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# FUNGAL PROTEIN FROM CORN WASTE EFFLUENTS

## A model study

(with a summary in Dutch)

J. A. SCHELLART

*Department of Microbiology, Agricultural University, Wageningen, The Netherlands*

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# 1. INTRODUCTION

## 1.1. General introduction

The literature of recent years reveals an increasing interest from the food, agricultural and chemical industries for special treatments of wastes and effluents to recover by-products by microbiological processes. One of the resulting products, the microbial biomass itself, can be used as a protein source – 'single cell protein' (SCP) – in animal and human nutrition. This idea of utilization of wastes as a substrate for the production of microbial cells is not new, but most processes have been too expensive in the past, and only a few of the processes developed in laboratory studies are being operated on a large scale in some countries.

The increasing pollution rate of surface waters has resulted in legislation and taxation that forced manufacturers to treat wastes and effluents which were previously dumped. Thus, these wastes have acquired a negative commercial value, which makes them at present more attractive as a substrate in microbiological processes than previously and such methods for solving waste disposal problems have an obvious advantage over purely destructive methods. At the same time, the rising protein prices in the world will make the production of single cell protein probably more economical in the near future.

Some of the most important wastes from the food and agricultural industries, suitable for production of SCP on operational scale, are summarized in Table 1.1 (e.g. Woldendorp, 1970; Worgan, 1973a).

The oldest established process of microbial production on a commercial scale is that of baker's yeast (*Saccharomyces cerevisiae*); nowadays usually on a molasses substrate with addition of an ammonium salt as the nitrogen source. This yeast is primarily produced for its baking properties. For SCP production the yeast *Candida utilis* is preferred because of its less exacting nutritional requirements. This yeast is produced as a food yeast for more than fifty years. In the first and second World Wars it has been used in Germany as a protein supplement for food. It is probably the only example of SCP, which has been produced on a commercial scale for use in human nutrition.

The chemical structure of the carbon sources which are present in the wastes largely determines which species of micro-organisms will be used. If starch is present, for instance, it may be difficult to grow yeasts. In the Symba-Yeast process (Tveit, 1967) this problem has been solved by the use of a starch-hydrolysing yeast (*Endomycopsis fibuligera*), in combination with *C. utilis*, the other yeast in the process. Another method for the use of potato processing wastes as a substrate for the growth of *C. utilis* has been described by Reiser (1954).

Food yeast is grown on sulphite waste liquor (from paper pulp manufacture) and other wastes in the USA (Wiley, 1954; Peppler, 1967), in Czechoslovakia (Fencl, 1969) and in other countries.

TABLE 1.1. Important wastes and by-products from food and agricultural industries suitable for the production of single cell protein (SCP).

| Producing industry | Waste or by-product | Countries |
|---|---|---|
| Paper manufacture | sulphite liquor (hexoses, pentoses) | Eastern Europe, Canada, USA, West-Germany |
| Sugar manufacture | molasses (glucose, saccharose) | Taiwan, South Africa, Cuba, Philippines |
| Alcohol fermentation | pentoses | Eastern Europe, France, Belgium |
| Cheese manufacture | cheese whey (lactose, lactate, amino acids) | USA, France, Eastern Europe, New Zealand |
| Potato starch manufacture | potato fruit water (proteins, amino acids, sugars, organic acids) | |

In the USSR, cellulose in forestry and agricultural wastes is hydrolysed to prepare substrates for the production of food yeast (BUNKER, 1963; HOSPODA, 1966). *Saccharomyces fragilis* is produced on cheese whey but only in a few small units in the USA and France (PEPPLER, 1967). This yeast is chosen because it can utilize lactose. The harvested product also contains protein inclusions from the whey.

Reviews of investigations on microbiological methods for utilizing wastes have been published by e.g. THATCHER (1954), STEEL (1958), HALL (1962) and WORGAN (1972).

An important reason for using yeasts in most investigations on the commercial production of SCP, probably is that toxic properties have never been observed in these micro-organisms. Especially *Candida utilis* ('food yeast'), has remained important for commercial production of SCP, because it has an established reputation as a safe food. Nevertheless, it has recently been found that several other micro-organisms have a higher nutritional value than food yeast, and may be grown on a wider variety of carbon sources.

Besides the application of carbohydrates, proteins etc. from industrial wastes, the utilization of cheap hydrocarbons as a substrate for the production of SCP has been frequently investigated during the last decades (e.g. YAMADA et al., 1968; WILKINSON, 1971; GOUNELLE DE PONTANEL, 1972).

## 1.2. GENERAL REQUIREMENTS TO BE MET BY MICRO-ORGANISMS USED FOR PRODUCTION OF SCP FROM WASTES

The micro-organism of choice has to satisfy the following conditions:

I. *Process requirements:*

a. Optimum utilization of the waste components and simple nutritional demands; any additive required may influence the process costs adversely.

b. High specific growth rate, and 'competitive ability'. This makes a non-sterile process possible.
c. Simple and cheap separation.

II. *Properties of the biomass in nutrition:*
a. High nutritional value.
b. Good digestibility.
c. Good taste; 'acceptability'.
d. Non-toxic; safety in animal (and human) diet.

### 1.3. Characteristic properties of micro-organisms in relation to the production of SCP

Bacteria, yeasts, fungi and algae have all been investigated as potential sources of food. The genera which have been most often used in studies on SCP production are (Kihlberg, 1972):
bacteria: *Bacillus, Hydrogenomonas, Methanomonas, Methylomonas;*
yeasts: *Candida, Rhodotorula, Saccharomyces;*
filamentous fungi: *Aspergillus, Fusarium, Penicillium;*
algae: *Chlorella, Scenedesmus, Spirulina.*

Important general characteristic properties of micro-organisms relevant to the production of SCP can be summarized as in Table 1.2 (Woldendorp, 1970; Wilkinson, 1971; Kihlberg, 1972). The following remarks can be made concerning these general properties:

a. *Growth rate*

The high maximum specific growth rate of bacteria, which can be approached in experiments using dilute media, has no advantage on a production scale where the oxygen transfer to maintain aerobic conditions usually becomes the limiting factor in the operation of the process. Processes using bacteria, fungi or

Table 1.2. General characteristic properties of micro-organisms, relevant to the production of SCP.

| Micro-organisms | Maximum specific growth rate | Diameter (μm) | Crude protein[1] (%Nx6,25) | Digestibility | Knowledge of toxicity |
|---|---|---|---|---|---|
| Bacteria | high | 0.5–2 | 50–80 | good | insufficient |
| Yeasts | moderate | 4 –8 | 40–65 | good | sufficient |
| Fungi | low | ca.10 | 20–50 | bad | insufficient |
| Algae | low | ca.10 | 30–60 | bad | insufficient |

[1] The minimum and maximum values from the reviews given by Woldendorp (1970), Wilkinson (1971) and Kihlberg (1972) are reported.
Only 70–80% of the crude protein values can be accounted for as amino acid N (Kihlberg, 1972).

yeasts will therefore probably operate at the same growth rate (WORGAN, 1972). It may be an advantage to use strains with optimum growth rates at high temperatures to prevent high costs of cooling.

b. *Separation of the biomass*

The diameter of a micro-organism will influence the costs of separation. In general, at higher diameter the separation costs will be lower. These costs will also be lower at higher concentrations of micro-organisms (WANG, 1968). Since the substrate concentration in waste effluents generally is rather low, the microbial density will also be low, so that for bacteria and yeasts the harvesting costs may be relatively high. If the primary consideration is reduction of BOD or COD and of the nitrogen content of the effluents, fungi have an advantage over bacteria, yeasts and algae, in that the mycelium can be separated by relatively simple and cheap filtration methods.

c. *Crude protein content of micro-organisms*

The crude protein content (%N $\times$ 6.25) is generally highest in bacteria. A content of 87% has been reported for *Lactobacillus fermentans* (BUNKER, 1963) and of 83% for *Salmonella typhimurium* at a doubling time of 5 hours (ELSWORTH et al., 1968). These values are influenced by the medium composition (as C/N ratio), other growth conditions and the growth rate; therefore, it can be said, that statements in the literature that certain species have high protein contents have little meaning.

The protein content of yeasts is generally lower than that of bacteria. Fungi are reported to produce biomass with much lower protein contents than bacteria or yeasts. Nevertheless, a number of fungal species have been reported to produce protein contents as high as those of yeasts. Mycelium of *Fusarium semitectum*, for instance, containing 60% crude protein has been obtained by growth in submerged liquid culture, with an ammonium salt as the N source (DELANEY and WORGAN, 1970). After acid hydrolysis, the recovery of amino acids was 90% of the mycelial nitrogen. A crude protein content of 56% has been reported for *Trichoderma* by RHODES et al. (1961). On the basis of amino acid analysis, the protein content of *Trichoderma viride*, grown on corn waste effluents, was 42–45%, which was about 80% of the crude protein content (CHURCH et al., 1972). Also SOLOMONS (1973) has reported high crude protein contents for fungi (up to 74%).

d. *Nucleic acid content of micro-organisms*

An important part of the cell nitrogen consists of nucleic acids (NA). Generally it is assumed that the NA content of fungi (ca. 5%) is lower than that of yeasts (ca. 10%) or bacteria (10–15%), but this content is also depending on growth rate and growth conditions; it increases at higher growth rates. Because of the difference in maximum specific growth rate between bacteria, yeasts and fungi it is questionable whether there will be any difference in NA content between these micro-organisms at equal specific growth rates.

A high amount of NA has to be avoided in human nutrition; accumulation of urates formed by breakdown of purine bases may cause gout and renal calculi in man. The NA content is of less importance for use of SCP in animal feed. Processes have been developed to reduce the NA content for application of SCP in human nutrition. This NA content can be lowered in a number of ways:

1. By limiting the growth rate, but this may be unattractive because of the general aim to obtain high yields and a rapid turnover.
2. By extraction of cell RNA with hot NaCl (10%). A commercial process has been developed (PEPPLER, 1970). The production on pilot plant scale of protein concentrates from yeast after mechanical desintegration and precipitation protein by heating at alkaline pH has been described by HEDENSKOG and MOGREN (1973).
3. Degradation of NA by endogenous enzymes. A three step heating process has been developed (MAUL et al., 1970).
4. Degradation of RNA with exogenous enzymes (ribonuclease). A method has been described by SCHLENK and DAINKO (1965), and developed further by CASTRO et al. (1971).

A review of NA reduction methods in SCP has been given by KIHLBERG (1972). In most NA extraction processes described in the literature loss of amino acids and proteins was a major problem. Another question is the economics of these processes, although several nucleotides, nucleosides and their derivatives may become of value for medicine and nutrition (DEMAIN, 1968). Degradation products of RNA are commercially produced as flavour-enhancing compounds, mainly by extraction of RNA from *C. utilis* strains with high NA content (KIHLBERG, 1972).

e. *Digestibility and net protein utilization (NPU) of microbial food*

Although generally the digestibility of fungi is bad, – chitin and/or cellulose are normal components of the cell wall –, a better digestibility and net protein utilization (NPU) than for food yeast was found for *Fusarium semitectum* (MILLER, 1968) and *Trichoderma viride* (CHURCH et al., 1972). Also other species of micro-organisms (*Escherichia coli, Aspergillus niger, A. oryzae* and others) should have a higher nutritional value than food yeast (WORGAN, 1973a), but the NPU values of only a few micro-organisms have been reported in the literature, and these values are always lower than those found for meat (FAO, 1964). The biological value can be increased by addition of deficient amino acids such as methionine to yeasts (NELSON et al., 1960) and fungi (RHODES et al., 1961) or tryptophan to bacteria (ANDERSON et al., 1958); increase of the biological value will result in a higher NPU.

f. *Texture and flavour of microbial food*

Bacteria and yeasts grow as individual cells without texture. After harvesting and drying, a powder is obtained, which can be added in small quantities as a protein-rich supplement to foods. Methods have been developed for obtaining protein concentrates from micro-organisms (e.g. HEDENSKOG et al., 1970). It is

also possible to spin protein from bacteria (HEDÉN et al., 1971) and yeast (HUANG and RHA, 1971) into fibres, which makes it possible to develop a product similar to textured soya bean protein. However, this will be more expensive than the direct use of microbial cells. For application of the microbial protein in human nutrition, fungi have the advantage of an acceptable flavour and a more palatable texture than food yeast, without further processing. The hyphae of fungi mat together when the material is harvested and give a product with a texture similar to moist chicken meat. In the conversion of wastes to protein for use in animal feed, especially the texture is of less importance.

g. *Toxicity of micro-organisms*

On toxicity of micro-organisms relatively little is known. The toxic properties will be depending on strain, growth media, and growth conditions. Numerous species do not appear to have any toxic effects. Many harmless micro-organisms occur in foods and are consumed in significant quantities. Cheese, oriental foods and numerous types of fermented liquors contain micro-organisms. A review of traditional fermented foods has been given by HESSELTINE and WANG (1967). Toxic effects of yeasts have never been published, contrary to fungi. However, a lot of edible species of larger fungi (mushrooms) have been reported in the literature. Also with several other fungi no toxic symptoms have been observed when the mycelium was fed to (weanling) rats (*Fusarium semitectum*, WORGAN, 1971; *Trichoderma viride*, CHURCH et al., 1972; PEITERSEN, 1975; *Penicillium chrysogenum*, DOCTOR and KERUR, 1968), or to mice (*Penicillium chrysogenum*, PATHAK and SESHADRI, 1965). Of all the hundreds of fungi screened by SOLOMONS (1973), only one proved to be toxic (a strain of *Alternaria*).

Simple processing methods such as heat treatment are known to destroy some of the toxins, which may be present in micro-organisms. Although it is probable that only few micro-organisms are toxic, it is difficult to establish that a new food is safe for human consumption. This consideration might prevent the introduction of microbial foods in human nutrition in the next future. For use in animal feed less rigorous tests for toxicity can be applied to the products, so that this application may have the best chance. Evaluations of the product safety have been described by e.g. OSER (1968) and KIHLBERG (1972).

Reviews

Reviews of investigations on SCP production and microbial foods have been published by e.g. THATCHER (1954), BUNKER (1963), GRAY (1966), MATELES and TANNENBAUM (1968), LIPINSKY and LITCHFIELD (1970), SNYDER (1970), BHATTACHARJEE (1970), and KIHLBERG (1972).

## 1.4. OUTLINE OF THE PRESENT INVESTIGATION

The manufacture of starch from maize is an example of a process with a waste effluent stream containing relatively low concentrations of valuable materials. The COD of the waste effluent is only about 6,000 (mg $O_2$/l). The average con-

TABLE 1.3. The average concentrations of N-free compounds in corn waste effluent expressed as mg/l[1].

| | |
|---|---|
| Glucose | 3000 |
| Starch | 1000 |
| Ethyl alcohol | 500 |
| Lactic acid | 500 |
| Acetic acid | 300 |
| Pentosans | 50 |

[1] Data from Scholten-Honig Research B.V., Foxhol (Gr.), The Netherlands.

centration of N-free compounds is given in Table 1.3. Other components of the effluent are present in about the same ratio as in corn steep liquor (CSL, cf. section 2.2.1). The average C:N:P ratio of such a waste effluent is about 100:6:1.

The low concentration of valuable materials made it unattractive in the past to recover by-products from this waste stream, because of the unfavourable costs.

Recent increase in the prices of proteins on the world market and the need to reduce pollution have led to a new interest in the possibilities of recovering materials from this waste stream, by physical and (bio)chemical processes. Besides, for instance, the concentration by reverse osmosis, which may make spray-drying of the concentrate economically feasible in the future, the production of SCP may be a possibility to solve the pollution problem economically.

At a COD level of 6,000 (mg $O_2$/l) and a yield constant ($Y$) of 0.4–0.5, the yield of micro-organisms will be only 2.5–3 g/l, if the polluting compounds are completely consumed. This implies that, without concentration of the waste stream, the production of yeast or bacterial SCP may be too expensive because of the high costs of separation of these micro-organisms in low concentration (WANG, 1968). For this reason, and for the reason that starch is an important component in the waste stream (Table 1.3), a fungus was chosen.

The influence of several factors (especially C/N ratio and pH) on growth rate, reduction of COD and composition of the mycelium (particularly the crude protein content), was investigated, in batch as well as in continuous culture.

Carbon sources, present in the waste stream, were tested as substrates for the selected fungus. However, the breakdown of pentosans has not been studied, because of the very low concentration in which this carbon source is present in the waste (Table 1.3). Nevertheless, the selected fungus, a strain of *Trichoderma viride*, may be able to utilize pentosans (SIMPSON, 1959).

Because starch and dextrins are besides glucose the most important carbon sources in the waste, special attention was given to the breakdown of these compounds. A study of the amylolytic activity was made with mainly well-defined mineral media as substrates, because it is unattractive to use a complex medium in enzyme regulation studies. For that reason the growth on mineral media was occasionally compared with that on complex media.

The potential possibility to use the harvested mycelium as a protein source in feed was investigated only by determination of the amino acid content and composition of the fungus.

## 1.5. The production and application of fungal mycelium as food or feed

*Introduction*

The use of fungi in human nutrition is not new. Higher fungi (mushrooms) have been used both as food and as food flavouring for centuries, and the mycelium of some of them (e.g. *Morchella sp.*) may be produced on an industrial scale (e.g. Litchfield, 1967a, b; Worgan, 1968). A problem in this mycelium production is the aroma retention of submerged cultured mycelium.

An advantage of moulds for food production is their ability to use not only inorganic nitrogen sources but also cheap macromolecular carbon sources, including even cellulose.

More than fifty years ago, Pringsheim and Lichtenstein (1920) reported the feeding of animals with the mould *Aspergillus fumigatus*, grown on straw supplemented with inorganic nitrogen fertilizer in Germany in World War I. In the same country several industrially produced fungi (*Fusarium, Candida, Rhizopus*) seem to have been incorporated into human diets with satisfactory results, in World War II (Thatcher, 1954).

The most investigated fungi as potential food are the fleshy Basidiomycetes (mushrooms) and the Deuteromycetes. The use of only a few Ascomycetes and Phycomycetes has been reported in the literature.

An interesting theoretical calculation and comparison of protein yields of fungi and cattle has been made by Gray (1962). The same author reviewed the potentiality of fungi as food, and applications of agricultural wastes as a substrate for production of fungi (1970). A few other examples of investigations on production (and application) of fungal SCP have been cited already in section 1.3.

*Influence of growth conditions on specific growth rate of fungi*

The specific growth rate of most fungi is low, but can increase considerably after addition of supplements, such as corn steep liquor (CSL), which is rich in vitamins and minerals (e.g. Litchfield et al., 1963; Gray, 1970). The selection of rapidly growing fungal strains will be necessary. The doubling times of cell mass of known edible species are between 4 and 12 hours (Litchfield, 1968), which means a much lower growth rate than that of many bacteria and yeasts. It is questionable, however, whether this is really a disadvantage (cf. section 1.3a). In addition, several filamentous fungi have much higher specific growth rates. Solomons (1973) reported a mould having a doubling time of 1.7 hr.

Fungi have been grown in submerged culture within a temperature range of 20–35°C, but little has been published on the effects of temperature on growth rate and yield of fungal mycelium. Nevertheless, it may be economical to grow micro-organisms at high temperatures because of the saving effect on cooling expenses. Some strains with a rather high optimum temperature have been isolated (e.g. Gray, 1962; Danielson and Davey, 1973b), but no investigator has ever claimed the isolation of a suitable fungus, being capable of growth within the range 45–55°C.

The most favourable pH range for fungal growth is between 4 and 7, but it is advantageous to grow at low pH, in view of the diminished risk of infections by bacteria.

Aeration and agitation have also important effects on growth rate, yield constant or economic coefficient, mycelial form (dispersed or pellet form) and composition, but these effects vary widely and are depending on organism and substrate. Although it may be attractive for separation to grow pellets, a disadvantage is the low mass transfer rate into pellets, accompanied by a decreasing growth rate (PHILLIPS, 1966; KOBAYASHI et al., 1973).

Another important factor which may affect the lag phase and morphology in the exponential phase are size and age of the inoculum.

*Protein content of fungi*

GRAY et al. (1964) screened a wide variety of imperfect fungi (175 strains) determining the crude and extractable protein produced in shake flasks after a 4-days incubation period. Also RHODES et al. (1961) determined the nitrogen (crude protein) content for a large number of fungal strains, and they have found the highest N content (9%) for a strain of the genus *Trichoderma*. SOLOMONS (1973) reported even a fungal N content of 11.8% but he did not mention the name of the fungus.

It is, however, difficult to compare the composition (protein content etc.) of several fungal species from literature, because of the variation in growth conditions (pH, temperature, aeration, C/N ratio of the nutrient medium etc.) and thus in growth rate. Since the optimum conditions in relation with protein content may be different for several species and strains, statements in the literature that certain species have high protein contents have little meaning. In addition, the protein content is mostly calculated as 'crude protein' (%N $\times$ 6.25), neglecting the fact that a considerable but unknown fraction of the nitrogen originates from nucleic acids and chitin. The nucleic acid content varies with the growth rate. The cell walls, especially those of imperfect fungi, contain important amounts of chitin; values in chitin content may range from 2.6–26.2% on dry weight and this content increases more rapidly with age in submerged culture than in still culture (BLUMENTHAL and ROSEMAN, 1957). A value of 2–5% chitin was observed for *Fusarium semitectum* at a doubling time of less than 5 hours (WORGAN, 1971).

*Amino acid composition of fungi*

The amino acid composition of most fungi shows the sulfur-containing amino acids – cystine and methionine – to be deficient (RHODES et al., 1961) as compared to the FAO reference pattern (1957). In addition, some amino acids – such as lysine – may be bound to the cell wall and as a consequence are biologically unavailable. The availability of lysine may be considerably decreased by processing of SCP, resulting in deficiency of this amino acid.

*Vitamin content of fungi*

Generally fungi are rich in vitamins of the B group (LITCHFIELD, 1967a), but they contain less of these vitamins than yeasts.

*Nutritional studies using fungi*

Only a few studies have been published on the nutritional value of fungal proteins for animals. Some examples have been cited already in section 1.3. A review has been given by LITCHFIELD (1968). Although the mycelia of some mushroom species appear to have acceptable flavours to man (LITCHFIELD, 1967b), human nutritional studies using fungal mycelium as a protein source have not been reported to our knowledge. Conclusions as to the value of fungal mycelium as food or feed, can only be drawn after extensive feeding studies with both domestic animals and man, to establish nutritional value and safety.

*Wastes as a substrate for fungal growth*

Several wastes have been proposed in the literature as a substrate for production of fungi such as molasses (GRAY and ABOU-EL-SEOUD, 1966), soybean whey (FALANGHE et al., 1964; CHURCH et al., 1972), corn waste (LITCHFIELD, 1964; CHURCH et al., 1972), cellulosic wastes (UPDEGRAFF, 1971; MAHLOCH, 1972; PEITERSEN, 1975), wastes from the coffee and rum-distilling industries (UPDEGRAFF et al., 1973), and other wastes of the food-processing industry, as summarized by GRAY (1970). Satisfactory results were obtained in pilot plant runs at low pH, without sterilization of the substrate (GRAY and ABOU-EL-SEOUD, 1966; CHURCH et al., 1972; UPDEGRAFF et al., 1973).

