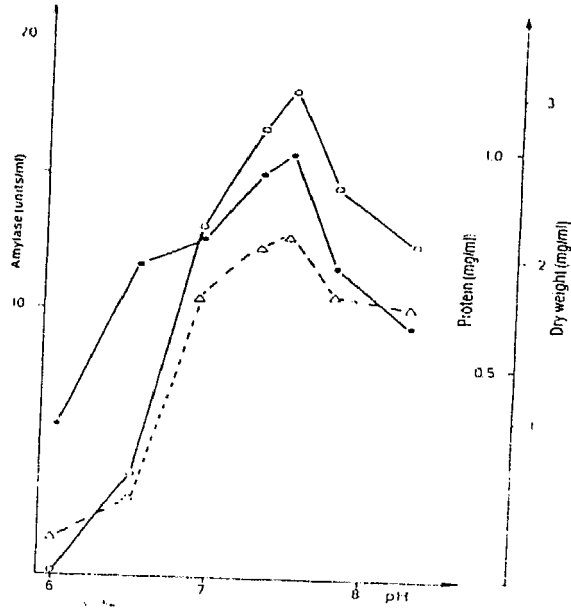


Figure 4.8 Effect of pH on  $\alpha$ -amylase production, extracellular protein and growth.

○—○  $\alpha$ -amylase; ●—● protein;  $\Delta$ — $\Delta$  cell dry weight (Form Glymph and Sutuzenberger, 1977)

Table 4.5 Effect of carbon source on amylase and protease production in *Trichoderma viride*

Carbon source	Amylase units/ml	Protease units/ml
Corn starch	235	351
Maltose	179	175
Glucose	52	243
Sucrose	17	350
Lactose	3	175
Control	0	324

(Upton and Fogarty, 1977)