## 1.6. *Trichoderma viride*, THE FUNGUS USED IN THIS STUDY

The genus *Trichoderma* includes very rapidly spreading and opportunistic soil fungi, having the ability to establish themselves very well, and surviving treatments of soils with $CS_2$ or formaldehyde better than other micro-organisms do (EVANS, 1955; SAKSENA, 1960; MOUBASHER, 1963; PARK, 1965).

The names of three species are frequently encountered in the literature: *T. viride* Persoon ex Fries; *T. lignorum* (Tode) Harz and *T. koningii* Oudemans. The differences between many strains of these species are very small, however, and therefore BISBY (1939) considers the genus to be monotypic and the correct name of the species to be *T. viride* Pers. ex Fries. The name of the perfect, ascomycetous, stage of *Trichoderma viride* is *Hypocrea rufa* Pers. ex Fries.

*T. viride* appears fairly frequently in the laboratory as an air-borne infection on a variety of materials, and grows well on most common media. Colonies spread rapidly, forming a thin mycelial layer with irregularly shaped verdigris green patches, being the colour of the ripe spores in mass. The spore masses in some strains remain white for some time, becoming green only tardily. The fragile, spherical spore heads each contain 10–20 conidia, globose or slightly ovate, 2.5–3 μm in diameter. Sometimes chlamydospores (7.5–15 μm) are formed

in the hyphae. For the coconut odour, produced by some strains, 6-phenyl-α-pyrone seems to be responsible, according to COLLINS and HALIM (1972).

The optimum pH of *Trichoderma* has been found to be rather low (3.7–4.7) for many strains (DANIELSON and DAVEY, 1973b), while even a lower optimum is cited by others (CHURCH et al., 1972). Consequently, species of *Trichoderma* have been frequently isolated from acid, especially forest, soils (DANIELSON and DAVEY, 1973a).

Other properties enhancing the competitive saprophytic ability of the fungus are the good enzymatic complement and potentiality and possibly the ability of antibiotic production.

The most investigated extracellular enzyme of the fungus is cellulase, which is produced in high quantities by many strains under inducing conditions (MANDELS and REESE, 1957; KATZ and REESE, 1968; GHOSE, 1969; MANDELS et al., 1971, 1974; MENEZES et al., 1973; MITRA and WILKE, 1975; PEITERSEN, 1975). Cellulose, hydrolysed by *T. viride* cellulases may be used as a substrate for SCP production (GHOSE and MANDELS, 1970; PEITERSEN, 1975). Other extracellular enzymes produced by the mould are amylases (MANDELS and REESE, 1957; MANDELS et al., 1971), glucanases (HASEGAWA and NORDIN, 1969; DE VRIES and WESSELS, 1973), polygalacturonases (RAGHEB and FABIAN, 1955), pentosanases (SIMPSON, 1959), chitinases (ALEXANDER, 1965; DE VRIES and WESSELS, 1973), xylanases (ALEXANDER, 1965) and others.

Many years ago *Trichoderma* has already been found to be a parasite of other soil fungi (WEINDLING, 1932). In later years toxic substances of *Trichoderma* cultures have been isolated (e.g. WEINDLING and EMERSON, 1936; WEINDLING, 1937). By its antagonistic action the fungus has sometimes been used for controlling plant pathogenic fungi (WRIGHT, 1956). The most well-known antibiotics produced by *Trichoderma* are gliotoxin (anti-bacterial and anti-fungal) and viridin (anti-fungal). Gliotoxin and viridin seem to be produced in media containing $NH_4^+$-tartrate as the N source. The gliotoxin production may be inhibited by the presence of complex N sources, but a low pH and aeration are favourable for its production (BILAI, 1963). The same author reports that also 'volatile antibiotic substances', are produced by *Trichoderma*, contributing to the antagonistic action of the fungus. In a Japanese investigation *T. viride* has been found to produce a polypeptide with antibiotic action (OOKA et al., 1966). Another, yellow-coloured, compound with antibiotic action (anti-bacterial and anti-fungal) has been found by PYKE and DIETZ (1966), while the substance was isolated and the structure characterized by MEYER (1966). A new mycotoxin has been recently isolated by HOU et al. (1972).

The production of antibiotics and mycotoxins may suggest *T. viride* to be unattractive for SCP production. However, one has to keep in mind that the production of such substances is depending on medium composition and conditions of growth, whereas many of these substances may be destroyed by special treatments. Some feeding trials gave satisfactory results (CHURCH et al., 1972; PEITERSEN, 1975). On the whole, *T. viride* seems to possess favourable properties for use in production of SCP; it has been used in investigations on treatments of

non-sterile wastes from food-processing industries (CHURCH et al., 1972; UPDEGRAFF et al., 1973). It has been found to be the predominant micro-organism during aerobic decomposition of paper wastes (MAHLOCH, 1972).

The high nitrogen content of *T. viride* (9%), as has been found by RHODES et al. (1961), may indicate a comparatively high protein content of this fungus.

# 2. MATERIALS AND METHODS

## 2.1. FUNGAL STRAIN

In the present investigation *Trichoderma viride* strain CBS 354.44 was used. It was maintained on malt agar slopes at room temperature. Conventional techniques were employed for the transfer of the stock cultures.

## 2.2. MEDIA

### 2.2.1. *Complex media*

Corn steep liquor (CSL, donated by Scholten-Honig Research B.V., Foxhol, Gr., The Netherlands) was used as the complex nitrogen source in most experiments; occasionally peptone (Oxoid Ltd., London, England) was used as the N source.

#### Model for corn waste effluent

For practical reasons a waste model was chosen, consisting of mixtures of corn steep liquor (CSL) diluted with tap water, and an additional carbon source. This carbon source was usually glucose (E. Merck, A.G., Darmstadt, W. Germany), in some instances soluble starch (Merck), whereas in a few experiments instead of a carbohydrate, ethanol, acetate or lactate were used. CSL components are present in the waste effluent in about the same ratio as in CSL itself. Generally a COD level was chosen of about 2500 mg $O_2$/l to prevent the oxygen transfer to become the limiting factor for growth (see also section 2.3). By changing the ratio CSL to additional carbon source the C/N ratio could be varied. The complete medium was autoclaved at 120°C for 20 minutes. No influence on growth rate and nitrogen uptake was found no matter whether CSL and carbohydrates were sterilized separately or together in a pH range 3–7. The initial pH was adjusted at the desired value with 1 N $H_2SO_4$ or 1 N NaOH.

#### Composition of corn steep liquor

Corn steep liquor (CSL) is a by-product of the starch production from corn. It is rich in amino acids, peptides, vitamins and minerals; different samples show only small variations (Table 2.1), which may be explained by differences in the origin of the raw material and changes during the steeping process.

The samples were found to have a chemical oxygen demand (COD) of about 485 mg $O_2$/g, and the C/N ratio was calculated to be about 5.1, if the C content of CSL protein (= %N × 6.25, after subtraction of 13% ammonium N) is assumed to be 53%. The total phosphorus content in CSL is 2–3% of the dry weight; it is partly present as phytic acid. During the steeping process proteins are hydrolysed to peptides and amino acids. The amino acid composition of

TABLE 2.1. Composition of different samples of corn steep liquor in %.

| | SOLOMONS (1969) | | Mean value of KSH samples[1] |
|---|---|---|---|
| | Sample A | Sample B | |
| Total solids | 52 | 50.7 | 54 |
| Total N (Kjeldahl) | 4.3 | 3.7 | 3.65 |
| Acidity as lactic acid | 15 | 17.4 | 12 |
| Total carbohydrates as glucose | –[2] | –[2] | 9.8 |
| Free reducing carbohydrates as glucose | 5.6 | –[2] | 4.5 |
| Ash | 7.9 | –[2] | 8.6 |
| Phytic acid | –[2] | –[2] | 3.25 |
| pH | 4.0 | 3.9 | 4.2 |

[1] KSH samples and some data were kindly provided by Scholten-Honig Research B.V., Foxhol, Gr., The Netherlands.
[2] not determined.

CSL (after complete hydrolysis) is given in Table 2.2. The variation is rather small.

Other essential or growth-stimulating compounds present in CSL are vitamins of the B complex such as inositol (partly present as phytic acid), choline, nicotinic acid amide, pantothenic acid, pyridoxine, riboflavin, *p*-amino benzoic acid, biotin, thiamine and others. CSL is used in the fermentation industry as a raw material for the production of penicillin and other antibiotics, vitamin $B_{12}$, lysine, glutamic acid, organic acids and enzymes. An important part is also used

TABLE 2.2. The amino acid composition of CSL in % of the nitrogen.

| Amino acid | RHODES and FLETCHER (1966) | Data from KSH[2] |
|---|---|---|
| Lysine | –[1] | 4.0 |
| Histidine | –[1] | 6.8 |
| Arginine | 8.0 | 8.2 |
| Tryptophan | –[1] | –[1] |
| Aspartic acid | –[1] | 1.7 |
| Threonine | 3.5 | 3.4 |
| Serine | –[1] | –[1] |
| Glutamic acid | 8.0 | 8.0 |
| Proline | 5.0 | 4.8 |
| Glycine | –[1] | –[1] |
| Alanine | 25.0[3] | 27.7 |
| Cystine | 1.0 | 1.2 |
| Valine | 3.5 | 3.9 |
| Methionine | 1.0 | 1.1 |
| Isoleucine | 3.5 | 3.4 |
| Leucine | 6.0 | 5.9 |
| Tyrosine | –[1] | 0.7 |
| Phenylalanine | 2.0 | 2.0 |

[1] Not determined.
[2] See Table 2.1.
[3] Maximum value.

directly in the feed industry. CSL has been used furthermore in investigation on fodder yeast production (e.g. LIGGET and KOFFLER, 1948; FODA et al., 1973). In laboratory experiments it is frequently added as a growth-promoting substrate component, especially for fungal cultures.

2.2.2. *Mineral media*

The growth in the complex CSL medium was compared occasionally with that in mineral media, but the last media were mainly used for amylolytic enzyme studies of the organism. The basal mineral medium contained per litre: $KH_2PO_4$, 0.5 g; $Na_2SO_4$, 0.2 g; $CaCl_2$, 0.1 g; $MgSO_4.7H_2O$, 0.05 g, and trace elements to a final concentration of $FeCl_3.6H_2O$, 0.2 mg/l; $MnCl_2.4H_2O$, 0.2 mg/l; $ZnCl_2$, 0.1 mg/l and $CuSO_4.5H_2O$, 0.1 mg/l. All chemicals were of reagent grade and were obtained from commercial sources. Unless otherwise indicated, 2.5 g/l glucose or soluble starch (Merck) were used as the carbon source, and 0.2 g/l urea as the nitrogen source, which brought the C/N ratio of the medium to about 10. Other carbon sources used for growth studies were: amylose and lactose (Merck); maltose (Brocades); an amylopectin preparation (waxy maize); pullulan; $\beta$-cyclodextrin (cyclomaltoheptaose); a maltotriose preparation and preparations of dextrins: 30 DE and 20 DE (dextrose equivalents) containing an average number of about 3.3 and 5 glucose units per molecule, respectively. Pullulan, $\beta$-cyclodextrin and the maltotriose and dextrin preparations were kindly donated by Drs Th. M. van Bellegem, 'Proefstation voor aardappelverwerking, T.N.O.', Groningen, The Netherlands. In some experiments ammonium sulphate or potassium nitrate were used as the nitrogen source.

The initial pH of the media was adjusted at the desired value with 1 N $H_2SO_4$ or 1 N NaOH. The basal medium, trace element solution and carbohydrate solution were autoclaved separately at 120°C for 20 minutes and mixed after cooling. The urea solution (20 g/l) was sterilized by Seitz filtration and stored at –20°C up to two weeks; one ml of this solution was used per 100 ml of medium.

Cycloheximide (Actidione), occasionally added to culture filtrates (25 mg/l) for preservation, was purchased from Koch-Light Laboratories, Colnbrook, Bucks, England.

## 2.3. CONDITIONS OF GROWTH

2.3.1. *Batch cultures*

Usually, cultures were grown in 300 ml conical flasks using a volume of 100 ml of medium. After sterilization, the flasks were incubated in Gallenkamp orbital shakers at 29°C and 200 rev/min. Under these conditions the oxygen transfer rate (OTR) was found to be 7–8 mmol $O_2$/l.hr, as measured by the sulphite method of COOPER et al. (1944). No pH control was applied in these experiments.

Occasionally, cultures were grown in Biotec fermentors of 3.2 l capacity, using working volumes of 3 l at a constant temperature of 29°C. In these ex-

periments the pH could be recorded and controlled automatically with 1 N $H_2SO_4$ and 1 N NaOH, using sterilizable glass electrodes.

When a mineral medium was used or if no COD had to be determined, occasional foam was suppressed discontinuously by the addition of a few drops of polypropylene glycol 2000 (Shell Chemie N.V., the Netherlands), which appears to be a useful antifoam for mould fermentations (SOLOMONS, 1967).

The air was moistened by passing it through stone spargers in flasks with water to decrease evaporation from the fermentors and sterilized by miniature line filters (Microflow Ltd., Fleet, Hants, England).

At 325 rev/min and an air flow of 0.5 litre per litre of medium per minute, the OTR was found to be 7.5–10 mmol $O_2$/l.hr., and at 520 rev/min 20–22.5 mmol $O_2$/l.hr.

Samples of usually 100 ml were taken with the help of a rather wide glass tube (⌀ 7 mm inside or more) to prevent clogging. This tube was fitted at the upper side of the fermentor.

2.3.2. *Continuous cultures*

For continuous culture experiments, Biotec fermentors of 3.2 l capacity were used, with working volumes varying from 1.5 to 3 l.

The medium was sterilized as described previously and stored in 10 l flasks, placed on 'Cenco' magnetic stirrers, to keep unsoluble particles in homogeneous suspension. It was pumped into the fermentors on the upper side using LKB varioperpex 12000 peristaltic pumps (LKB-produkter AB, Bromma, Sweden) and sterilizable silicone rubber tubes. Usually the medium was aerated at a stirring rate of 325 rev/min and an air flow of 0.5 litre per litre of medium per minute (OTR 7.5–10 mmol $O_2$/l.hr). The pH was controlled automatically and air was moistened and sterilized as described previously (2.3.1). By changing the flow rate $F$ (ml/hr) and/or the working volume $V$ (ml) the dilution rate $D = F/V$ ($hr^{-1}$) could be varied.

The working volume was controlled by over-pressure and the mycelium suspension could flow out through a glass tube, which was flexibly fitted on the upper side of the fermentor. Rather wide glass tubes were used (⌀ 7 mm inside or more) to prevent clogging. The mycelium suspension was collected in sterile 10 l flasks. Samples were taken as described previously (see 2.3.1).

To prevent wall growth, a typical small-scale problem especially with fungi, several precautions were taken. A small teflon bar magnet was sterilized with the fermentor, and by the use of a powerful horse-shoe magnet at the outside, the fermentor glass wall was cleaned several times a day (RIGHELATO and PIRT, 1967). Also a teflon film (Hilflon, P.T.F.E. Sales division, Middlesex, England) was sprayed on the glass wall of the fermentor, the shaft and the electrodes. However, the film came gradually off in small particles after a few days, probably caused by water penetrating between the glass and the teflon film.

Foam formation was another problem. Although anti-foams such as polypropylene glycol 2000 (see 2.3.1) are very effective, they could not be used since

the COD level is increased by anti-foams, and calculations had to be made from the reduction values of the COD, because of the complexity of the medium. The presence of wall-grown mycelium or abundant foam adversely affects attainment of a 'steady state' (cf. section 5.2.2).

For these reasons, experiments were carried out under the following conditions:

a. only short-run experiments were performed (maximum ca. 2 weeks)
b. low substrate concentrations were used (about 2,500 COD units per litre of medium in most experiments; higher COD levels were applied at low dilution rates only)
c. a low rate of mixing and aeration was applied (325 rev/min; OTR 7.5–10 mmol $O_2$/l.hr)
d. no chemical anti-foams were added, but foam was suppressed mechanically by the use of additional blades on the fermentor shaft.

Under these conditions occasional foam was no serious problem.

## 2.4. Methods of inoculation

The inoculum was prepared from 2–3 weeks old slant cultures. The spores were suspended in sterile distilled water and the spore suspensions contained $10^6$–$10^7$ spores per ml. Per 100 ml of medium 1 ml of such a standard spore suspension was used as an inoculum.

In a number of experiments the inoculum consisted of a 24 hr old homogenized culture, prepared as described above. In that case 0.5 ml of the mycelium suspension per 100 ml of medium was transferred by means of a wide-mouth pipette.

The last method was preferred when results of experiments, carried out at different times and/or with different inocula, had to be compared. Inoculation by a mycelium suspension avoided a prolonged lag phase which is depending on the sporulation and germination conditions and on the age of the spore suspension.

Continuous culture experiments were always started as batch cultures, inoculated with standard spore suspensions (1 %; v/v).

## 2.5. Analytical methods

### 2.5.1. *Determination of dry weight of mycelial mass*

From duplicate samples of the culture (generally 100 ml) the mycelium was harvested by filtration on Whatman no. 4 filter paper tared after drying at 103 °C. Having been washed twice with distilled water, the filter papers containing the mycelium were dried at 103 °C to constant weight.

2.5.2. *Determination of oxygen transfer rate*

The oxygen transfer rate (OTR) was determined by the sulphite method of COOPER et al. (1944). The OTR was expressed in mmol $O_2$/l.hr.

2.5.3. *Determination of oxygen concentration and oxygen uptake rate*

The oxygen concentration and uptake rate were measured occasionally using a non-sterilizable galvanic oxygen probe of the Ag-Pb type.

The oxygen uptake rate was determined at 29 °C using the oxygen probe fixed in a closed vessel, in which the mycelium was kept in suspension by a magnetic stirrer. The decrease of the oxygen concentration was recorded as a function of time.

For calibration a sulphite solution and air-saturated distilled water were used.

2.5.4. *Determination of chemical oxygen demand*

The chemical oxygen demand (COD) was determined by the bichromate-sulphuric acid method (ANONYMUS, 1960) in the modification described by SPAANDER and PRINS (1965). The COD was expressed in mg oxygen per litre of medium or culture filtrate.

2.5.5. *Determination of reducing sugars in the culture medium*

Reducing sugars were estimated by the method of Somogyi-Nelson (SOMOGYI, 1952), as described by HODGE and HOFREITER (1962), and expressed in glucose or maltose equivalents (mg/l).

2.5.6. *Determination of total carbohydrates in the culture medium*

Total carbohydrates were estimated by the anthrone method as described by HODGE and HOFREITER (1962), using glucose as a standard (mg/l). The method was especially used for estimation of dextrins, produced during hydrolysis of starch (see chapter 7).

2.5.7. *Determination of starch in the culture medium*

Starch was determined as its blue-coloured iodine complex.

Reagents: The iodine reagent was prepared according to ZEVENHUIZEN (1966). Citrate buffer (100 ml; pH 6.0; 0.1 M), water (170 ml) and $I_2$-KI solution (20 ml; 2 g/l and 4 g/l, respectively) were mixed.

Procedure: To 1 ml of the sample (containing 100–500 μg starch) 3 ml of water and 2 ml of the iodine reagent were added. The absorbancy was measured within a few minutes at 623 nm against a control.

The wavelength of 623 nm lies between the maxima produced by the starch components amylose (660 nm) and amylopectin (530–550 nm). Although amylose makes up only about 20% of the soluble starch, it is responsible for about 80% of the absorbancy at 623 nm, after reaction with iodine reagent.

The relationship between starch concentration and absorbancy, which appeared to be linear, was exactly determined each time a fresh reagent was prepared.

In experiments with either amylose or amylopectin as the single (enzyme) substrate, the absorbancy was measured at 660 and 532 nm, respectively.

2.5.8. *Identification of sugars resulting from starch hydrolysis*

For purpose of identification, the sugars were separated by thin layer chromatography in n-butanol-pyridine-water-benzene (5:3:3:1 by volume) using cellulose thin-layer sheets (Merck) and detected by spraying the chromatograms with *p*-anisidine hydrochloride. Glucose, maltose and maltotriose were used as the reference compounds.

2.5.9. *The assay of amylolytic activity*

The rate of starch hydrolysis by culture filtrates was determined as the saccharifying and as the dextrinizing activity.

Procedure: A sample of the culture filtrate (1–4 ml, depending on the activity to be expected), and 1 ml of a sodium acetate buffer (0.2 M; pH 5.0) were pipetted into a test tube. After mixing, 4 ml of a freshly prepared soluble starch solution (4 g/l) was added. This starch solution had been heated for 5 min. at 100 °C and cooled just prior to its addition. The reaction mixture was made up to 10 ml with distilled water and immediately incubated for 1–6 hr at 30 °C. The reaction was stopped by immersing the glass-stoppered test tubes in boiling water for 5 minutes. Incubation mixtures of the same composition but heated immediately after mixing served as the controls.

The dextrinizing and saccharifying activities were calculated from the decrease of the starch concentration and the increase of the reducing compounds respectively, and are defined as follows, except where otherwise indicated:

- dextrinizing activity (DA): the decrease in mg of soluble starch in the reaction mixture, as determined with the iodine reagent, expressed per ml of culture filtrate per hour.
- saccharifying activity (SA): the increase, equivalent to mg maltose, of reducing carbohydrates in the reaction mixture, expressed per ml of culture filtrate per hour.
- specific activities are defined as above, but expressed per mg of dry biomass per hour.

As regards the validity of the enzyme assay method, satisfactory results (reproducibility within 15%) were obtained when, determined with the iodine blue reaction, more than 10% but less than 70% of the starch concentration initially present (1.6 g/l) was hydrolysed at the end of the enzyme incubation. However, in no case more than 50% of the starch initially present was allowed to be hydrolysed during the enzyme assays. Under these conditions the amounts of soluble starch hydrolysed and the amounts of reducing compounds produced were proportional to the enzyme concentration and to the incubation time.

Other enzyme substrates such as amylose, amylopectin, maltotriose, maltose and lactose, were used occasionally and in the same concentrations as soluble starch. The origin of these compounds has been mentioned under 2.2.2. Where necessary, the definitions of the activities given above have been adapted to these substrates.

In a few experiments the amylolytic activities of *T. viride* were compared with those of a relatively pure α-amylase preparation obtained from *A. oryzae* (Fungamyl 1600, NOVO-industri A/S, Copenhagen, Denmark).

2.5.10. *Determination of lactic acid, acetic acid and ethanol*

a) Lactic acid

For the determination of lactic acid the *p*-hydroxydiphenyl method (BARKER and SUMMERSON, 1941) was used, as described by ALLPORT and KEYSER (1957). Under the conditions described, the relation between the quantity of lactic acid and the resulting absorbancy is linear.

Acetic acid, ethanol and urea do not interfere with the analysis under the conditions specified (BARKER and SUMMERSON, 1941).

b) Acetic acid and ethanol

Acetic acid and ethanol were determined gas chromatographically according to REICHELT and DOELLE (1971).

2.5.11. *Determination of total nitrogen and crude protein*

Total nitrogen was determined by the Kjeldahl method. Crude protein was calculated as total nitrogen × 6.25.

Reagents: sulphuric acid (96%); sodium hydroxide (40 g/100 ml); boric acid (4 g/100 ml). As a catalyst was used 2 g of a mixture of $Na_2SO_4$ (250 g), $CuSO_4.5H_2O$ (4 g) and selenium nigrum (2.5 g). Indicator: 150 mg methyl red and 100 mg methylene blue were dissolved in 200 ml ethanol (96%) and the solution filtered before use.

Procedure: Dried filter papers with mycelium, or samples of the medium or culture filtrate were digested in 250 or 500 ml Kjeldahl flasks during about 2 hr after addition of concentrated sulphuric acid (10–15 ml) and the catalyst mixture.

If necessary, the heating treatment was continued until the solution became clear. After being cooled the destruction mixture was diluted with 50 ml of distilled water. Subsequently 50 ml NaOH was added and ammonia was distilled into 15 ml boric acid solution containing a few drops of the indicator; the ammonia was estimated by titration with 0.05 N sulphuric acid.

In each series a control was run simultaneously.

2.5.12. *Determination of amino acids*

a) Hydrolysis of proteins in the dried biomass and in concentrated culture filtrates

Samples were diluted 20-fold with 6 N HCl. Hydrolysis was carried out in a sealed glass tube during 20 hours at 110°C. The hydrolysed samples were evaporated under vacuum at 50°C until dry and the residue was dissolved in a buffer solution of pH 2.2, the composition of which has been given by VAN EGERAAT (1972).

By acid hydrolysis, tryptophan, asparagine and glutamine are lost completely while threonine and serine are partly destroyed, depending on the time of hydrolysis (SLUMP, 1969).

b) Isolation of free amino acids from the culture filtrate

Free amino acids in the culture filtrate were separated from salts and proteins using the cation-exchange resin Amberlite IR-120($H^+$). The ninhydrin-positive compounds were eluted from the resin with 0.5 N $NH_4OH$ and the eluate was evaporated under vacuum at 50°C until dry. The residue was dissolved in a buffer solution of pH 2.2 (see under a).

c) Estimation of ninhydrin-positive compounds

The analyses were made with the aid of a Biocal-200 amino acid analyser, following the procedure of MOORE and STEIN (1954), as described by VAN EGERAAT (1972).

# 3. FACTORS AFFECTING THE GROWTH, COD REDUCTION AND NITROGEN UPTAKE IN CSL GLUCOSE MEDIA IN BATCH CULTURE

## 3.1. INTRODUCTION

For the examination of several factors such as pH, C/N ratio, incubation time etc. affecting the growth rate, yield, COD reduction, fungal protein content and nitrogen uptake from the medium, batch culture experiments had to be carried out. Generally these experiments were done in shake flasks, without pH control, but occasionally 'Biotec' fermentors were applied for pH-controlled experiments. The results were intended to serve as basic data for continuous culture experiments. Occasionally, results found with the complex CSL glucose media were compared with those obtained with mineral media. From the given composition of CSL the C/N ratio was calculated to be about 5.1. Addition of a carbon source such as glucose increases the C/N ratio to values as calculated in Table 3.1.

## 3.2. COD REDUCTION AND MYCELIUM YIELD AS A FUNCTION OF THE C/N RATIO AND THE pH OF THE FRESH MEDIUM

Conical flasks of 300 ml containing 100 ml of CSL glucose media at different pH values and various C/N ratios, were sterilized and inoculated with 0.5 ml of a standard mycelium suspension. The initial COD of the media was chosen at a level of about 2500 mg $O_2$/l and later determined exactly by experiments.

The COD reduction after 24 hr incubation as a function of the C/N ratio at different initial pH values (Fig. 3.1), shows that the lowest final COD is found in filtrates of mycelium suspensions grown at C/N ratio 12.6 and initial pH values 4.2 and 5.5 (90% reduction in COD or more).

The final pH after 24 hr incubation was found to depend on the initial pH and the C/N ratio of the fresh medium as well, as can be concluded from Fig. 3.2. If CSL was used as the sole source of nitrogen and carbon, the pH increased during incubation by the production of ammonia from CSL proteins. With an initial pH of 7.0 a lower final pH was found for C/N ratios $> 10.2$, which suggests the production of organic acids from the added glucose.

The mycelium yield corresponds to the COD reduction values (cf. Figs. 3.1 and 3.3); the highest yields were found at the lowest final COD levels.

The weight of biomass produced per unit of carbon source consumed can be formulated by the equation $X = Y\Delta S$, where $X$ is the dry weight of biomass produced, $\Delta S$ is the amount of C source utilized and $Y$ is a constant. Generally $Y$ is called the 'yield constant' (HERBERT et al., 1956), although also the term 'economic coefficient' has been used (FOSTER, 1949). Cultivation in complex media

TABLE 3.1. The C/N ratio of CSL media after addition of different amounts of glucose to 1 g CSL.

| Gram glucose. $H_2O$ added per gram of CSL | C/N ratio (calculated) |
|---|---|
| 0 | 5.1 |
| 0.125 | 6.3 |
| 0.375 | 8.9 |
| 0.500 | 10.2 |
| 0.750 | 12.6 |
| 1.000 | 15.2 |
| 1.250 | 17.6 |
| 1.500 | 20.1 |
| 2.000 | 25.2 |

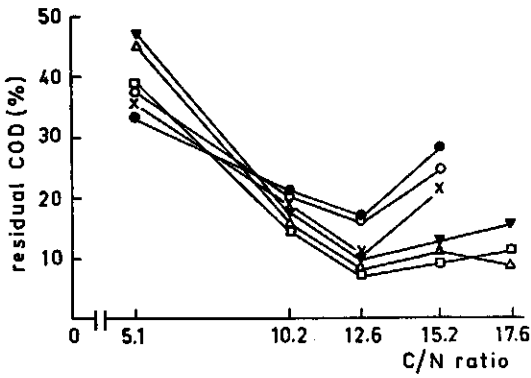


FIG. 3.1. Percentage residual COD after 24 hr of incubation of *T. viride* in CSL glucose media at various C/N ratios and pH values of the fresh medium. The media were inoculated with a standard mycelium suspension (0.5%; v/v). Initial pH: • 3.0; ○ 3.2; × 3.5; □ 4.2; △ 5.5; ▼ 7.0

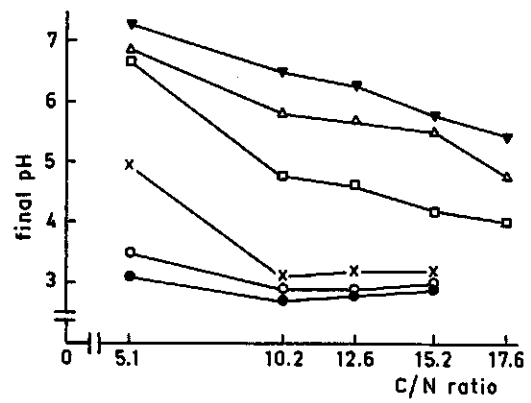


FIG. 3.2. Final pH after 24 hr of incubation of *T. viride* in CSL glucose media a t various C/N ratios and pH values of the fresh medium. The media were inoculated with a standard mycelium suspension (0.5%; v/v). Initial pH: • 3.0; ○ 3.2; × 3.5; □ 4.2; △ 5.5; ▼ 7.0.

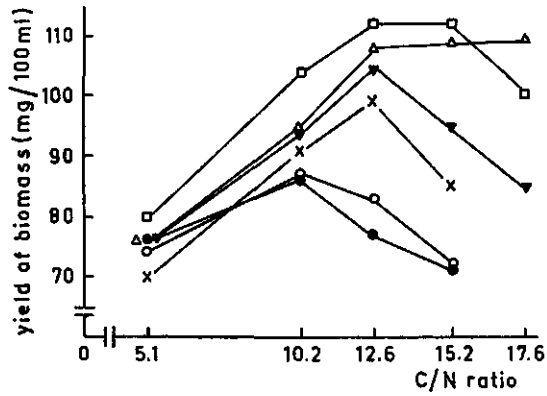


FIG. 3.3. Yield of biomass in mg dry weight per 100 ml of medium after 24 hr of incubation of *T. viride* in CSL glucose media at various C/N ratios and pH values of the fresh medium. Initial COD level of the medium 2665 mg/l. The media were inoculated with a standard mycelium suspension (0.5%; v/v). Initial pH: ● 3.0; ○ 3.2; × 3.5; □ 4.2; △ 5.5; ▼ 7.0.

does not permit calculation of a yield constant otherwise than on the basis of COD reduction. In this connection one should realize that glucose is the main carbon source in the medium at C/N ratios $>$ 10.2 and that 1 mg glucose is equivalent to 1.06 COD units, as the yield constant is usually expressed as units of biomass produced per unit of glucose consumed. At lower C/N ratios, however, the COD is besides by lactate, mainly caused by the CSL proteins, of which one unit of weight is equivalent to about 1.4 COD units (PORGES et al., 1950). The observed yield constant $Y$ was found to be about 0.45 after 24 hr incubation under optimum conditions. This is a quite acceptable value. A more detailed discussion on this matter will be given in chapter 5.

In the experiments the reducing sugars decreased to less than 3% of the initial value after 24 hr incubation at C/N ratios $<$ 15.2.

### 3.3. COD REDUCTION AND MYCELIUM YIELD AS A FUNCTION OF THE INCUBATION TIME

Comparison of results after one and the same incubation time may lead to wrong conclusions. Thus, the COD reduction after 24 hr of incubation was found to be about 90% at the C/N ratio 12.6 and an initial pH of 4.2, i.e. under optimal conditions. This figure of 90% increased to about 95% after 30 hr of incubation (Fig. 3.4). In addition to the data presented in the figures, it should be mentioned that 90% COD reduction could also be obtained at C/N ratios $>$ 12.6, but only at incubation times longer than 24 hr.

However, in media with C/N ratios lower than 10.2, the COD reduction was always smaller than 90% irrespective of the incubation time, while at C/N ratio 5.1 (CSL without glucose added) the reduction was never more than 70%. The

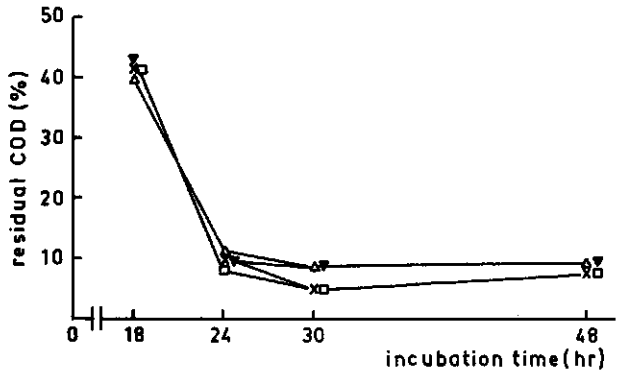


FIG. 3.4. Percentage residual COD as a function of the incubation time after growth of *T. viride* in CSL glucose media with C/N ratio 12.6 and various initial pH values. The media were inoculated with a standard mycelium suspension (0.5%; v/v). Initial pH: × 3.8; □ 4.2; △ 5.0; ▼ 5.5.

initial pH value (in relation with the C/N ratio) also influenced the results; generally highest COD reductions and yields of biomass were found when the pH during growth remained between 3.5 and 5.5 (see also section 3.4).

After incubation times longer than 30 hr the colour of the culture filtrate as a rule became more yellow, especially at high C/N ratios (C/N $\geqslant$ 15.2) and at high final pH values. When the pH after incubation times longer than 36 hr increased to values higher than 6.5, frequently a pink colour was observed in the medium. These phenomena have also been observed by others (DANIELSON and DAVEY, 1973c).

The yield constant $Y$ (mg fungal dry weight produced per mg COD reduced) decreased gradually during the incubation time (Fig. 3.5). Values for $Y$ higher than 0.50 were found after incubation times shorter than 18 hr, but this may have been partially due to solid CSL inclusions between the hyphae, especially at low C/N ratios; such inclusions were seen under the microscope. Probably the yield constant decreases during the incubation time because of the utilization of less easily assimilable substrates (such as lactic acid from CSL; cf. chapter 4) after glucose has been consumed, which may be accompanied by an increasing use of energy for maintenance (cf. chapter 5). Final yield constants were found of about 0.40 after 48 hr incubation.

In the case of exponential growth the specific growth rate $\mu$ ($hr^{-1}$) is related to doubling time of the biomass $t_d$ (hr) by the equation $\mu = t_d^{-1}.\ln 2$ (MONOD, 1942). Generally a filamentous form of mycelium was observed, and therefore growth according to the exponential law might be assumed (PIRT and CALLOW, 1960). A more detailed discussion on this matter will be given in chapter 5.

It is difficult to determine the growth yield in the initial phase of growth because, as indicated above, also undigested CSL particles (inclusions) are filtered off. If the lag phase is neglected after inoculation by a mycelium suspension, about 7.5 doubling times are necessary to increase the mycelium concentration

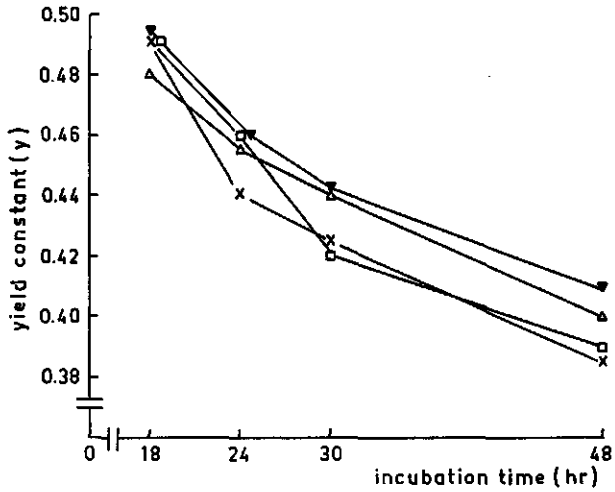


FIG. 3.5. Yield constant ($Y$), calculated as mg biomass produced per mg COD reduced, as function of the incubation time of *T. viride* in CSL glucose media with C/N ratio 12.6 and various initial pH values. The media were inoculated with a standard mycelium suspension (0.5%; v/v). Initial pH: × 3.8; □ 4.2; △ 5.0; ▼ 5.5.

from 0.5 mg/100 ml to a concentration of about 100 mg/100 ml. Since the last value is reached within 24 hr of incubation, $t_d$ is calculated to be no more than 3.2 hr and $\mu$ will be at least 0.21 $hr^{-1}$. However, as is generally the case in complex media, in CSL glucose media a gradually decreasing growth rate was found during the incubation time. Experimentally an initial specific growth rate was found of 0.28 $hr^{-1}$ ($t_d = 2.4$ hr). It is possible that the initial specific growth rate is still higher than 0.28 $hr^{-1}$, because no correction was made for undigested CSL particles present between the hyphae during initial growth.

### 3.4. THE pH DURING GROWTH AS A FUNCTION OF THE C/N RATIO AND pH OF THE FRESH MEDIUM

Although CSL glucose medium like many complex media has some buffer capacity, the pH changed during growth, depending on both the C/N ratio and the initial pH. From these changes in pH some information on the metabolic activities of the fungus might be obtained. In Fig. 3.6 the pH is shown as a function of the incubation time for various initial pH values at an initial C/N ratio of 12.6. The drop of the pH is smaller when the initial pH is lower, which may suggest that less organic acid is produced, the lower the pH (cf. residual sugars in Table 3.4; section 3.9)

The pH as a function of the C/N ratio of the fresh medium is given (Fig. 3.7) for an initial pH value of 4.2. The different initial drops of the pH suggest a higher production of organic acids at higher C/N ratios (see also section 3.2).

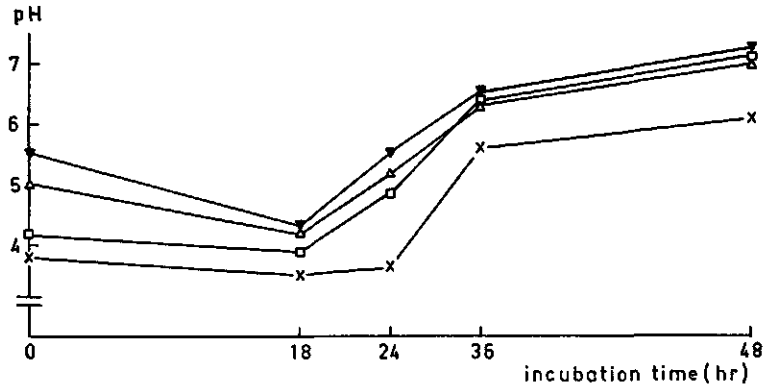


FIG. 3.6. The pH of the culture medium as function of the incubation time at various initial pH values of CSL glucose media with C/N ratio 12.6. The media were inoculated with a standard mycelium suspension (0.5%; v/v). Initial pH: × 3.8; □ 4.2; △ 5.0; ▼ 5.5.

Probably gluconic acid is produced, an acid formed by many species of fungi at a high glucose concentration, a high pH and aeration. Also citric acid may accumulate in the medium, especially at low pH (COCHRANE, 1958); a patent claims the production of citric acid using a strain of *Trichoderma viride* (KINOSHITA et al., 1961). Later on most of the excreted acids will be consumed again. The consumption of organic acids causes an increase of pH, while finally, when autolysis occurs, also basic compounds may be excreted by breakdown of fungal proteins, causing a further increase of the pH.

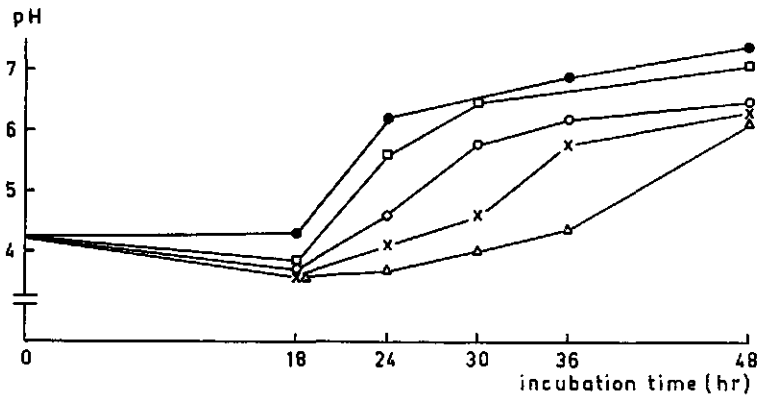


FIG. 3.7. The pH of the culture medium as function of the incubation time at various C/N ratios of CSL glucose media and an initial pH value of 4.2. The media were inoculated with a standard mycelium suspension (0.5%; v/v). C/N ratio: ● 6.3; □ 8.9; ○ 12.6; × 15.2; △ 20.1.

### 3.5. THE CRUDE PROTEIN CONTENT OF THE MYCELIUM AS A FUNCTION OF THE INCUBATION TIME AT VARIOUS C/N RATIOS OF THE FRESH MEDIUM

For the economic production of SCP the protein content and the composition of the mycelium are very important. Although the commercial value of the mycelium is determined by the amino acid composition (each essential amino acid has its own price), generally only the crude protein content is determined. As a rule, the same method was used in the present investigation. Data on the fungal protein content and the amino acid composition as found in the present investigation will be given later on (see chapter 6).

The crude protein content of the mycelium as a function of the incubation time at various C/N ratios of the fresh medium is given in Fig. 3.8. At low C/N ratios and short incubation times the harvested product contained inclusions from the CSL (probably rich in proteins), which is indicated by the broken lines. The nitrogen content (calculated on dry weight of biomass) decreased during the incubation time by the formation of N-free polymers (carbohydrates and fat) in the cell. During the initial phase of growth the assimilation rate of glucose is lower than that of various amino acids, which causes temporarily high initial fungal nitrogen contents. It may be concluded that the C/N ratio of the medium has an important influence on the nitrogen content of the mycelium, which is known to vary considerably with the growth conditions in all fungi and also in heterotrophic bacteria. Fungal mycelium of the same species for example can be produced containing 24.4% fat and 18.6% protein or 4.2% fat and 52.1% protein (WORGAN, 1968).

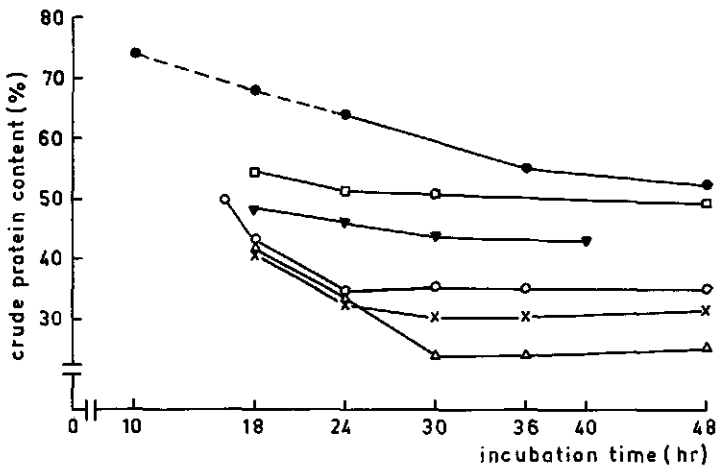


FIG. 3.8. Crude protein content of the biomass (% of dry matter) as function of the incubation time after growth of *T. viride* in CSL glucose media with various C/N ratios: ● 6.3; □ 8.9; ○ 12.6; × 15.2; △ 20.1, and after growth in a mineral glucose urea medium with C/N ratio 10 (▼). The initial pH was 4.2. The media were inoculated with a standard mycelium suspension (0.5%; v/v). The broken line indicates the presence of CSL inclusions between the hyphae.

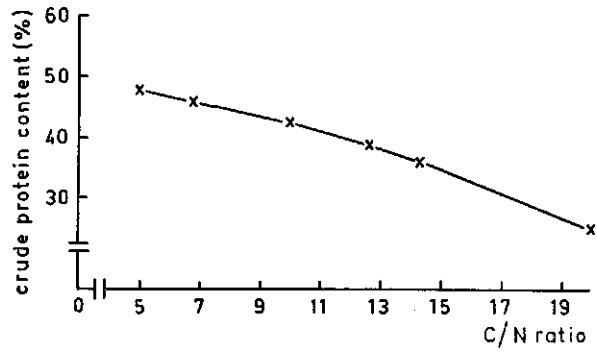


FIG. 3.9. Crude protein content of the biomass (% of dry matter) as function of the C/N ratio of the medium after 40 hr of incubation of *T. viride* in mineral glucose urea media. The initial pH was 4.0. The media were inoculated with a standard spore suspension (1%; v/v).

The lowest observed fungal nitrogen content was about 3% at the end of the growth in media with C/N ratios higher than 25. With synthetic glucose urea media a comparable trend was found in fungal crude protein content, both as function of the incubation time (Fig. 3.8) and as function of the C/N ratio (Fig. 3.9). The growth rate in mineral media is much lower than in complex media with the same COD, so that after equal incubation times smaller yields were found. At the end of the growth, however, yields of biomass were generally somewhat higher in the mineral media.

### 3.6. NITROGEN UPTAKE FROM THE MEDIUM AS A FUNCTION OF THE INCUBATION TIME AT VARIOUS C/N RATIOS OF THE FRESH MEDIUM

Besides the COD reduction, the decrease in nitrogen content is an important aspect in treatments of waste effluents. In the experiments of Fig. 3.10 it was

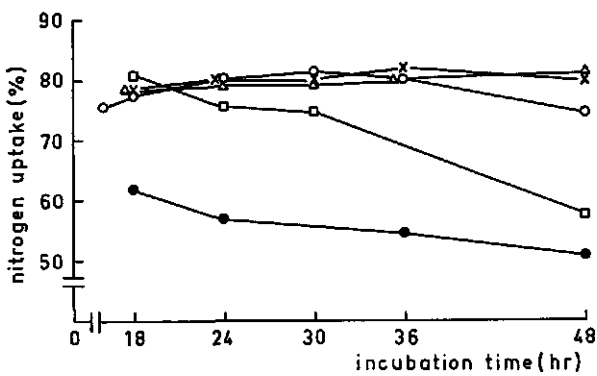


FIG. 3.10. Percentage nitrogen uptake from the medium as function of the incubation time of *T. viride* in CSL glucose media with various C/N ratios and an initial pH value of 4.2. The media were inoculated with a standard mycelium suspension (0.5%; v/v). C/N ratio: • 6.3; □ 8.9; ○ 12.6; × 15.2; △ 20.1.

generally impossible to reduce the nitrogen content of the medium for more than 80–85% at C/N ratios $\geqslant$ 8.9, while at C/N ratios $<$ 8.9 these percentages were still lower. Only at C/N ratios $>$ 20 sometimes 85–90% reduction of nitrogen content was observed. No difference was found whether the substrate was sterilized at pH 3 or pH 7, and whether glucose and CSL were sterilized together or separately. Neither did the pH during growth have any influence. When glucose was added to the exhausted medium (culture filtrate) only some secondary growth occurred. Much better growth on the exhausted medium was observed, however, when besides glucose, ammonia was added, so that the conclusion may be drawn that less easily assimilable nitrogen compounds remained in the medium after growth in CSL media. Indeed, many amino acids, such as L-lysine, L-cysteine, L-leucine, L-histidine and others (DANIELSON and DAVEY, 1973c) are unable to function as the sole N source for *Trichoderma* species. It was observed in the present investigation that peptides were the main nitrogen compounds remaining in the exhausted medium although a small amount of amino acids (and some ammonia) were also present (see chapter 6). This suggests, that part of the CSL peptides cannot be used by the fungus. However, MORTON and BROADBENT (1955) have found that filtrates of mycelium suspensions from *Trichoderma viride* and other fungi contained 14–24% of the original nitrogen content of the fresh medium, no matter whether organic nitrogen sources (amino acids) were used or inorganic ones ($NH_4^+$; $NO_3^-$), and independently of the nitrogen level of the fresh medium. The last-mentioned authors have also found that less than 10% of the remaining nitrogen was present in free amino acids and about 90% in peptides excreted by the fungus. The amino acids could be utilized as a nitrogen source again, the peptides after acid hydrolysis only. The concentration of extracellular nitrogen was lower when adequate amounts of trace elements had been added to the mineral medium.

The nitrogen contents of the culture filtrates increased after longer incubation times, but more rapidly at low C/N ratios (Fig. 3.10). In these phases of growth the culture filtrates became turbid and the COD increased. After exhaustion of the medium, compounds of the mycelium itself will be used as energy sources for maintenance. Which compounds are used as substrates for endogenous metabolism depends on the composition of the mycelium. MIZUNUMA (1963) has found that, at least with fungi, at high C/N ratios of the fresh medium, endogenous carbohydrates and lipids are used first, while at low C/N ratios, fungal amino acids, proteins and nucleic acids are used. The subsequent ammonia production explains the further increase in nitrogen content and pH of the culture filtrate.

### 3.7. THE OXYGEN UPTAKE RATE OF THE MOULD AT DIFFERENT PHYSIOLOGICAL AGES

During the previous experiments the question arose under which circumstances oxygen could become a limiting factor for growth. The oxygen transfer rate

(OTR) as measured by the sulphite method of COOPER et al. (1944) was found to be 7–8 mmol/l.hr under the usual experimental conditions. According to PIRT and CALLOW (1958), the OTR as measured by the sulphite method has to be at least 1.4–2.0 times the oxygen demand (OD) in order to ensure a sufficient oxygen supply to cultures of bacteria. For fungal cultures a factor 3 was applied by the same authors in a later report (PIRT and CALLOW, 1960). Much higher substrate concentrations and generally antifoams have been used, however, in their studies. In microbial cultures, OTR may be influenced by various factors. Antifoams may reduce the OTR, but foam itself may reduce the OTR also. The viscosity of fungal cultures will decrease the OTR, while the morphology (the presence of filamentous mycelium or pellets and the pellet size) may influence the oxygen uptake rate considerably (KOBAYASHI et al., 1973). The formation of pellets will be influenced by medium composition, environmental conditions and method of inoculation (cf. section 3.10).

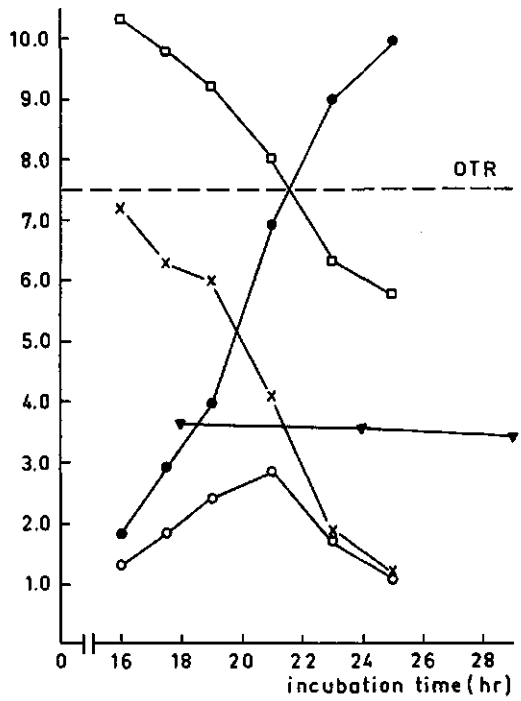


FIG. 3.11. The oxygen uptake rate of *T. viride*, growing in a CSL glucose medium with C/N ratio 12.6, as function of the incubation time. The COD level of the fresh medium was 2425 mg/l and the initial pH 4.2. The medium was inoculated with a standard spore suspension (0.5%; v/v). Experiments were carried out by the use of an oxygen probe.
• yield of biomass (mg/10 ml); × specific rate of respiration (mmol $O_2$/g dry wt.hr); ○ rate of respiration of total culture (mmol $O_2$/l. hr); □ fungal nitrogen content (%); ▼ specific rate of respiration (mmol $O_2$/g dry wt.hr) in a mineral glucose urea medium with C/N ratio 10 and COD 2500 mg/l; initial pH 4.2. The broken line indicates the oxygen transfer rate (OTR), measured by the sulphite method (mmol $O_2$/l.hr).
N.B. The figures on the vertical axis apply to all data as specified above.

To determine the relation between the OTR and the OD of cultures as used in the present investigation, experiments were carried out with mycelia, growing in a CSL glucose medium at the optimal C/N ratio of 12.6 and at pH 4.2 of the fresh medium. In different phases of growth the OD and the biomass were determined. The specific rate of respiration of the cultures (mmol $O_2$/g dry weight.hr) was determined by the use of a galvanic oxygen probe as described in section 2.5.3. The results were compared with those obtained from experiments in Warburg manometers. Occasionally a mineral glucose urea medium (COD ca. 2500 mg/l; C/N ratio 10; pH 4.0) was used for comparison with the complex CSL glucose medium. The results found by the use of the oxygen probe are expressed in Fig. 3.11. The figures resulting from the Warburg experiments were about the same. The ratio OTR/OD reached a minimum value of 2.7 after 21 hr of incubation. A rapidly decreasing specific rate of respiration was found during growth in the complex medium and a similar trend was found in the fungal nitrogen content, while the specific rate of respiration decreased more slowly in the mineral medium. The maximum value in the initial phase of growth (7.1 mmol $O_2$/g dry biomass.hr) was approximately the same as the maximum values found in young cultures of filamentous mycelia by some other authors (MEYRATH and MCINTOSH, 1963; KOBAYASHI et al., 1973). Generally lower values have been found, however, depending on medium composition, growth rate and the presence of pellets (PIRT and CALLOW, 1960; MEYRATH and MCINTOSH, 1963; RIGHELATO et al., 1968; KOBAYASHI et al., 1973).

From the growth rate and the COD reduction rate in some preliminary experiments with higher initial COD levels, it was concluded that under the usual conditions oxygen would not be a limiting factor for growth in CSL glucose media up to COD levels of 4500–5000 mg/l.

### 3.8. GROWTH OF THE MOULD AFTER PARTIAL SUBSTITUTION OF CSL BY UREA AND GLUCOSE

In waste effluents with C/N ratios higher than 12.6 (cf. section 1.4) it may be attractive to decrease the C/N ratio by addition of urea or ammonia rather than CSL if no other components are limiting. Addition of CSL will increase the COD level of the effluent, since, as has been found in section 3.3, about 30% of the COD originating from CSL remains in the medium.

In the following experiment various volumes (from 10 up to 100 per cent) of a CSL glucose medium (COD 2425 mg/l; C/N ratio 12.6; pH 4.5) were substituted by equal volumes of a solution of glucose and urea in tap water, resulting in the same COD, C/N ratio and pH. The mycelia were grown under standard conditions and inoculated with 0.5% (v/v) of a standard spore suspension. From the results (Fig. 3.12) it may be concluded that a maximum of 70% substitution did not reduce the growth yield after 26 hr incubation. No higher yields were found after longer incubation times. Since the experiment gave no information on the effect of the substitution on the specific growth rate, the yield was determined as

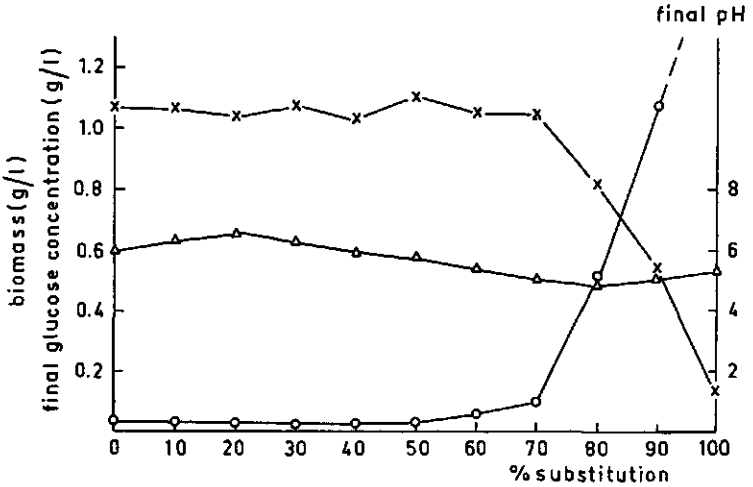


FIG. 3.12. Influence on the yield of biomass after substitution of CSL glucose medium with COD 2425 mg/l, C/N ratio 12.6 and pH 4.5, by a urea glucose solution with equal COD, C/N ratio and pH. The media were inoculated with a standard spore suspension (0.5%; v/v). Results after 26 hr of incubation: × biomass (g/l); ○ final concentration of reducing sugars in glucose equivalents (g/l); △ final pH.

a function of the incubation time at 2 substitution percentages (30 and 50%) and a blank (no substitution). It can be seen from the results (Table 3.2), that the difference in specific growth rate between the medium with 50% substitution and the blank is very small. Considerable differences in yield were found, however, after 70% substitution, suggesting a decreased specific growth rate. It may be further concluded from the results, that amino acids from CSL are more rapidly consumed than urea, giving higher initial fungal protein contents and a rapid nitrogen uptake from the medium. After 26 hr incubation no significant

TABLE 3.2. Yield and crude protein content of the biomass; nitrogen uptake from and final pH of the medium as a function of the incubation time at various substitution percentages of a CSL glucose medium (COD 2425 mg/l; C/N ratio 12.6; pH 4.5) by a urea glucose solution with equivalent COD, C/N ratio and pH. Media were inoculated with 0.5% (v/v) of a standard spore suspension.

| Incubation time (hr) | Yield of biomass (mg/100 ml) | | | Crude protein content of the biomass in % | | | Nitrogen uptake from the medium in % | | | Final pH of the medium | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | % substitution | | | % substitution | | | % substitution | | | % substitution | | |
| | 0 | 50 | 70 | 0 | 50 | 50 | 0 | 50 | 70 | 0 | 50 | 70 |
| 17 | 28 | 25 | 26 | 58 | 52 | 43 | 36 | 29 | 25 | 4.5 | 4.3 | 4.4 |
| 20 | 68 | 64 | 42 | 42 | 35 | 36 | 64 | 50 | 34 | 4.3 | 4.2 | 4.3 |
| 24 | 102 | 104 | 94 | 35 | 33 | 34 | 78 | 76 | 71 | 5.2 | 4.8 | 4.4 |
| 26 | 104 | 107 | 101 | 35 | 34 | 34 | 81 | 82 | 77 | 6.1 | 5.7 | 4.6 |

differences were found, however, neither in protein content of the biomass, nor in total nitrogen recovery from the medium.

One may suppose, that addition of urea or ammonia instead of CSL to waste effluents may give higher protein contents of the mycelia, without decreasing COD reduction rates, or even may give increased COD reduction rates. Furthermore, one must keep in mind that the optimum C/N ratio of 12.6 was determined for CSL glucose media and was related to the COD reduction rate (cf. section 3.2). Theoretically, it can be calculated that if mycelium has to be produced with a nitrogen content of 10% on dry weight, and a carbon content of 50% at a carbon recovery of 50%, a substrate must be supplied with a C/N ratio of 10 if nitrogen is recovered completely. If the nitrogen recovery is only 80%, the resulting optimum C/N ratio is 8. At carbon recoveries lower than 50%, higher optimum C/N ratios will result. Anyhow, addition of inorganic nitrogen may theoretically increase the fungal protein content, also if mycelium is grown in CSL carbohydrate media with an original C/N ratio of 12.6.

Some previous batch experiments with mineral media (see Figs. 3.8 and 3.9) indicated, that the crude protein content could be increased by the addition of inorganic nitrogen but much more urea had to be added than was calculated on the theoretical basis. In preliminary experiments with CSL media, it was found that the C/N ratio had to be decreased by nitrogen addition to even less than 5 in order to obtain a fungal crude protein content of about 60%, but this value was only observed after initial growth. A surplus of nitrogen remained in the culture filtrate under these conditions. However, the addition of inorganic nitrogen increased the growth rate, in CSL glucose media as well as in mineral media.

In addition, the nitrogen metabolism may be also influenced by the size of the inoculum. McINTOSH and MEYRATH (1963) have found a large inoculum to assimilate nitrogen more efficiently than a small inoculum did. The inocula used in the present investigation were large as compared to those in the experiments of these authors. This condition may have influenced the results, but the effect of the inoculum size was not studied in detail.

### 3.9. FUNGAL GROWTH IN 'BIOTEC' FERMENTORS COMPARED TO SHAKE FLASK CULTURES

Before carrying out continuous culture experiments, batch experiments were performed in a 'Biotec' fermentor with and without control of pH, to compare the growth in the fermentor to that in shake flasks. In addition, a better impression of the optimum pH can be obtained from pH-controlled experiments. These experiments were carried out only in the pH range 3.5–5.5. Values of pH lower than 3.5 or higher than 5.5 were less attractive because of the decreasing growth rate, while at pH values higher than 5.5 the risk of bacterial contamination increases considerably. The C/N ratio of 15.2, used in the experiments of this section is about equal to that of the industrial corn waste effluent (C/N ratio

about 16; see section 1.4), while the COD level was chosen at 2910 mg/l.

In addition, the influence of the stirring rate on growth rate and morphology was studied by using two rates (325 and 520 rev/min; no baffles were used). If the culture is aerated by 0.5 litre air/litre of medium.minute (standard condition) and the density of air (containing about 20% oxygen) is approximately 1.2 g/l, the aeration efficiency is calculated to be only 3.3–4.4% at an OTR of 7.5–10 mmol $O_2$/litre of medium.hr (stirring rate 325 rev/min) and about 10% at an OTR of 22.5 mmol $O_2$/litre of medium.hr (stirring rate 520 rev/min).

No differences were found (after 24 hr incubation) between the two stirring rates used, neither in COD reduction, sugar consumption and yield of biomass (see Table 3.3), nor in morphology of the mould; changes in pH did not differ from those in shake flask experiments. These results suggest that there is no oxygen limitation at a stirring rate of 325 rev/min. In further experiments this stirring rate was used. The oxygen concentration in the suspension, occasionally measured by the use of an $O_2$-probe at different times during the incubation period, was never found to be lower than 70% of the saturation value at 29°C. Somewhat lower COD reduction values were found, however, than in shake flask experiments after 24 hr of incubation (cf. section 3.2). Although there may be some differences in inoculation, lag phase and/or growth rate, and also in rate of evaporation between fermentor and shake flask cultures, the different results are expected to be caused mainly by the formation of foam in the fermentor towards the end of the growth (after about 20 hr of incubation), a phenomenon never observed in the shake flask experiments. A flotation tendency of the mycelium was observed in this phase, possibly causing a decreased growth rate by a limiting diffusion of nutrients from the bulk. Gas bubbles fixed on the hyphae in the viscous suspension are probably responsible for the flotation

TABLE 3.3. The influence of the stirring rate in a 3 litre 'Biotec' fermentor on COD reduction, sugar consumption and yield of biomass after 24 hr of incubation in a CSL glucose medium at an initial COD level of 2910 mg/l, a C/N ratio of 15.2 and an initial pH of 4.2 (no pH control). The cultures were aerated at a level of 0.5 litre of air/litre of medium.minute. The media were inoculated with a standard mycelium suspension (0.5%; v/v).

| | stirring rate (rev/min) | |
|---|---|---|
| | 325 | 520 |
| OTR (mmol $O_2$/l.hr; sulphite method) | 7.5–10 | 22.5 |
| Yield of biomass (mg dry weight/100 ml) | 94 | 90 |
| Crude protein content (%) | 39.6 | 40.7 |
| Final COD of the culture filtrate (mg/l) | 725 | 755 |
| COD reduction (%) | 75 | 74 |
| Yield constant ($Y$) | 0.47 | 0.44 |
| Reducing compounds in the culture filtrate (as mg glucose/litre) | 360 | 340 |
| Decrease in reducing compounds (%) | 82 | 84 |
| Final pH | 3.8 | 3.8 |

effect. Microscopic examination showed a rather homogeneous mycelium and closely branched hyphae surrounded by gas bubbles. Later on, the foam disappeared, while the viscosity of the suspension decreased, the pH rose and the mycelium gradually sedimented when stirring was stopped. Microscopic examination now showed more flocks to be present and the hyphae to be broken into smaller units, which seems to be a symptom of old age of a non-growing mould (RIGHELATO et al., 1968). This effect was not clearly observed in shake flask experiments, which suggests the shearing action of the stirrer to be also responsible for this phenomenon. The nitrogen content of the mycelium was similar to that observed in shake flask experiments, thus the breaking up of the mycelium did not result in leakage of an appreciable amount of nitrogen into the medium. The COD of the culture filtrate in this phase dropped further to about 10% of the initial value and thereafter it increased again.

Experiments with pH control were carried out at the following pH values: 3.5; 4.2; 4.5; 5.5. The fermentors were inoculated with 0.5% (v/v) of a standard mycelium suspension. The results after 24 hr incubation (see Table 3.4) suggest the optimum pH to be about 4.5. DANIELSON and DAVEY (1973b) have found the optimum pH of *Trichoderma* to range from 3.7 to 4.7. Rather similar phenomena were observed towards the end of the growth as described above for experiments without pH control.

Representative sampling from the fermentor cultures towards the end of the growth was difficult because of the presence of wall growth and inhomogeneous mycelium. This may have influenced the determination of fungal yields, and thus the yield constants.

TABLE 3.4. The influence of pH on COD reduction, sugar consumption and yield of biomass after 24 hr incubation of *T. viride* in a CSL glucose medium at an initial COD level of 2910 mg/l and a C/N ratio of 15.2. The cultures were grown in 3 l 'Biotec' fermentors and aerated at a level of 0.5 litre of air/litre of medium.minute at a stirring rate of 325 rev/min. The media were inoculated with a standard mycelium suspension (0.5%; v/v).

| pH controlled at | 3.5 | 4.2 | 4.5 | 5.5 |
|---|---|---|---|---|
| Yield of biomass (mg dry weight/100 ml) | 92 | 108 | 120 | 96 |
| Final COD of the culture filtrate (mg/l) | 830 | 435 | 440 | 600 |
| COD reduction (%) | 71.5 | 85 | 85 | 79.5 |
| Yield constant ($Y$) | 0.44 | 0.43 | 0.48 | 0.42 |
| Reducing compounds in the culture filtrate (as mg glucose/litre) | 485 | 240 | 205 | 180 |
| Decrease in reducing compounds (%) | 76 | 88 | 90 | 91 |

## 3.10. INFLUENCE OF GROWTH CONDITIONS AND PHYSIOLOGICAL AGE ON THE MORPHOLOGY OF THE FUNGUS

Fungi in submerged culture may grow either in the form of compact spherical masses of mycelium known as 'pellets', or as a homogeneous suspension of

filaments, but generally a mixture of these extremes is encountered. In most of the experiments described in this chapter, growth was mainly filamentous with the observation that some more pellets were present at higher pH values (pH > 4.5) than at lower pH values. A similar result has also been observed for *Penicillium chrysogenum* by PIRT and CALLOW (1959). These authors have found the morphology also to be influenced by the medium composition: less swollen cells and less branched hyphae were found when ammonium sulphate was substituted by CSL as the nitrogen source. They suggested that the cell wall structure or composition depends on the pH and other environmental conditions during growth, and that the resistance of the cells to shear increases with decrease in pH value.

In the present study the C/N ratio of the CSL glucose medium was also found to influence the size of the hyphae. A high C/N ratio (C/N > 10.2) resulted in more swollen and closely branched hyphae, while at lower C/N ratios (especially when CSL was used without addition of carbohydrates) long thin sparsely branched hyphae were produced. The last form of growth was also observed in mineral media with lactate as the sole carbon source (see section 4.3). Generally a pellet form of growth was observed with acetate as the sole C source (see section 4.3), which suggests the morphology to depend on the composition of the medium.

After the growth ceased and the medium was exhausted, a considerable increase in hyphal vacuolation was observed and conidia appeared in the culture, which phenomena have also been found by RIGHELATO et al. (1968) for *Penicillium chrysogenum*. Later on the mould autolysed.

The breakdown of the hyphae into smaller units, as described in section 3.9, and the simultaneous decrease in viscosity seemed to occur to a larger extent in fermentor cultures than in shake flask cultures under the usual conditions, presumably due to the shearing action of the impeller. However, also in shake flask experiments the incubation method and inoculation were found to influence the morphology. If smaller working volumes were used (50 ml instead of 100 ml) in the 300 ml conical flasks, and the flasks were incubated in a shaker at much higher rates than usual (250–300 rev/min instead of 200 rev/min), a pellet form of growth was observed with regular pellets of ca. 3 mm diameter, suggesting growth in pellet form to be promoted by the vigorous agitation of the culture. The phenomenon of pellet growth was considerably better observed, however, after inoculation with a standard mycelium suspension (0.5%; v/v) than after a heavy inoculation with a standard spore suspension (1%; v/v). In the last case mainly homogeneous filamentous mycelia with some very small pellets were found. Smaller COD reductions were observed in filtrates of pellet-containing cultures, than in filtrates of cultures with filamentous mycelia, after similar incubation times. This suggests a decreasing rate of respiration by oxygen limitation within the pellets, a phenomenon observed by several investigators (PHILLIPS, 1966; KOBAYASHI et al., 1973). However, at high mycelium concentrations the culture may become more viscous with filamentous mycelium than with pellets, and in such highly viscous filamentous cultures, rate of mass transfer

and efficiency of mixing may even become more reduced than in pellet-containing cultures.

Filtration of the mycelial mass after growth in CSL glucose media was generally found to be adversely affected by the same conditions that were unfavourable to growth (very low pH; low C/N ratio) and by incubation of the culture until autolysis started. Anyhow, the best filtration was found after a pellet form of growth.

### 3.11. Summary and conclusions

The present chapter deals with factors influencing the growth of *T. viride* and the accompanying COD reduction and nitrogen recovery in CSL glucose media in batch culture.

Maximum COD reduction rates have been found in media with an initial C/N ratio of 12.6 and initial pH values between 3.5 and 5.5. The highest COD reduction observed was about 95%. The yield constant ($Y$), based on mg COD reduction, decreased from about 0.5 to 0.4 during the incubation time. The maximum specific growth rate ($\mu_{max}$) observed was 0.28 $hr^{-1}$. Changes in pH of the culture depended strongly on the C/N ratio and pH of the fresh medium and on the incubation time. The fungal nitrogen content decreased with increasing C/N ratio of the medium and increasing incubation time; the value varied from about 3% to 10%. The maximum nitrogen uptake from the medium was more than 80%. The maximum specific respiration rate observed was 7.1 mmol $O_2$/g dry biomass.hr (equivalent to $Qo_2 = 160$ µl $O_2$/mg dry biomass.hr). Probably the aeration was sufficient under the growth conditions used. When CSL glucose medium was substituted by a urea glucose solution (with equal COD, C/N ratio and pH) the specific growth rate decreased significantly only at substitutions over 50%; the yield of biomass decreased when more than 70% of the CSL glucose medium was substituted. Differences in results between shake flask cultures and fermentor cultures had to be attributed to the different aeration methods and the shearing action of the impeller in the fermentor. The morphology of the mould depended on a large number of factors such as growth medium, growth conditions and age of the culture.

# 4. THE UTILIZATION OF ETHANOL, LACTIC ACID AND ACETIC ACID

## 4.1. INTRODUCTION

Ethanol, lactic acid and acetic acid are present in corn waste effluents in low concentrations only (see chapter 1.4). Although they scarcely contribute to the COD of the waste effluent, they may have an inhibiting effect on growth of micro-organisms in rather low concentrations. Especially undissociated acetic acid and other lower fatty acids such as formic and propionic acids are as a rule hardly utilizable or even toxic to yeasts (SAMSON et al., 1955) and fungi (JARVIS and JOHNSON, 1947; FENCL and LEOPOLD, 1959). Lower fungi may utilize acetate more rapidly than higher fungi do (COCHRANE, 1958).

In the present chapter experiments on the utilization of ethanol, lactic and acetic acids will be described in which CSL as well as urea were used as the nitrogen source. Not only the yield constants on these compounds as sole sources of carbon and energy were determined, but also the sequence in consumption from mixtures with at least two of these compounds.

## 4.2. EXPERIMENTS WITH CSL MEDIA

Conical flasks of 300 ml containing 100 ml CSL medium with additional amounts of carbon source were inoculated with a standard spore suspension (1%; v/v) and incubated in the Gallenkamp shaker as described in section 2.3.1.

### 4.2.1. *Growth as function of the ethanol concentration*

When ethanol was added in concentrations lower than 0.5% (v/v; about 80 mmol/l) to a basal medium consisting of CSL (2 g/l) diluted with tap water and brought to pH 4.0, an increase in yield was observed (Fig. 4.1). Higher concentrations of ethanol did not result in higher yields and after 48 hr of incubation yields were even lower. This points to some toxic effect. From the response to low concentrations of ethanol it may be concluded that the yield constant ($Y$) must be at least 0.55; this will be determined more exactly in section 4.3.1.

### 4.2.2. *Growth as function of the lactate concentration*

Different quantities of lactate were added to a basal CSL medium (2 g/l; pH 4.0); their influence on the mycelium yield is given in Fig. 4.2. It seems from these results that the yield constant on lactate is about 0.25, which is markedly lower than the yield constants on glucose and on ethanol. The yield constant on lactate will be determined more exactly in section 4.3.1.

A slightly inhibiting effect of higher lactate concentrations on the growth rate was found, especially at lower pH; this toxic effect apparently increases when

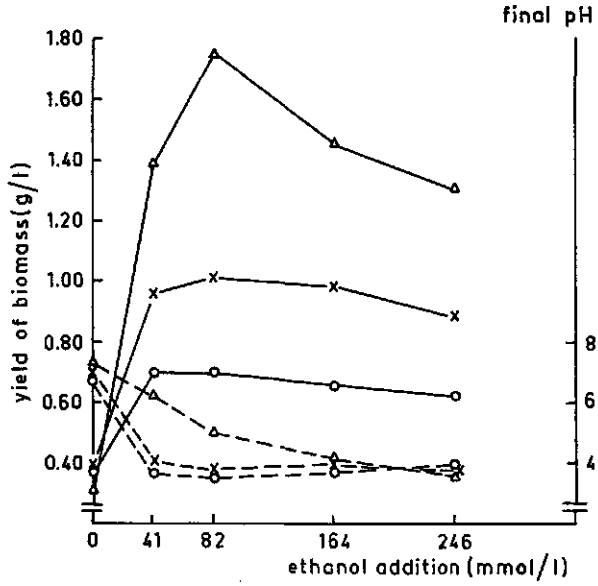


FIG. 4.1. The influence of ethanol addition on the yield of biomass and pH in a basal CSL medium with a COD of 970 mg/l. The initial pH was 4.0. The media were inoculated with a standard spore suspension (1%; v/v). The broken lines indicate the pH values. Incubation times: ○ 18 hr; × 24 hr; △ 48 hr.

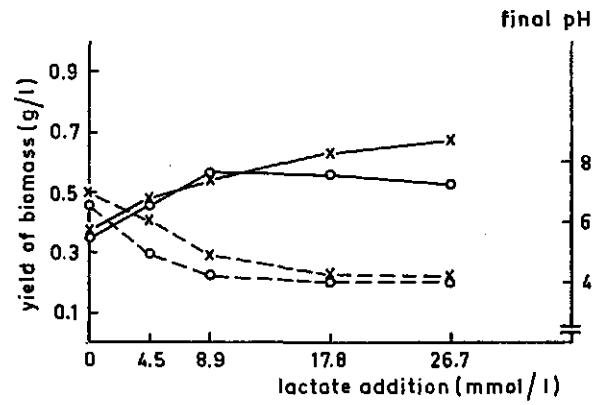


FIG. 4.2. The influence of lactate addition on the yield of biomass and pH in a basal CSL medium with a COD of 970 mg/l. The initial pH was 4.0. The media were inoculated with a standard spore suspension (1%; v/v). The broken lines indicate the pH values. Incubation times: ○ 18 hr; × 24 hr.

more undissociated acid is present. During the growth this inhibition decreased by an increase of pH, resulting from acid consumption.

4.2.3. *Growth as function of the acetate concentration*

Acetate, added to a basal CSL medium (2 g/l; pH 4.0) was utilized as a carbon source, although very low concentrations already had a toxic effect (Fig. 4.3). Acetic acid caused much more inhibition of growth rate than lactic acid at equal molarity and pH; probably it is taken up by the cell faster than lactic acid, since acetic acid is a weaker acid than lactic acid (pK acetic acid = 4.74; pK lactic acid = 3.86).

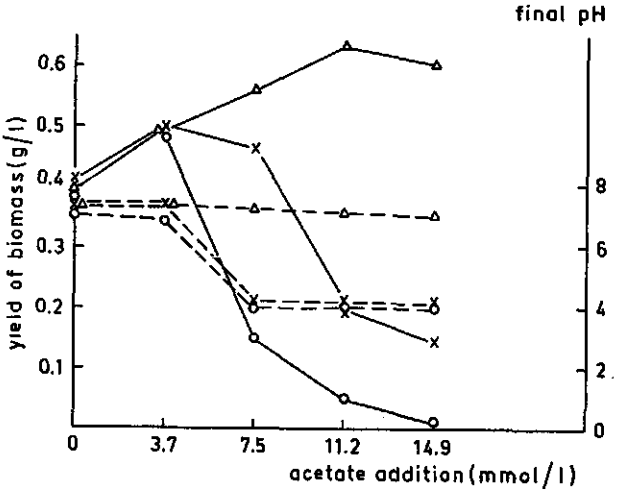


FIG. 4.3. The influence of acetate addition on the yield of biomass and pH in a basal CSL medium with a COD of 970 mg/l. The initial pH was 4.0. The media were inoculated with a standard spore suspension (1%; v/v). The broken lines indicate the pH values. Incubation times: ○ 18 hr; × 24 hr; △ 38 hr.

From the additional yield it can be calculated that the yield constant $Y$ on acetic acid is about 0.36.

It could be concluded from additional experiments that the toxic effect increased with increasing concentration of undissociated acetic acid. Utilization of the acid caused a rising pH and a decreasing inhibition of the growth rate. In the presence of acetic acid (> 7.4 mmol/l) generally growth in the pellet form was found, especially at lower pH values, a phenomenon not observed in media containing ethanol or lactic acid.

## 4.3. EXPERIMENTS WITH MINERAL MEDIA

4.3.1. *The yield constants on ethanol, acetate, lactate and glucose*

Since it is practically impossible to determine the yield constant ($Y$) on each individual C source present in complex media, the $Y$ values were determined in

mineral media, using urea as the nitrogen source and glucose, ethanol, lactate or acetate as the sole carbon sources.

In the following experiments glucose monohydrate; ethanol (96%; d = 0.789 g/ml); Na-acetate.$3H_2O$ or Na-lactate (0.6 g/ml) were added as the sole C source to the standard mineral medium with urea (2 g/l) as the N source; the initial pH was adjusted at 4.5.

Conical flasks of 300 ml capacity were supplied with 100 ml of medium containing ethanol (0.1%; v/v), glucose, acetate or lactate (1 g/l). These flasks were incubated during at most 60 hr in a Gallenkamp orbital shaker (200 rev/min). Other flasks, containing a double concentration of the C source (except glucose) were incubated during 44 and 60 hr. The cultures were inoculated with a standard spore suspension (1%; v/v).

The results of these experiments are summarized in Table 4.1. The yield constant $Y$ was calculated as: g biomass produced per g C source consumed. The final acetate concentrations could not be calculated exactly, because peaks with divergent tails were obtained on the (gas-liquid) chromatograms. The highest yield constant was found with ethanol as the carbon source; the lowest with lactate. Generally, a pellet form of growth was observed when the fungus was grown at pH $\leqslant$ 4.5 in a medium containing 7.4 mM acetate or more. Long thin sparsely-branched hyphae were found, however, after growth in media containing lactic acid, especially at pH $\leqslant$ 4.5. After growth in media containing glucose

TABLE 4.1. The yield constants of *T. viride* in mineral media with urea (2 g/l) as the nitrogen source, and glucose, ethanol, acetate or lactate as the sole carbon source and limiting nutrient. The initial pH was adjusted at 4.5. The cultures were inoculated with a standard spore suspension (1%; v/v) and incubated in Gallenkamp shakers.

| Carbon source | Incubation time (hr) | Final pH | Dry weight of the biomass produced (g/l) | Final substrate concentration as percentage of the initial concentration | Yield constant $Y$[1] |
|---|---|---|---|---|---|
| Glucose.$H_2O$ (1 g/l) | 24 | 4.4 | 0.36 | 27.5 | 0.50[2] |
| Glucose.$H_2O$ (1 g/l) | 36 | 7.3 | 0.47 | < 1 | 0.52[2] |
| Glucose.$H_2O$ (1 g/l) | 44 | 7.4 | 0.45 | < 1 | 0.50[2] |
| Ethanol 96% (0.1%) | 60 | 7.0 | 0.44 | < 1 | 0.58[3] |
| Ethanol 96% (0.2%) | 44 | 5.1 | 0.25 | 74 | 0.63[3] |
| Ethanol 96% (0.2%) | 60 | 6.0 | 0.63 | 29 | 0.58[3] |
| Na-lactate (1 g/l) | 60 | 5.9 | 0.16 | 30 | 0.29[4] |
| Na-lactate (2 g/l) | 44 | 4.6 | 0.11 | 79 | 0.32[4] |
| Na-lactate (2 g/l) | 60 | 5.0 | 0.18 | 65 | 0.32[4] |
| Na-acetate.$3H_2O$ (1 g/l) | 60 | 8.4 | 0.16 | < 5 | ca. 0.37[5] |
| Na-acetate.$3H_2O$ (2 g/l) | 44 | 7.5 | 0.30 | < 3 | ca. 0.35[5] |
| Na-acetate.$3H_2O$ (2 g/l) | 60 | 8.5 | 0.31 | < 3 | ca. 0.35[5] |

[1] $Y$ = g biomass produced/g C source utilized; [2] calculated on anhydrous glucose (0.91 g/l); [3] calculated on absolute ethanol (0.76 and 15.2 g/l, resp.); [4] calculated on lactic acid (0.805 and 1.61 g/l, resp.); [5] calculated on acetic acid (0.44 and 0.88 g/l. resp.).

or ethanol, these extremes were not observed but 'normal' branched hyphae without pellets were found.

4.3.2. *The utilization of ethanol, acetate, lactate and glucose from mixtures of these compounds*

In similar experiments as described in section 4.3.1 the sequence in utilization of C sources from mixtures was examined. The results (Table 4.2) suggest that at an initial pH of 4.5 glucose and acetate are utilized simultaneously before ethanol, whereas lactate is consumed after ethanol. Lactic acid must be considered to be a poor C source for *T. viride;* a slow utilization and a rather low yield constant were found with this acid. It is suggested that the remaining COD in the experiments of section 3.3 is caused to a large extent by lactate from CSL.

## 4.4. Discussion and conclusions

When the yield constants $Y$ as found for *T. viride* in the present investigation are compared with those found by Hernandez and Johnson (1967) for *Candida utilis* and *Pseudomonas fluorescens* (Table 4.3), some similarities can be seen. The final concentration of biomass in the experiments of these authors was approximately equal to that in the present investigation (0.5–1.0 g dry weight per litre) and they observed for *Candida utilis* equal growth rates on acetate and glucose ($t_d = 2.3$ hr), whereas on ethanol a lower growth rate was found ($t_d = 3.5$ hr). For *Pseudomonas fluorescens*, however, these authors found the highest growth rate on lactate ($t_d = 0.8$ hr) followed by acetate ($t_d = 0.9$ hr), glucose ($t_d = 1.3$ hr) and ethanol ($t_d = 2.1$ hr).

In the present study it was found that *T. viride* consumed glucose and acetate simultaneously and before ethanol. The growth rate of *T. viride* in mineral media was lower than that of *Candida utilis* in the cited report for each C source used. Although higher specific growth rates have been observed for *Pseudomonas fluorescens*, higher yield constants have been found, however, for *Candida utilis* and, as was the case in the present study, for *T. viride*.

A yield constant varying from 0.2–0.4 (g dry wt/g acetic acid) has been found for *Candida utilis* by Edwards et al. (1970). They observed a maintenance coefficient (see chapter 5.2.2) of 0.17 g acetic acid/g dry wt.hr.

The utilization of acetate is strongly pH-dependent and the toxic effect of the acid increases with decreasing pH. Samson et al. (1955) have shown that phosphate assimilation and oxidative phosphorylation in yeasts are inhibited by acetic acid and they presume that this inhibition is caused by a high concentration of acetate ion resulting from the undissociated acid that was able to penetrate into the cell. A bond was assumed by these authors to be formed between acetate and proteins, causing inhibition of enzymatic systems. Fencl and Leopold (1959) have found that spore germination of the mould *Aspergillus niger* depends directly on the presence of undissociated acetic acid in the medium. The limiting effective concentration of the undissociated acid lies somewhat above

TABLE. 4.2. The sequence in utilization by *T. viride* of glucose, acetate, ethanol and lactate from mixtures of these C sources in mineral media with urea (2 g/l) as the N source. The initial pH of the media was adjusted at 4.5. The cultures were inoculated with a standard spore suspension (1%; v/v) and incubated in Gallenkamp shake incubators.

| C sources | | | | Incubation time (hr) | Final pH | Dry weight of biomass (g/l) | Final substrate concentration as percentage of the initial concentration | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Glucose. $H_2O$ (1 g/l) | Ethanol 96% (0.1%; v/v) | Na-acetate. $3H_2O$ (1 g/l) | Na-lactate (1 g/l) | | | | glucose | ethanol | acetate | lactate |
| | x | x | | 48 | 7.2 | 0.32 | | 67 | < 5 | |
| | x | x(2 g/l) | | 48 | 6.7 | 0.33 | | 94 | < 3 | |
| | | x | x | 48 | 8.1 | 0.21 | | | < 5 | 79 |
| | | x(2 g/l) | x | 48 | 8.3 | 0.34 | | | < 3 | 92 |
| | x | | x | 48 | 6.5 | 0.54 | | < 1 | | 78 |
| | x | x | x | 60 | 7.0 | 0.67 | | < 1 | < 5 | 86 |
| | x | x(2 g/l) | x | 36 | 6.5 | 0.34 | | 96 | < 3 | 94 |
| x | x | | | 24 | 4.5 | 0.24 | 51 | 98 | | |
| x | x | | | 36 | 5.2 | 0.50 | 2.5 | 86 | | |
| x | | x | | 24 | 4.9 | 0.27 | 62 | | ca. 30 | |
| x | | x | | 36 | 7.4 | 0.61 | 2 | | < 5 | |
| x | | | x | 24 | 4.4 | 0.38 | 31.5 | | | 92 |
| x | | | x | 36 | 7.3 | 0.48 | 2 | | | 86 |
| x | x | x | | 24 | 5.0 | 0.22 | 65.5 | 101 | ca. 35 | |
| x | x | x | | 36 | 6.8 | 0.72 | 2.5 | 82 | < 5 | |
| x | x | | x | 24 | 4.5 | 0.37 | 33 | 94 | | 96 |
| x | x | | x | 44 | 5.8 | 0.99 | < 1 | < 1 | | 77 |
| x | | x | x | 24 | 4.8 | 0.30 | 57 | | ca. 25 | 98 |
| x | | x | x | 36 | 7.8 | 0.68 | < 1 | | < 5 | 87 |
| x | x | x | x | 24 | 4.8 | 0.27 | 62 | 98 | ca. 30 | 94 |
| x | x | x | x | 44 | 6.9 | 1.02 | < 1 | 19 | < 5 | 83 |

TABLE 4.3. The yield constants ($Y^1$) on various C sources as found by HERNANDEZ and JOHNSON (1967) for *Candida utilis* and *Pseudomonas fluorescens* compared to those found for *T. viride* in the present investigation.

| C source | HERNANDEZ and JOHNSON (1967) | | this study |
|---|---|---|---|
| | *Pseudomonas fluorescens* | *Candida utilis* | *T. viride* |
| Glucose | 0.38 | 0.51 | 0.50–0.52 |
| Ethanol | 0.49 | 0.68 | 0.58–0.63 |
| Acetate | 0.28 | 0.36 | 0.35–0.37 |
| Lactate | 0.32 | – | 0.29–0.32 |

[1] $Y$ calculated as g biomass produced per g C source utilized.

$2.10^{-2}$ M. At this concentration the spore germination was reversibly inhibited, by inhibition of the uptake of phosphate, sulphate and succinate anions. The authors concluded from these findings that accumulation of acetic acid inside the cell, either as such or in the form of acetate ion, influences the permeability thereby causing a lack of anions inside the cell. PÖHLAND et al. (1966) have found that *Candida utilis* utilized acetate simultaneously with glucose at pH 4, whereas at pH 6 glucose was consumed before acetate. This may suggest that the uptake rate of acetic acid depends on the pH, and that the undissociated acid is utilized more rapidly than the acetate ion.

As to lactic acid, it has been observed that this acid is generally utilized by yeasts after acetic acid (KAMPF and BEHRENS, 1966). From a mixture of acetate, lactate, lactose and glucose, *Penicillium chrysogenum* utilized acetate before glucose and lactate, whereas lactose was utilized last (JARVIS and JOHNSON, 1947).

Considering the composition of corn waste effluent (see section 1.4), it can be concluded that the influence of ethanol, lactate and acetate will be very small because of the low concentrations (about 11, 5.5 and 5 mmol/l, resp.) of these C sources in the effluent. Only acetate may cause some growth rate inhibiting effect at low pH (lower than pH 4), but probably only in batch processes. Lactic acid was suggested (4.3.2) to be an important residual compound after growth of *T. viride* in CSL-containing media. Compared to industrial corn waste effluents a similar concentration of lactate but negligible concentrations of ethanol and acetate are present in CSL glucose media at C/N ratio 12.6.

Remarkable in this study was the observed pellet form of growth in the presence of acetate concentrations of 7.5 mmol/l or more at pH $\leqslant$ 4.5; the causes of this pellet type of growth are obscure.

The position of starch in the utilization sequence of the different carbon compounds present in corn waste effluent was not investigated in this study, although starch is one of the main components of this effluent. In chapter 7, however, attention has been given to the hydrolysis of starch and to the regulation of amylolytic enzyme production in *T. viride*.

# 5. CONTINUOUS PRODUCTION OF MYCELIUM IN CSL CARBOHYDRATE MEDIA

## 5.1. General introduction

For the application of processes intended to produce biomass on a large scale, it can be theoretically calculated that continuous production is cheaper than batch production. However, the lack of experience and the risks of contamination have limited the number of continuous fermentation processes to only a few. Only food yeast, beer and ethanol are continuously produced on a commercial scale in some countries. Anyhow, from a scientific view, continuous culture experiments may be interesting because of the possibility to examine physiological processes and the morphology of micro-organisms under 'steady state' conditions.

## 5.2. General theory

### 5.2.1. *Growth models for fungi*

Studies on the kinetics of growth of fungi in submerged culture show two apparently conflicting observations. Emerson (1950) has found empirically that the growth of *Neurospora* in a submerged culture follows a cube root relation:

$$X_t^{1/3} = K_c t + X_o^{1/3} \tag{1}$$

where, $X_t$ = biomass (g/l); $t$ = time (hr); $X_o$ = biomass at $t = o$; $K_c$ = a constant.

On the other hand Pirt and Callow (1960) have found that various fungi may grow according to the exponential law (an autokatalytic reaction of the first order) like other micro-organisms (Monod, 1942; Herbert et al., 1956):

$$X_t = X_o e^{\mu t} \tag{2}$$

where, $\mu$ = a constant known as the specific growth rate ($hr^{-1}$). Other symbols have the same sense as above.

Emerson (1950) argued that the law (1) was the natural result of apical growth, only at the tips of the hyphae. However, through branching of the hyphae, apical growth is compatible with exponential growth (Pirt and Callow, 1960). The two types of growth kinetics may be accounted for by the two extreme modes of growth which fungi show in submerged culture: the 'stromatic' or 'pellet' form, in which the culture consists of a number of roughly spherical, compact masses of hyphae and the 'filamentous' form in which the mycelium consists of a mass of branched hyphae, homogeneously dispersed in the medium. In the last form all of the hyphae are capable of contributing to the fungal growth, which is the requirement of exponential growth. It should be taken into account that much

of the physiological research on filamentous fungi has been performed with the pellet type of growth; the causes of this type of growth are still obscure, however. A theory of the mode of growth of fungi in the form of pellets in submerged culture has been given by PIRT (1967). This author and others (YANO et al., 1961; PHILLIPS, 1966; KOBAYASHI et al., 1973) have shown oxygen to be a limiting factor for exponential growth in the pellet form.

Sometimes exponential growth has been found for pellets (CHOUDHARY and PIRT, 1965). In this case it has been assumed that the pellets were loose in texture and not compact enough for cube root growth to occur (PIRT, 1967). Generally, however, the growth varies between the two extremes (pellets-filamentous) as described above, and in practice the range in experimental results may often make both models useful. It is assumed in the present study, that growth occurs according to the exponential law, because generally homogeneous filamentous mycelium suspensions were obtained.

5.2.2. *Background of the continuous culture*

a. General

Besides equation (2) (see section 5.2.1) a few other equations are generally used for the mathematical formulation of growth.

The relation between the specific growth rate $\mu$ ($hr^{-1}$) of a micro-organism and the concentration of the growth-limiting substrate $S$ (g/l) can be given by an equivalent of the Michaelis-Menten equation for enzyme kinetics (MONOD, 1942):

$$\mu = \mu_{max} S/(S+K_s) \tag{3}$$

where, $\mu$ = specific growth rate ($hr^{-1}$); $\mu_{max}$ = maximum specific growth rate ($hr^{-1}$); $S$ = concentration of the growth-limiting substrate (g/l); $K_s$ = a constant (g/l).

When equation (2) is written in the form $dX/dt = \mu X$ (2a), then it follows from (2a) and (3):

$$dX/dt = \mu_{max} X S/(S + K_s) \tag{2b}$$

The relation between increase of biomass and decrease of growth-limiting substrate per unit of time can be given by:

$$dX/dt = -Y\, dS/dt \tag{4}$$

where, $Y$ = yield constant (g biomass produced per g substrate utilized).

b. The chemostat

The 'chemostat' is defined as a one-stage, homogeneously mixed, open continuous microbial production system. For a chemostat model with low substrate concentration the following substrate balance can be given (HERBERT et al., 1956):

Change of limiting substrate in the culture vessel = inflow – outflow – consumption, or in symbols:

$$V\, dS/dt = F S_r - F S - V \mu X/Y \tag{5}$$

where, $V$ = working volume of the chemostat; $F$ = flow rate (l/hr); $S_r$ = concentration of growth-limiting substrate in the fresh medium, flowing into the chemostat (g/l).

When the dilution rate $D$ is defined as $F/V$ ($hr^{-1}$), then it follows from (5):

$$dS/dt = D(S_r - S) - \mu X/Y \tag{5a}$$

Combination of (5a) and (3) gives:

$$dS/dt = D(S_r - S) - \mu_{max} X S/Y(K_s + S) \tag{5b}$$

In accordance with the substrate balance the biomass balance can be given by:

$$V\, dX/dt = \mu XV - FX \tag{6}$$

or (with $D = F/V$):

$$dX/dt = X(\mu - D) \tag{6a}$$

Combination of (6a) and (3) gives: $dX/dt = \mu_{max} X S/(K_s + S) - DX$, and thus:

$$dX/dt = X[\mu_{max} S/(K_s + S) - D] \tag{6b}$$

The condition of 'steady state' is defined by $dX/dt = 0$ and $dS/dt = 0$. Under steady state conditions it follows from (6a):

$$D = \mu \tag{7}$$

and from (5a):

$$X = Y(S_r - S) \tag{8}$$

During a steady state it follows from (6b):

$$S = K_s D/(\mu_{max} - D) \tag{9}$$

Substituting S (equation (9)) in equation (8), it follows that:

$$X = Y[S_r - K_s D/(\mu_{max} - D)] \tag{8a}$$

The output of biomass per unit volume of culture (the productivity), is the product of biomass concentration and dilution rate ($DX$) and thus from equation (8a):

$$\text{output} = DX = DY[S_r - K_s D/(\mu_{max} - D)] \tag{10}$$

At the optimum value of $D$ (maximum productivity) $dDX/dD = 0$ and thus:

$$D_m = \mu_{max}[1 - \sqrt{K_s/(S_r + K_s)}] \tag{11}$$

In practice, other values of $D$ may be considered as optimum, due to costs of substrate and other factors.

The equations can be applied at low substrate concentrations for $0 < D \leqslant \mu_{max} S_r/(S_r + K_s) = D_c$, where $D_c$ indicates the 'critical' dilution rate. For values of $D > D_c$ the micro-organism will be washed out and a sterile system is described.

c. The maintenance coefficient

Many authors have shown experimentally that at lower growth rates lower yield constants are found than theoretically expected (DAWES and RIBBONS, 1964; SCHULZE and LIPE, 1964; PIRT, 1965). This phenomenon has been attributed to the need of maintenance energy of micro-organisms. In general, metabolic energy turnover may be resolved into growth rate dependent and growth rate independent terms. Growth rate independent maintenance functions probably include resynthesis of macromolecules, osmotic regulation, cellular organization and special activities such as the production of secondary metabolites. Although several authors have proposed models with correction factors for maintenance, only PIRT's equation (1965) is cited here. PIRT started from the substrate balance in differentiated form:

total rate of substrate utilization = use for cell growth + use for cellular maintenance,

or in symbols: $dS/dt = (dS/dt)_G + (dS/dt)_m$.

With $(-dS/dt)_G = \mu X/Y_G$ and $(-dS/dt)_m = mX$, the substrate balance can be written as: $-dS/dt = \mu X/Y_G + mX = \mu X/Y_{sub}$ and thus at steady state ($\mu = D$):

$$1/Y_{sub} = m/D + 1/Y_G \tag{12}$$

where, $Y_{sub} = dX/-dS$ = observed growth yield constant (g biomass produced/g C source consumed); $Y_G$ = 'true' (maximum) growth yield constant, if no energy were required for maintenance; $m$ = maintenance coefficient (g C source consumed/g biomass.hr). The values of $m$ and $Y_G$ are depending on the choice of the micro-organism, the substrate composition and the growth conditions and they are of considerable importance in the quantitative formulation of growth processes, in particular for SCP production, as was shown theoretically by ABBOTT and CLAMEN (1973).

## 5.3. CHEMOSTAT CULTURES WITH CSL GLUCOSE MEDIA AT VARIOUS C/N RATIOS

### 5.3.1. *Introduction*

The equipment used for these experiments was described in section 2.3.2. Experiments were done with media consisting of diluted mixtures of CSL and glucose in various ratios (generally $10.2 \leqslant$ C/N ratio $\leqslant 15.2$). The pH in the fermentor was controlled within the range 4.2–4.5. The pH of the influent was adjusted to 4.2 before sterilization.

Generally experiments were carried out at a COD level of 2425 mg/l. A COD level of 4850 mg/l was used at $D \leqslant 0.12$ hr$^{-1}$ and a COD level of about 9700 mg/l at $D = 0.05$ hr$^{-1}$, but only at a C/N ratio of 12.6.

Steady state conditions were assumed to have been achieved after a time $t = 3/D$, after adjustment of a dilution rate. This is in accordance with an assumption of others (NG et al., 1973), based on experimental results. During the assumed steady state at least 5 duplicate samples were taken, as much as possible after periods calculated from $n/D$, with $n = 3, 4, 5, 6$, or 7.

5.3.2. *Results and discussion*

a. COD reduction, yield constant and nitrogen uptake as a function of the dilution rate

Although the determination of the COD itself was found to be highly reproducible (a difference between duplicates of generally less than 3%), less reproducible results were obtained from samples taken at various times during a steady state. The relative range in reproducibility varied from about 10% for $0.08 < D\ (hr^{-1}) \leqslant 0.18$ to about 20% or more at $D > 0.18\ hr^{-1}$ and $D \leqslant 0.08\ hr^{-1}$. As causes are suggested: influence of wall growth, differences in dilution rate by occasional foam and small differences in the medium during a run.

The determination of dry weight indicated a difference of up to about 10% between duplicate samples, while between samples taken at various times during an (assumed) steady state a still larger range in results was observed. A better reproducibility was obtained in the determination of the nitrogen content of the biomass and of the culture filtrate (a range within 5% between different samples during a steady state). For this reason it was decided to determine the concentration of biomass not directly by dry weight measurements but from the nitrogen balance. Such a calculation was supposed to be valid as the total nitrogen content of the culture was assumed to be constant because of the low pH which prevents loss of nitrogen in the form of ammonia. The biomass concentration ($X$) was calculated from the nitrogen balance as follows:

$$X(g/l) = 100\,\frac{[\text{N in substrate (g/l)} - \text{N in culture filtrate (g/l)}]}{\text{N content of the biomass (\%)}}$$

The biomass concentrations as a function of the dilution rate are summarized in the figures 5.1a-c. Although it is difficult to determine the critical dilution rate ($D_c$) exactly because of the effect of wall growth, the maximum specific growth rate will have been about similar to that found in batch culture ($\mu_{max} = 0.25$–$0.28\ hr^{-1}$). This may be surprising, because several authors (CARTER and BULL, 1969; PIRT, 1972) have observed that fungi are washed out at much lower dilution rates than corresponding with the maximum specific growth rate ($\mu_{max}$), found in batch cultures (up to about 50%). As a cause of this phenomenon has been suggested (CARTER and BULL, 1969) the destructive effect of a high shearing rate of the impeller in highly viscous cultures. In the present study a low stirring rate was used, whereas also only relatively low viscosities were reached. In addition, a much higher substrate concentration than in the present study has been applied by other authors and under such conditions more toxic metabolic products may be present. A similar critical dilution rate has been found for *T. viride* by MITRA and WILKE (1975) at higher substrate concentrations (up to 10 g glucose per litre).

The maximum biomass concentration, found by calculation from the N balance, was about 0.85 g/l for C/N ratio 12.6 at $D = 0.18\ hr^{-1}$. However, higher biomass concentrations were found occasionally by weighing the biomass.

The lowest COD levels in the culture filtrate, observed at dilution rates of

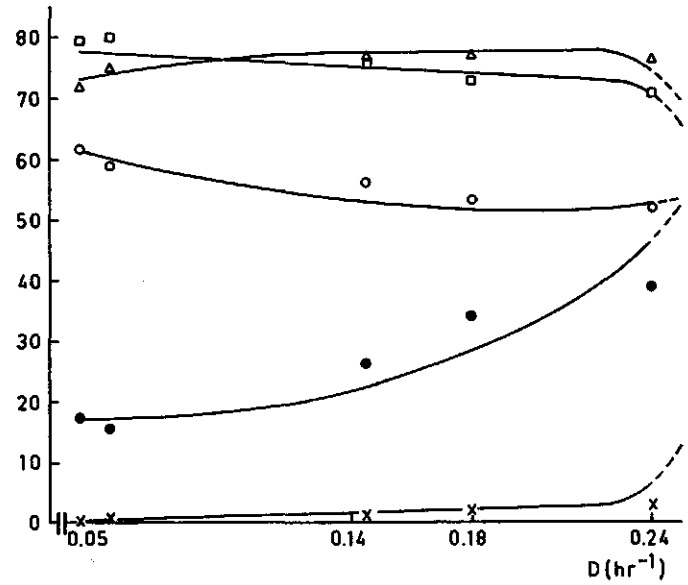


FIG. 5.1a. Chemostat cultures of *T. viride* at various dilution rates. The fungus was grown in a CSL glucose medium with an initial COD level of 2425 mg/l and C/N ratio 10.2.
● final COD as percentage of the initial; △ concentration of biomass in mg/100 ml; □ nitrogen uptake from the medium in per cent; ○ crude protein content of the biomass in per cent; × final concentration of reducing compounds as percentage of the initial.
N.B. The figures on the vertical axis apply to all data as specified above.

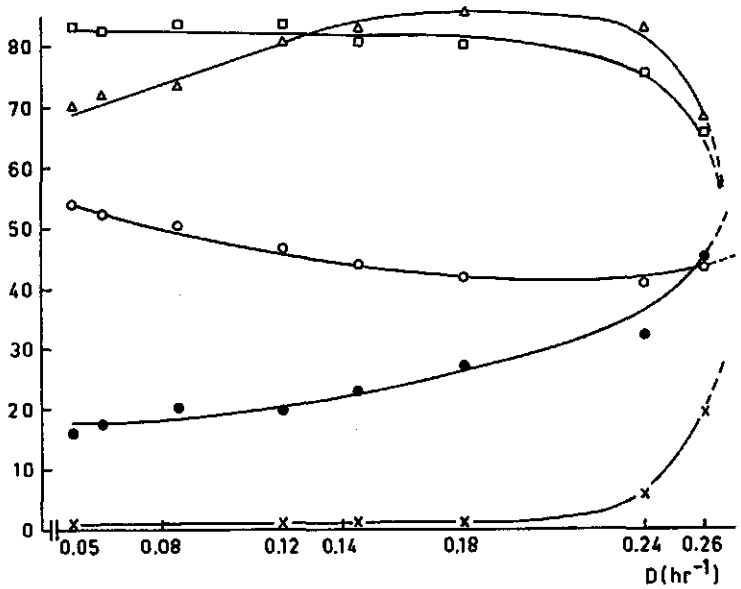


FIG. 5.1b. As 5.1a, but C/N ratio 12.6.

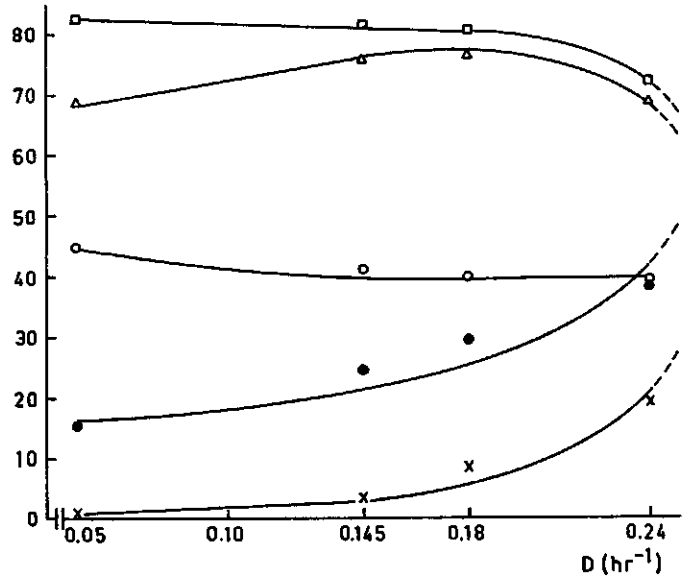


FIG. 5.1c. As 5.1a, but C/N ratio 15.2

about 0.05, where about 15% of the COD level of the fresh medium, independently of the C/N ratio within the range $10.2 \leqslant C/N \leqslant 15.2$. At dilution rates lower than 0.05, still less reproducible results were obtained, probably caused by autolysis of the mycelium. Generally, turbid culture filtrates were observed with an increased COD and N content. Therefore these results were not recorded.

The figures 5.1a-c indicate a decreasing yield constant at dilution rates lower than about 0.18 $hr^{-1}$, which might be caused by an increasing influence of the maintenance energy. A maintenance coefficient will be calculated later on (this section under b) using PIRT's equation (1965). At equal dilution rates higher crude protein contents were found for media with lower C/N ratios; the crude protein content increased also at lower dilution rates (Fig. 5.1). Because the nucleic acid (NA) content decreases at lower growth rates (SCHAECHTER et al., 1958; ELSWORTH et al., 1968), the results suggest an increasing 'true' protein content (cf. chapter 6) at decreasing dilution rates, for the indicated range of C/N ratios. However, the chitin content may also increase with age i.e. with lower dilution rates (BLUMENTHAL and ROSEMAN, 1957). Generally, low NA contents have been observed in fungi. DELANEY and WORGAN (1970) reported values of 4–6% in *Fusarium semitectum* at $t_d < 5$ hr. Higher NA values have generally been found in yeasts and bacteria. It is questionable, however, whether there will be any difference in NA content between micro-organisms, growing at equal dilution rates.

That the figures obtained depend on the C/N ratio (or CSL content) of the medium may be indicated by some preliminary experiments at a C/N ratio of 20.1. At this ratio a still increasing mycelium concentration and a decreasing

crude protein content were observed at decreasing dilution rates below 0.18 $hr^{-1}$.

The observed maximum nitrogen uptake in continuous culture (80–85%) was about the same as found in batch cultures (cf. section 3.6). The composition of the remaining N-containing compounds is reported in the next chapter. In preliminary experiments using media with higher CSL contents (C/N ratio < 10.2), much lower N uptake values than 80% were observed. The carbon and energy source (glucose) will probably become the limiting component of the substrate at C/N ratio < 10.2, whereas at C/N ratio 20.1 another component, present in CSL, will be the limiting factor. CSL glucose mixtures may be considered to be 'in balance' for 10.2 < C/N ratio < 15.2.

b. Yield constant and maintenance coefficient

For comparison of the yield constant with values in the literature, one has to keep in mind that 0.94 mg glucose (and lactic acid) corresponds to a COD of 1.0 mg; therefore in the following 1.0 mg COD will be considered to be equivalent to 0.94 mg glucose. In this study the contribution of glucose to the COD varies from 50% at C/N ratio 10.2 to 67% at C/N ratio 15.2.

The observed yield constant $Y_{sub}$, calculated on glucose equivalents, varies from about 0.38 at $D = 0.05\ hr^{-1}$ to about 0.51 at $D = 0.24\ hr^{-1}$. Somewhat lower maximum yield constants (about 0.45) have been observed for *T. viride* (MITRA and WILKE, 1975), for *Penicillium chrysogenum* (PIRT and CALLOW, 1960), for *Aspergillus nidulans* (CARTER and BULL, 1969) and for *Aspergillus niger* (NG et al., 1973). However, these workers used mineral media with glucose as the sole C source, whereas a complex medium was used in the present study. To all probability due to this difference, generally also higher growth rates were observed in this study. In addition, undissolved CSL components may have influenced the dry weight determinations, especially at high dilution rates.

In Fig. 5.2 the reciprocal yield constant (g decrease of COD per g biomass produced) is expressed as a function of the reciprocal dilution rate. From the slope of the line obtained, the maintenance coefficient ($m$) was calculated to be 0.025–0.030 g glucose/g biomass.hr. Similar values have been observed for other fungi: 0.022 for *Penicillium chrysogenum* (RIGHELATO et al., 1968); 0.018 for *Aspergillus niger* (NG et al., 1973). Generally, higher values have been reported for bacteria and yeasts. A maintenance coefficient of 0.094 g glucose/g biomass.hr was reported for *Aerobacter cloacae* under aerobic conditions (PIRT, 1965) and a similar value for baker's yeast (HARRISON, 1967). According to the literature, maintenance coefficients for Gram-negative organisms grown in defined glucose media vary from about 0.055 to 0.25 g glucose/g biomass.hr (SCHULZE and LIPE, 1964; PALUMBO and WITTER, 1969).

c. Visual observations

Higher viscosities were generally observed at higher dilution rates. An increased number of small pellets (1–2 mm) and clumps of mycelium were observed at low dilution rates. In addition, at low dilution rates, especially below

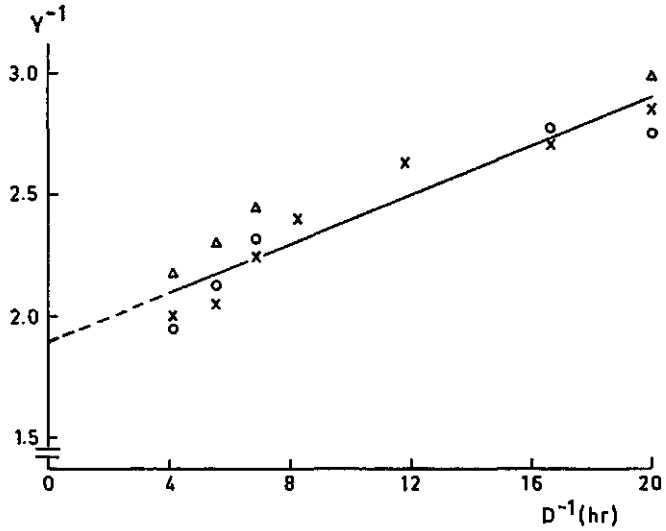


FIG. 5.2. Calculation of the maintenance coefficient ($m$) from the equation of PIRT (1965): $Y_{sub}^{-1} = m.D^{-1} + Y_G^{-1}$ where, $Y_{sub}$ = observed yield constant; $Y_G$ = 'true' yield constant; $m$ = maintenance coefficient; $D$ = dilution rate ($hr^{-1}$). The yield constant $Y$ was calculated as: g biomass produced per g COD reduced. Data were obtained from the results recorded in Fig. 5.1a-c. C/N ratios: ○ 10.2; × 12.6; △ 15.2.

0.05 $hr^{-1}$, the culture filtrate became more turbid, caused by autolysis of (wall-grown) mycelium. Without pH control the pH dropped at high dilution rates, but it rose at low dilution rates. However, this effect depended also on the C/N ratio of the medium; at equal dilution rates the pH dropped to lower values at higher C/N ratio.

When the pH increased to about 7, sometimes a pink-coloured culture was observed. At a high C/N ratio of the medium sometimes a yellow pigment was produced at the usual pH 4.2. Formation of some foam was especially observed at high dilution rates and during the time between two steady states.

Microscopic examination showed undigested CSL particles to be filtered off at high dilution rates. At dilution rates of 0.05 $hr^{-1}$ and below, an increasing vacuolation of the hyphae was found. The observed increase of wall-grown and autolysing mycelium was probably responsible for the badly reproducible results under these conditions. Conidia were sometimes observed at these low dilution rates. It has been found by RIGHELATO et al. (1968) with *Penicillium chrysogenum* and by NG et al. (1973) with *Aspergillus niger*, that under glucose limitation the maximum conidiation occurred at growth rates just above zero. No difference in conidiation was observed in the present study between the various C/N ratios used. However, as already stated above, only a few experiments were carried out at dilution rates below 0.05 $hr^{-1}$.

Occasionally contaminating micro-organisms, generally small rods and yeasts, were observed under the microscope, in particular at low dilution rates. Some-

times, however, especially after an increase of the pH during disturbances of the pH control, an increasing number of contaminants was observed, causing a turbid culture filtrate. In such a case the run was stopped and a new one started after sterilization of the equipment. Even when the culture filtrate was clear, contamination was indicated occasionally by plate count tests.

d. Oxygen demand of the culture

Occasionally the oxygen concentration was determined during experiments at (the optimum) C/N ratio 12.6 and dilution rate 0.24 $hr^{-1}$. The observed oxygen concentration was at least 50% of the saturation value at 29°C (7.7 mg/l), which suggests sufficient oxygen to be dissolved in the medium, also at lower dilution rates and other C/N ratios.

From the results (COD reduction maximum 70%; biomass concentration about 0.85 g/l) under the conditions specified above, it can be calculated that the oxygen demand of the rapidly growing mould must be about 7.4 mmol $O_2$/g biomass.hr. This is approximately the same value as was found after initial growth in batch cultures (cf. section 3.7). Experimentally somewhat lower values were observed, however, possibly caused by deviations in dry matter content of the samples and by the presence of CSL particles in the biomass.

e. Results with higher COD levels

Only a few experiments were done with higher COD levels (see section 5.3.1). On the basis of equation (3) (section 5.2.2) a higher COD reduction, expressed as percentage of the initial COD value, can be expected at equal dilution rates, when higher initial COD levels are used, provided that the substrate offered is completely assimilable. However, the observed COD reduction in % was about the same at the various initial COD levels. At a dilution rate of 0.05 $hr^{-1}$ about 85% of the COD was removed. At higher mycelium concentrations the culture becomes more viscous and consequently the efficiency of mixing and rate of oxygen transfer may be reduced, which should influence the results. In addition, inhibiting compounds may be excreted or may already be present in the medium. Inhibition models have been developed by several authors. AIBA et al. (1968) studied product inhibition in alcohol fermentation. MEYRATH and ROTH (1973) found self-inhibiting extracellular proteins in culture filtrates of *Aspergillus oryzae*, which may cause great difficulties in maintaining a true steady state in homogeneous single stage continuous cultures. OOKA et al. (1966) found *T. viride* to produce a polypeptide with antibiotic activity, but a self-inhibiting action of this substance is not known. The critical dilution rate ($D_c$) may decrease by toxic metabolic products, when high substrate concentrations are used. This was not examined in the present study because of the risk of oxygen limitation in the culture at higher dilution rates. Theoretical substrate inhibition models have been discussed by EDWARDS (1970). The range in results and the complex composition of the medium in the present study made it difficult, however, to examine the problem of inhibition in continuous cultures.

f. Influence of the process scale on the results

The results obtained in this study may be highly influenced by the volume of the fermentor used. Especially the influence of wall growth on the results should be considerable. A true steady state is impossible under such conditions and the range in results in the present study probably has to be explained mainly by this effect. A laboratory process with a high surface to volume ratio can give optimistic predictions of both necessary residence time and stability of the large scale process unless wall growth is accounted for (HOWELL et al., 1972). A discussion on the influence of wall growth on results has also been given by WATSON (1972). On the other hand on a large scale the lower degree of mixing achieved does affect the cell yield at low dilution rates adversely; appreciable quantities of energy source can be utilized for maintenance in comparison to those utilized for making cell mass. Poor distribution of the limiting component may give results different from those, calculated theoretically for well-mixed systems (HANSFORD and HUMPHREY, 1966). In addition, the degree of mixing will influence the washout and steady state analyses (FAN et al., 1970).

## 5.4. SINGLE-STREAM DUAL-STAGE CULTURES

### 5.4.1. *Introduction*

It is frequently assumed that for biomass production, a dual-(or multi-)stage system may be advantageous compared to a single stage system, as a smaller total working volume is expected to give equal or better results i.e. high yield and exhaustion of the medium.

A multi-stage system has been described by FENCL and BURGER (1958) for the utilization of mixtures of hexoses and pentoses in sulphite liquor and pulp wastes. They demonstrated a two stage system to have a higher productivity than one fermentor, or two single fermentors in parallel, with the same total working volume.

In addition, it has been suggested by several authors, that multi-stage systems are also advantageous in producing extracellular metabolites of commercial interest (antibiotics, enzymes etc.), a problem discussed by FENCL et al. (1972). Generally, in the first fermentor optimum conditions are created for growth and in the second one for production of the extracellular metabolites.

From the physiological viewpoint, a multi-stage system may be advantageous if the final metabolite has a toxic effect on micro-organisms or if a substrate itself is toxic. The treatment of (toxic) industrial wastes in a multi-stage system has been discussed by WILLIMON and ANDREWS (1969). A review of multi-stage systems has been given by ŘIČICA (1970).

This chapter is only concerned with preliminary experiments on a single-stream dual-stage system, although other systems (multi-stream, multi-stage, recirculation of the biomass) may be also interesting. However, with filamentous organisms, even the simplest systems meet already with large difficulties on a small scale.

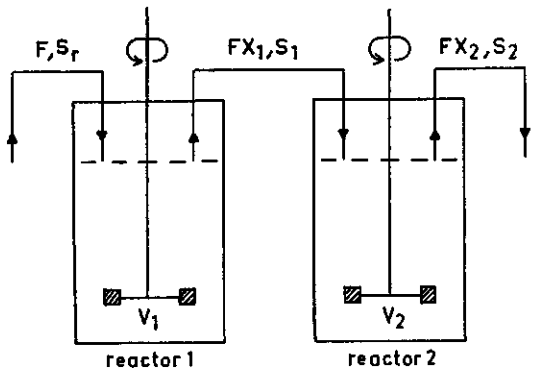


FIG. 5.3. Single-stream dual-stage culture system. $F$ = flow rate (l/hr); $S$ = concentration of growth-limiting substrate (g/l); $X$ = concentration of biomass (g/l); $V$ = working volume of the fermentor (1).

5.4.2. *Theory*

The first theoretical approach to a multi-stage continuous culture has been given by MAXON (1955). His principles have been developed further, amongst others, by ŘIČICA (1970).

Only the theory of a single-stream dual-stage system will be considered in this section, based on the principles given by the authors mentioned above.

If a single medium is assumed to flow into the first reactor and the whole outflow is passed on into the second stage, then $F = D_1V_1 = D_2V_2$. The dilution rate $D$ in each of both stages depends besides on $F$, only on the working volume of the reactors ($D = F/V$). This is schematically represented by Fig. 5.3.

Cell mass and substrate balance equations for the first stage have already been given in section 5.2.2. Considering only steady states ($dX_2/dt = 0$) for the system in the diagram (Fig. 5.3) the cell balance equation in the second stage is as follows:

$$D_2X_1 + \mu_2X_2 = D_2X_2 \tag{13}$$

In accordance the substrate balance is given by:

$$D_2S_1 - \mu_2X_2/Y_2 = D_2S_2 \tag{14}$$

Combination of equation (3) (cf. section 5.2.2) and equation (13) gives:

$$\mu_2 = \mu_{max}S_2/(K_s + S_2) = D_2(X_2 - X_1)/X_2 = D_2Y_2(S_1 - S_2)/X_2 \tag{15}$$

Thus $\mu_2 < D_2$ when $X_1 > 0$, and washout is impossible in the second stage, independent of the dilution rate $D_2$, if in the first stage $D_1 < D_c$.

The increase of the biomass concentration in the second stage can be written as:

$$X_2 - X_1 = Y_2(S_1 - S_2) \tag{16}$$

From: $X_1 = Y_1(S_r - S_1)$ (equation 8 and 16), it follows that:

$$(X_2 - X_1)/X_2 = Y_2(S_1 - S_2)/[Y_2(S_1 - S_2) + Y_1(S_r - S_1)] \tag{17}$$

or with $Y_2 = Y_1$

$$(X_2 - X_1)/X_2 = (S_1 - S_2)/(S_r - S_2) \quad (18)$$

and with, e.g., $Y_2 = 0.5\ Y_1$

$$(X_2 - X_1)/X_2 = (S_1 - S_2)/(2S_r - S_1 - S_2) \quad (19)$$

The yield constant ($Y$) is usually rather constant indeed for a considerable part of the growth curve. It may decrease at low concentrations of the limiting substrate due to increasing importance of the maintenance energy or by a changed metabolism resulting from the accumulation of metabolites. This would result in a lower yield constant in the second stage.

### 5.4.3. *Experiments with CSL carbohydrate media*

#### Introduction

The experiments were done with two 'Biotec' fermentors in series. The whole outflow of the first stage (mycelium and substrate) was passed on into the second stage, by means of air pressure resulting from the aeration. Other details (pH control etc.) were already described in section 2.3.2.

In the single stage experiments glucose was the only carbohydrate in the substrate; in the experiments described in this section starch was also used, instead of or partially substituting glucose. In one experiment, the organic nitrogen provided by CSL was partially substituted by $(NH_4)_2SO_4$. Such substitutions gave a closer approach to the composition of the waste effluent in study, and the problems connected with its upgrading.

The following media were used:

a. CSL (2 g/l), glucose.$H_2O$ (1 g/l) and starch (0.5 g/l), dissolved in tap water; COD ca. 2510 (mg/l); C/N ratio ca. 12.8; pH = 4.2.
b. CSL (1 g/l), glucose.$H_2O$ (1 g/l), starch (1 g/l) and $(NH_4)_2SO_4$ (0.17 g/l) dissolved in tap water; COD ca. 2595 (mg/l); C/N ratio ca. 13.1; pH = 4.2.
c. CSL (2 g/l) and starch (1.5 g/l) dissolved in tap water; COD ca. 2680 (mg/l); C/N ratio ca. 13.4; pH = 4.2.

It is frequently suggested to use a smaller volume in the first fermentor ($V_1$) and a larger in the second one ($V_2$). After a rapid growth in the first stage the medium should be exhausted in the second one. Therefore, in all of the experiments the dilution rate in the second stage was adjusted equal to, or smaller than that in the first stage ($D_2 \leqslant D_1$).

#### Results and discussion

While it was already improbable that a steady state was approached in the second stage, it was even rather doubtful whether a steady state was obtained in the first stage. As causes are suggested the influence of the retrogradation of starch after sterilization of the medium, and the effect of wall growth. As a result of the retrogradation of starch after sterilization, this substrate becomes gradually more difficult to attack for the amylolytic enzymes. Wall growth results in discontinuous overflow of biomass from the first fermentor into the second.

The results obtained at presumed steady states are summarized in Table 5.1. In this connection it should be mentioned that $D_{total} = F/(V_1 + V_2)$; $Y_2 = (X_2 - X_1)/(S_1 - S_2)$ and $Y_{total} = X_2/(S_r - S_2)$ where $S_r$ and $S_2$, as usual in this study, are expressed in COD units. Generally in the first stage somewhat lower COD reductions were observed at dilution rate 0.24 $hr^{-1}$ than obtained in previous experiments with CSL glucose media at equal C/N ratio (cf. section 5.3.2). It may be suggested that the production of utilizable sugars from starch by amylolytic enzymes controls the growth rate at high dilution rates. Although even at $D_1 = 0.24$ $hr^{-1}$ in the culture filtrates a considerable decrease of the intensity of the blue colour with iodine reagent was observed (Table 5.2), indicating a dextrinizing activity, the concentration of reducing carbohydrates was always lower than 100 mg glucose equivalents per litre. Apparently the concentration of assimilable sugars was very low (under the level above which cata-

TABLE 5.1. COD reduction, yield constant and nitrogen uptake in a single-stream dual-stage culture system with different media as described in the text.

| Medium composition (see text) | Dilution rate ($hr^{-1}$) | | | Total COD reduction | Observed yield constant | | | Crude protein content of the biomass | Total nitrogen uptake from the medium |
|---|---|---|---|---|---|---|---|---|---|
| | $D_1$ | $D_2$ | $D_{total}$[1] | | $Y_1$ | $Y_2$[2] | $Y_{total}$[3] | | |
| | | | | (%) | | | | (%) | (%) |
| a | 0.24 | | | 63 | 0.49 | | | 46 | 80 |
| | 0.24 | 0.24 | 0.12 | 74 | | 0.28 | 0.45 | 46 | 84 |
| | 0.24 | 0.18 | 0.10 | 79 | | 0.32 | 0.44 | 43 | 84 |
| | 0.24 | 0.12 | 0.08 | 82 | | 0.18 | 0.41 | 46 | 87 |
| | 0.18 | | | 71 | 0.47 | | | 44 | 82 |
| | 0.18 | 0.18 | 0.09 | 81 | | 0.06 | 0.42 | 44 | 82 |
| | 0.18 | 0.12 | 0.07 | 83 | | 0.13 | 0.42 | 44 | 86 |
| b | 0.24 | | | 56 | 0.50 | | | 51 | 82 |
| | 0.24 | 0.24 | 0.12 | 72 | | 0.45 | 0.46 | 45 | 86 |
| | 0.24 | 0.18 | 0.10 | 77 | | 0.42 | 0.45 | 45 | 90 |
| | 0.24 | 0.12 | 0.08 | 81 | | 0.30 | 0.42 | 46 | 90 |
| | 0.18 | | | 71 | 0.46 | | | 47 | 88 |
| | 0.18 | 0.18 | 0.09 | 82 | | 0.39 | 0.45 | 43 | 92 |
| | 0.18 | 0.12 | 0.07 | 86 | | 0.15 | 0.41 | 46 | 92 |
| c | 0.24 | | | 56 | 0.49 | | | 51 | 84 |
| | 0.24 | 0.24 | 0.12 | 77 | | 0.47 | 0.46 | 41 | 86 |
| | 0.24 | 0.18 | 0.10 | 80 | | 0.48 | 0.46 | 39 | 86 |
| | 0.24 | 0.12 | 0.08 | 83 | | 0.35 | 0.42 | 41 | 86 |
| | 0.18 | | | 68 | 0.47 | | | 45 | 84 |
| | 0.18 | 0.18 | 0.09 | 81 | | 0.35 | 0.45 | 40 | 86 |
| | 0.18 | 0.12 | 0.07 | 85 | | 0.22 | 0.41 | 41 | 86 |
| | 0.14 | | | 73 | 0.46 | | | 44 | 86 |
| | 0.12 | | | 77 | 0.43 | | | 44 | 86 |

[1] $D_{total} = F/(V_1 + V_2)$.
[2] $Y_2 = (X_2 - X_1)/(S_1 - S_2)$.
[3] $Y_{total} = X_2/(S_r - S_2)$.

TABLE 5.2. Residual starch-like compounds determined with iodine reagent as percentage of initial concentration at various dilution rates in a single-stream dual-stage continuous system. The medium compositions a, b and c are described in the text.

| Dilution rate ($hr^{-1}$) | | Residual starch-like compounds (%) at medium composition | | |
|---|---|---|---|---|
| $D_1$ | $D_2$ | a | b | c |
| 0.24 | – | 34 | 66 | 11 |
| | 0.24 | < 10 | 22 | < 5 |
| | 0.18 | – | 8 | – |
| | 0.12 | – | < 5 | – |
| 0.18 | – | < 10 | 19 | 8 |
| | 0.18 | – | < 5 | < 5 |

bolite repression occurs; cf. section 7.3) and chiefly dextrins may have been present. The amylolytic activity of the fungus will be studied in more detail in chapter 7.

At a dilution rate $D_1 = 0.12\ hr^{-1}$ about equal COD reductions were observed as in previous single-stage experiments with CSL glucose media (cf. section 5.3). The observed yield constant in the first stage was about equal to that in the previous experiments.

After the second stage, a higher total COD reduction than in the previously described single-stage system with equal total working volume was never found. However, a somewhat higher total yield constant ($Y$ total) was calculated with two stages. The nitrogen content of the biomass was independent of the dilution rate $D_2$, whereas it increased in a single-stage system at lower dilution rates.

The low remaining nitrogen concentration in the culture filtrate (8–10% of the initial concentration), after partial substitution of CSL by ammonia (medium composition b) is surprising. This may indicate that ammonia is more readily utilized than some nitrogen compounds from CSL. This low remaining nitrogen concentration has, remarkably, not been observed in previous experiments with batch cultures after partial substitution of CSL by urea (cf. section 3.8).

Visual observations always showed higher viscosities in the first stage than in the second. The mycelium suspension in the first fermentor was rather homogeneous; clumps of mycelium were generally observed in the second one. In samples taken from the first fermentor the biomass showed a flotation tendency, whereas mycelium from the second stage generally sedimented, especially at the lower dilution rates. More gas bubbles were observed to be retained between the hyphae in samples from the first stage than in those from the second. Culture filtrates from the second stage were generally more yellow coloured, than those from the first.

It is impossible to conclude from these results whether or not a dual-stage system is advantageous to a single-stage one. In addition, other factors than yield and COD reduction at a certain dilution rate may influence the choice of

the process conditions, such as filtration of the biomass (morphology of the mycelium) and other properties of the harvest. Production costs, market price of the product and the negative value of the remaining COD of the effluent have also to be considered in commercial processes.

5.4.4. *A numerical approach of the optimum ratio of the effective reactor volumes in a single-stream dual-stage system*

In the following, an attempt will be made to calculate under which conditions and to what extent a single-stream dual-stage system might be advantageous to a single-stage system, from the viewpoint of biomass production and COD reduction. Instead of mathematical-analytical methods a numerical approach is chosen to answer these questions.

We assume the source of carbon and energy to be the limiting factor of the substrate, and $Y$ = constant (no maintenance energy required). For the first stage it follows at steady-state ($D_1 = \mu_1$) from equation (3): $\mu_{max}/D_1 = V_1\mu_{max}/F = (S_1 + K_s)/S_1$. For the second stage follows from equation (13): $D_2 = \mu_2 X_2/(X_2 - X_1)$ and, since $Y_1 = Y_2$ = constant, from equation (18): $D_2 = \mu_2 (S_r - S_2)/(S_1 - S_2)$. With substitution of equation (3) for the second stage, it follows that: $\mu_{max}/D_2 = V_2\mu_{max}/F = (S_1 - S_2)(S_2 + K_s)/S_2(S_r - S_2)$. For various values of the ratios $S_2/S_r$ and $K_s/S_r$, the ratios $\mu_{max}/D_1$, $\mu_{max}/D_2$ and thus $V_1/V_2$ and $(V_1 + V_2)\mu_{max}/F$ can be calculated as a function of $S_1/S_r$. The minimum value of $(V_1 + V_2)\mu_{max}/F$ indicates the optimum ratio $V_1/V_2$ and the corresponding $S_1/S_2$ ratio for the chosen set of values for $S_2/S_r$ and $K_s/S_r$. In Table 5.3 an example is given of the calculated values when $S_2 = K_s = 0.01\ S_r$. It can be seen that in this case a dual-stage system is only advantageous to a single-stage one when $V_1/V_2 > 1.0$, and under such conditions $S_1/S_r < 0.5$. The Table shows that the optimum ratio $V_1/V_2 = 9.5$, and this corresponds to a value of $S_1/S_r = 0.07$. Notice, that the first stage must have a much larger volume than the second, to obtain optimum results and that the second stage reduces the substrate concentration only a further 6%, after the reduction of 93% obtained already in the first stage.

The results obtained with some other sets of values for $S_2/S_r$ and $K_s/S_r$ are

TABLE 5.3. Numerical calculation of the optimum ratio $V_1/V_2$ in a single-stream dual-stage culture system in the case that $S_2 = K_s = 0.01\ S_r$ and $Y_1 = Y_2$.

| $S_1/S_r$ | $V_1\mu_{max}/F$ | $V_2\mu_{max}/F$ | $V_1/V_2$ | $(V_1 + V_2)\mu_{max}/F$ |
|---|---|---|---|---|
| 0.01 | 2.00 | 0 | $\infty$ | 2.00 |
| 0.03 | 1.34 | 0.04 | 33 | 1.38 |
| 0.05 | 1.20 | 0.08 | 15 | 1.28 |
| 0.07 | 1.14 | 0.12 | 9.5 | 1.26 |
| 0.1 | 1.10 | 0.18 | 6.1 | 1.28 |
| 0.3 | 1.03 | 0.59 | 1.8 | 1.62 |
| 0.5 | 1.02 | 0.99 | 1.0 | 2.01 |
| 0.7 | 1.01 | 1.39 | 0.73 | 2.40 |
| 0.9 | 1.01 | 1.80 | 0.56 | 2.81 |

TABLE 5.4. Numerical calculation of the optimum ratio $V_1/V_2$ and the $V_1/V_2$ ratio above which a dual-stage system is advantageous to a single stage one, at various values for $S_2/S_r$ and $K_s/S_r$ and when $Y_1 = Y_2$.

| $S_2/S_r$ | $K_s/S_r$ | $S_2/K_s$ | Value of $V_1/V_2$ above which a dual-stage system is advantageous to a single-stage system [$(V_1 + V_2) < V$ single stage] | Maximum value of $S_1/S_r$ when a dual-stage system is advantageous to a single-stage system | Optimum value of $V_1/V_2$ (at minimum of $V_1 + V_2$) | $S_1/S_r$ at optimum $V_1/V_2$ |
|---|---|---|---|---|---|---|
| 0.01 | 0.001 | 10 | 11 | 0.08 | 47 | 0.03 |
| 0.01 | 0.005 | 2 | 2.1 | 0.30 | 15 | 0.06 |
| 0.01 | 0.01 | 1 | 1.1 | 0.45 | 9.5 | 0.07 |
| 0.01 | 0.05 | 0.2 | 0.22 | 0.80 | 3.2 | 0.09 |
| 0.01 | 0.1 | 0.1 | 0.11 | 0.85 | 2.2 | 0.095 |
| 0.05 | 0.001 | 50 | –[1] | –[1] | –[1] | –[1] |
| 0.05 | 0.01 | 5 | 8.5 | 0.15 | 20 | 0.095 |
| 0.05 | 0.05 | 1 | 1.2 | 0.50 | 6.3 | 0.15 |
| 0.05 | 0.1 | 0.5 | 0.63 | 0.60 | 3.2 | 0.20 |
| 0.1 | 0.001 | 100 | –[1] | –[1] | –[1] | –[1] |
| 0.1 | 0.01 | 10 | –[1] | –[1] | –[1] | –[1] |
| 0.1 | 0.05 | 2 | 3.5 | 0.30 | 12 | 0.18 |
| 0.1 | 0.1 | 1 | 1.6 | 0.45 | 7 | 0.2 |

[1] No advantage of the dual-stage system to the single-stage one.

summarized in Table 5.4. It may be concluded that only when $S_2/K_s$ is relatively small, the dual-stage system is advantageous to the single stage. There are conditions, where a dual-stage system is advantageous to a single-stage one and $V_1/V_2 < 1$, but under optimum conditions always $V_1/V_2 > 1$.

In the calculations above, it was always assumed, that $Y_1 = Y_2$ and $\mu_{max} =$ constant. In practice, however, $Y_2$ may be smaller than $Y_1$ (influence of maintenance). For some particular cases the ratio $V_1/V_2$ will be calculated at the assumption that one single carbon and energy source is present ($\mu_{max1} = \mu_{max2}$; $K_s =$ constant) and $Y_1 > Y_2$. When $Y_1 \neq Y_2$ it follows from equation (3) for the first stage: $V_1 = F(S_1 + K_s)/\mu_{max}S_1$ and from the equations (15) and (17) for the second stage: $V_2 = FY_2(S_2 + K_s)\ (S_1-S_2)/S_2\mu_{max}\ [Y_2(S_1-S_2) + Y_1(S_r-S_1)]$. In Table 5.5 the ratio $V_1/V_2$ is calculated for various assumed values of $Y_1/Y_2$ at constant values for the ratios $D_1/\mu_{max}$, $S_1/S_r$ and $S_2/S_r$. It appears that the ratio $V_1/V_2$ increases, with increasing $Y_1/Y_2$ ratio.

TABLE 5.5. Calculation of the ratio of the reactor volumes ($V_1/V_2$) in a single-stream dual-stage culture system for various assumed values of $Y_1/Y_2$ at constant values for $D_1/\mu_{max}$, $S_1/S_r$ and $S_2/S_r$.

| $D_1/\mu_{max}$ | $S_1/S_r$ | $S_2/S_r$ | $Y_1/Y_2$ | $V_1/V_2$ |
|---|---|---|---|---|
| 0.9 | 0.1 | 0.01 | 1 | 5.9 |
| 0.9 | 0.1 | 0.01 | 1.5 | 8.4 |
| 0.9 | 0.1 | 0.01 | 2 | 11 |

It can be calculated that only in particular cases (relatively high $K_s/S_r$ ratio and when is required $S_2 << K_s$) a ratio $V_1/V_2 < 1$ may be advantageous. It can also be demonstrated that the optimum $V_1/V_2$ ratios in Table 5.4 increase when the yield constant decreases in the second stage ($Y_2 < Y_1$).

As was stated already, in practice the problem will generally be more complicated, by the presence of mixtures of carbon sources with different values for $\mu_{max}$ and for $K_s$. It has been demonstrated in this section, however, that a dual-stage system is not necessarily advantageous to a single-stage one and if so, that the second stage must not necessarily have a larger volume than the first one.

## 5.5. SUMMARY AND CONCLUSIONS

In the present chapter the continuous production of mycelium on a model waste effluent has been studied, both in a single stage system and in a single-stream dual-stage system.

The maximum specific growth rate ($\mu_{max}$) in continuous culture will have been about the same as that found in batch culture ($\mu_{max} = 0.25-0.28\ hr^{-1}$). At an equal COD level (2.5 g/l) of the fresh medium, the maximum yield of biomass (ca. 0.85 g/l) was lower than that obtained in batch cultures (ca. 1.0 g/l), while also the maximum COD reduction observed (85%) was lower than that found in batch experiments (90–95%). At dilution rates lower than 0.05 $hr^{-1}$ autolysis

of mycelium was observed, accompanied by an increased COD of the culture filtrate. The yield constant ($Y$) varied from about 0.51 at $D = 0.24$ $hr^{-1}$ to about 0.36 at $D = 0.05$ $hr^{-1}$. Using media with a C/N ratio between 10.2 and 15.2, the crude protein content increased with dilution rates under $D = 0.18$ $hr^{-1}$ and also with lower C/N ratios of the fresh medium; this content ranged from about 40% to about 60%. The maximum nitrogen recovery from media with a ratio C/N $>$ 10.2 was about the same as observed in batch culture (80–85%). Using the equation of PIRT (1965), the maintenance coefficient was calculated to be 0.025–0.030 g glucose/g biomass.hr. A higher COD level of the fresh medium did not result in a higher COD reduction than about 85%.

Single-stream dual-stage cultures were not markedly advantageous to a single-stage system. In a numerical approach it has been shown theoretically that a single-stream dual-stage system is not necessarily advantageous to a single-stage system, and if so, that the second stage must not have necessarily a larger volume than the first one.

The results obtained experimentally in this study may have been highly influenced by the process scale used, especially because of the considerable wall growth.

# 6. AMINO ACID COMPOSITION OF THE BIOMASS AND THE NATURE OF THE NITROGEN COMPOUNDS IN FILTRATES FROM DIFFERENT CULTURES

## 6.1. INTRODUCTION

The quality of a protein to be used as a food is determined by its amino acid composition. The term 'true protein' in nutrition science usually includes free amino acids in the cell in addition to those in peptides and protein molecules. For the nutritional value it makes no difference whether amino acids are present as such or in protein molecules; therefore only the total amino acid content and composition has to be determined.

Because in previous chapters only the 'crude protein' content (%N × 6.25) was determined, a correction factor for non-protein nitrogen present as cell wall nitrogen (chitin) and nucleic acid N, has to be applied in calculations of the 'true protein' content. Since the microbial nucleic acid content depends on the growth rate (SCHAECHTER et al., 1958) and the chitin content on the age of the mycelium (BLUMENTHAL and ROSEMAN, 1957), the correction factor to be used is not constant but depends on the conditions of growth. Only 70–80% of the nitrogen content of micro-organisms would be accounted for as amino acid N (KIHLBERG, 1972). On the basis of amino acid analysis the 'true protein' content of *T. viride* has been found to be 42–45% which was about 80% of the crude protein content. (CHURCH et al., 1972). WORGAN (1971) has reported a crude protein content of 60% for *Fusarium semitectum*, while after acid hydrolysis 90% of the mycelial N was recovered as amino acid N.

As regards its nutritional value, fungal protein is generally deficient in the sulphur-containing amino acids (NELSON et al., 1960; RHODES et al., 1961), whereas bacterial protein is often deficient in tryptophan (ANDERSON et al., 1958). However, during processing of SCP at high temperatures and at high pH values in the presence of reducing sugars, also the availability of lysine may decrease considerably as a result of Maillard reactions. The influence of these reactions on the availability of amino acids in foods has been studied by SLUMP (1969).

In this chapter experiments on the influence of the incubation time and the C/N ratio of CSL-containing media on the amino acid content of *T. viride* will be reported. In addition, the influence of the nitrogen source (CSL or urea) will be described, especially with regard to the nature of the nitrogen compounds in the culture filtrate and the amino acid composition of the biomass.

## 6.2. Experiments

Fungal biomass was grown, as described in section 2.3.1, in conical flasks, containing CSL glucose media, at various C/N ratios and incubation times. The COD of all of the media was about 2500 mg/l. The fungus was also grown in standard mineral glucose urea medium and harvested after 36 hr incubation. The cultures were inoculated with a standard spore suspension (1%; v/v).

## 6.3. Results and discussion

The results, except the amino acid analyses, are summarized in Table 6.1. The final concentration of reducing sugars decreased to less than 2% of the initial concentration in each of the experiments.

The results of the amino acid analyses are summarized in Table 6.2. It may be concluded that about 80% of the mycelial N can be accounted for as amino acid N. The values may be still higher, as tryptophan was not determined and another amino acid peak present on the chromatogram (probably γ-aminobutyric acid) was neglected. In addition, part of the threonine and serine fractions may have been destroyed, while asparagine and glutamine were deaminated to aspartic acid and glutamic acid, respectively. In each of the biomass analyses, the proteins were deficient in the sulphur-containing amino acids (cf. the FAO reference pattern in Table 6.3).

Because cystine can be synthesized from methionine, only the latter amino acid has to be added to fungal protein foods, and this amino acid can be produced cheaply by synthetic methods.

Table 6.1. Data of shake flask cultures used for the examination of the amino acid content of the fungal biomass and the culture filtrates. The COD level of the media was about 2500 mg/l.

| | Medium | | | |
|---|---|---|---|---|
| | a | b | c | d |
| C and N sources in the medium (g/l) | | | | |
| CSL | 2 | 2 | 3 | |
| urea | | | | 0.2 |
| glucose.$H_2O$ | 1.5 | 1.5 | 1 | 2.5 |
| C/N ratio | 12.6 | 12.6 | 8.5 | 10.0 |
| Incubation time (hr) | 24 | 36 | 18 | 36 |
| Initial pH | 4.2 | 4.2 | 4.2 | 4.2 |
| Final pH | 4.5 | 5.3 | 5.2 | 5.6 |
| Biomass (g/l) | 1.04 | 1.01 | 0.99 | 1.13 |
| Fungal crude protein content (%) | 35.5 | 34.9 | 56.2 | 46.3 |
| Nitrogen uptake from the medium (%) | 82 | 78.5 | 82 | 89.5 |
| Ammonia (urea) N in the culture filtrate as % of total N in the culture filtrate | 5 | 11 | 11 | 57 |

TABLE 6.2. Amino acid composition and 'true protein' content of mycelia and culture filtrates expressed as g amino acid/16 g N (= 100 g crude protein). For other experimental data see Table 6.1.

| | BIOMASS | | | | CULTURE FILTRATE | | | |
|---|---|---|---|---|---|---|---|---|
| | medium | | | | medium | | | |
| | a | b | c | d | a | b | c | d |
| Lysine | 6.6 | 6.7 | 7.7 | 6.6 | 6.6 | 6.1 | 6.2 | 2.8 |
| Histidine | 3.0 | 3.0 | 3.8 | 2.6 | 6.8 | 6.3 | 6.1 | 0.7 |
| Arginine | 6.0 | 5.5 | 6.8 | 5.8 | 1.8 | 0.4 | 0.7 | 0.6 |
| Tryptophan | – | – | – | – | – | – | – | – |
| Aspartic acid | 7.4 | 7.6 | 8.6 | 8.2 | 7.4 | 10.4 | 7.2 | 3.8 |
| Threonine | 5.6 | 5.2 | 5.4 | 6.1 | 5.8 | 6.6 | 5.7 | 3.6 |
| Serine | 5.1 | 5.0 | 5.0 | 5.3 | 5.8 | 6.9 | 5.9 | 3.4 |
| Glutamic acid | 9.7 | 9.8 | 10.4 | 9.9 | 12.3 | 14.3 | 11.2 | 4.6 |
| Proline | 6.0 | 5.0 | 5.5 | 6.3 | 15.6 | 13.2 | 14.3 | 1.6 |
| Glycine | 4.3 | 4.4 | 4.3 | 4.6 | 7.0 | 7.3 | 7.0 | 1.8 |
| Alanine | 5.5 | 5.5 | 5.6 | 5.8 | 6.4 | 7.3 | 6.5 | 3.2 |
| Cystine | 1.0 | 1.2 | 1.5 | 0.9 | 7.3 | 5.1 | 5.8 | 0.5 |
| Valine | 5.7 | 5.5 | 5.6 | 5.9 | 5.9 | 6.5 | 5.8 | 3.2 |
| Methionine | 1.6 | 1.9 | 2.3 | 2.2 | 1.8 | 1.2 | 1.4 | 0.9 |
| Isoleucine | 5.2 | 4.7 | 5.5 | 5.9 | 3.4 | 4.4 | 3.9 | 1.4 |
| Leucine | 7.6 | 7.1 | 8.0 | 8.1 | 8.4 | 7.2 | 7.3 | 2.0 |
| Tyrosine | 4.1 | 4.0 | 4.0 | 4.4 | 4.4 | 3.9 | 4.0 | 1.0 |
| Phenylalanine | 5.2 | 4.6 | 5.3 | 5.9 | 3.3 | 3.4 | 3.5 | 2.4 |
| Recovery of amino acid N (as % of total N in biomass or culture filtrate) | 78 | 76 | 84 | 81 | 93 | 91 | 86 | 31 |
| 'True' fungal protein content in % of biomass (recovered amino acid N × 6.25) | 28 | 27 | 47 | 38 | | | | |

The high 'true protein' content in experiment c may suggest that solid CSL particles were filtered off with the mycelium. However, also with a mineral medium (experiment d) the amino acid N content was found to be more than 80% of the total mycelial N. It seems, that a low C/N ratio of the medium not only gives a high crude protein content of the fungus but also a high proportion of amino acid N of total nitrogen. Although the chitin content may increase with fungal age (BLUMENTHAL and ROSEMAN, 1957), it depends probably hardly or not at all on the C/N ratio of the medium.

About 90% of the nitrogen present in the culture filtrates of CSL media was recovered as amino acid N after acid hydrolysis. Less than 5% of this amino acid N was recovered as free amino acids. The main free amino acids in the culture filtrates were glutamic acid and aspartic acid. Furthermore, especially alanine, glycine, serine, leucine, and arginine were recovered, and only small differences were observed between culture filtrates of CSL media and those of the mineral medium. More than 95% of the amino acid N in the culture filtrates must be attributed to peptides. These peptides have been partly present in CSL

TABLE 6.3. Essential amino acids in *T. viride* protein and in some other fungal proteins, several (food) proteins and the FAO provisional pattern of essential amino acids in human nutrition. The values are expressed as g amino acid/16 g N (= 100 g crude protein).

| | *Trichoderma viride* (this study; CSL-glucose medium; C/N ratio 12.6) | *Fusarium semitectum* (Worgan, 1973b) | *Gliocladium deliquescens* (Church et al., 1972) | Corn steep liquor (this study) | Hen's egg (FAO, 1968) | Beef (Slump, 1969) | Casein (Slump, 1969) | Skim milk powder (Slump, 1969) | Isolated soy bean protein (Slump, 1969) | Reference protein (FAO, 1957) |
|---|---|---|---|---|---|---|---|---|---|---|
| Isoleucine | 5.2 | 4.2 | 4.1 | 5.3 | 6.3 | 4.8 | 5.9 | 5.5 | 5.0 | 4.2 |
| Leucine | 7.6 | 6.5 | 6.2 | 9.0 | 8.8 | 7.6 | 10.2 | 9.8 | 7.7 | 4.8 |
| Lysine | 6.6 | 6.6 | 6.2 | 3.5 | 7.0 | 8.4 | 8.6 | 8.1 | 5.8 | 4.2 |
| Methionine + Cystine | 2.6 | 2.2 | 2.6 | 3.9 | 5.8 | 3.5 | 3.4 | 3.6 | 2.0 | 4.2 |
| Methionine | 1.6 | 1.7 | 1.2 | 2.0 | 3.4 | 2.5 | 3.3 | 2.7 | 1.2 | 2.2 |
| Cystine | 1.0 | 0.5 | 1.4 | 1.9 | 2.4 | 1.0 | 0.4 | 0.9 | 0.8 | 2.0 |
| Phenylalanine + Tyrosine | 9.3 | 7.2 | 7.3 | 6.3 | 9.9 | 7.4 | 11.9 | 10.1 | 9.1 | 5.6 |
| Phenylalanine | 5.2 | 3.9 | 4.0 | 4.0 | 5.7 | 4.0 | 5.6 | 5.0 | 5.4 | 2.8 |
| Tyrosine | 4.1 | 3.3 | 3.3 | 2.3 | 4.2 | 3.4 | 6.3 | 5.1 | 3.7 | 2.8 |
| Threonine | 5.6 | 4.4 | 4.9 | 5.2 | 5.1 | 4.6 | 4.6 | 4.3 | 3.5 | 2.8 |
| Tryptophan | –[1] | –[1] | 2.3 | – | 1.5 | 1.1 | 1.3 | 1.3 | 1.1 | 1.4 |
| Valine | 5.7 | 5.2 | 4.9 | 5.4 | 6.8 | 5.1 | 7.5 | 6.5 | 5.0 | 4.2 |

[1] A value of 0.9 has been found for *Trichoderma* and a value of 1.4 for *Fusarium* by Rhodes et al. (1961).

media and partly have been excreted by the fungus. When the fungus was grown on a mineral medium, amino acid N, largely present as peptides, was also excreted (Table 6.2 exp. d). Some of the excreted peptides of fungi may have antibiotic properties (OOKA et al., 1966), while self-inhibiting proteins may also be excreted by fungi (MEYRATH and ROTH, 1973). The results show much accordance with those of MORTON and BROADBENT (1955). These authors found in addition that no more than 5% of the extracellular nitrogenous material was protein originating from extracellular enzymes.

The difference in amino acid composition of the culture filtrates of used CSL and mineral media (Table 6.2) demonstrates that in particular proline but also cystine and histidine are present in CSL-containing media in much larger amounts than are utilized by the fungus (for the amino acid compositions of CSL see section 2.2.1). DANIELSON and DAVEY (1973c) have found that alanine, aspartic acid and glutamic acid are generally utilized as the sole N source by *Trichoderma sp.*, which is in agreement with the general pattern of amino acid utilization by a wide variety of fungi (COCHRANE, 1958). Other amino acids seem to be less efficiently utilized, e.g. cystine, leucine and lysine; DANIELSON and DAVEY (1973c) considered these amino acids to be moderate or poor sources of nitrogen for *Trichoderma.*

In Table 6.3 figures are given for the contents of essential amino acids in fungal proteins and in proteins from other sources, together with the FAO reference pattern. These figures are related to total (Kjeldahl) nitrogen content and would have been higher if they had been based on amino acid nitrogen content only; this applies in particular to the fungal proteins, since depending upon growth conditions, the non-protein nitrogen may contribute considerably to the total nitrogen found in fungi.

# 7. STARCH HYDROLYSIS BY *TRICHODERMA VIRIDE* AND PROPERTIES OF THE AMYLOLYTIC ENZYMES

## 7.1. INTRODUCTION

Although numerous reports exist on the cellulolytic enzyme production of *T. viride*, little information is available on the amylolytic enzymes of the fungus. Only a few authors (e.g. MANDELS and REESE, 1957; MANDELS et al., 1971) have reported on the amylolytic activity, besides the cellulolytic one, of the culture filtrate. Since starch is one of the predominant carbon compounds in corn waste effluents (cf. section 1.4), an investigation of its degradation by *T. viride* is justified.

Moulds are known to produce two types of amylase, α-amylase [α-D-(1 → 4) – glucan glucanohydrolase, E.C.3.2.1.1] and glucoamylase or amyloglucosidase [α-D-(1 → 4) – glucan glucohydrolase, E.C.3.2.1.3]. These enzymes differ with respect to their mechanism of action and their stability at low pH. α-Amylase rapidly converts starch into dextrins and maltose, resulting in a rapid loss of the ability to produce a blue colour with iodine reagent. Glucoamylase hydrolyses starch, splitting off glucose units from the non-reducing end of the chain. The dextrinizing power of the latter enzyme is so low, that the iodine reaction does not disappear until over 80% of the starch has been set free as glucose equivalents. Generally α-amylases are rapidly inactivated at low pH values, although exceptions have been described (MINODA and YAMADA, 1963). Glucoamylases are more stable under acid conditions than α-amylases. Besides glucoamylase, or α-amylase and α-glucosidase, a debranching activity, splitting the α-1.6 glycosidic bonds is required for the complete hydrolysis of starch. In fungi the α-amylase activity generally is predominant.

Starch degradation by *Trichoderma viride* was expected to differ from that by other moulds because of the low pH optimum of the former (pH 3.7–4.7; DANIELSON and DAVEY, 1973b; this study) and the inability of several strains of *T. viride* to assimilate maltose, which is the main end product of α-amylase action on starch.

It is generally assumed that the synthesis of (gluco)amylases is induced by starch, dextrins or maltose. Amylolytic enzyme activities in cultures of *Aspergillus niger* growing on these compounds are high; in cultures with glycerol, lactose and other sugars (especially monosaccharides), only trace amounts of the amylolytic enzymes are excreted (e.g. BARTON et al., 1969, 1972). In cultures with two carbon sources, an inducing compound and a non-inducing one (e.g. glucose, fructose, glycerol, α-ketoglutarate, pyruvate, organic N compounds), low amylolytic enzyme activities are found (e.g. BARTON et al., 1972). Apparently, the induction of amylases by starch or its degradation products is counteracted by catabolite repression exerted by glucose and other readily metabolized carbon sources. Besides the carbon source, the nitrogen source is known to

affect the production of starch-hydrolysing enzymes in moulds. Effects of the C/N ratio in starch media (LINEBACK et al., 1966; BARTON et al., 1969) as well as the nature of the N source have been reported. The (gluco)amylase production by *A. niger* in starch media has been found to be higher with a complex organic nitrogen source than with an inorganic one (LINEBACK et al., 1966) but also the opposite has been observed with this organism (BARTON et al., 1972), and with other organisms such as *Bacillus polymyxa* (GRIFFIN and FOGARTY, 1973). Preliminary experiments revealed that complex organic nitrogen sources (e.g. corn steep liquor and peptone) partly inhibit (gluco)amylase production. These observations and the observed inability to metabolize maltose prompted us to a more detailed study of the regulation of the synthesis of amylolytic enzymes in the strain of *T. viride*, used in this study.

The second section of this chapter deals with the excretion of amylolytic enzymes during the growth in a mineral medium and with some properties of these enzymes. In the third section the regulation of amylolytic enzyme production is reported.

## 7.2. PRODUCTION AND PROPERTIES OF AMYLOLYTIC ENZYMES

### 7.2.1. *Excretion of the enzymes*

The maximum specific growth rate of *T. viride* observed in mineral urea medium with starch as the sole carbon source ($\mu_{max} = 0.14\ hr^{-1}$) was found to be somewhat lower than that with glucose ($\mu_{max} = 0.18\ hr^{-1}$). Data on the excretion of amylolytic enzymes by *T. viride* and the yield of biomass during growth in a mineral starch urea medium are presented in Fig. 7.1. Both the dextrinizing and the saccharifying activities of the culture filtrate still increased after maximum cell density was already reached which suggests that enzyme synthesis takes place to a considerable extent in the postexponential phase of growth. An increasing amylolytic activity in the postexponential phase has been also observed in experiments with *T. viride* reported by MANDELS and REESE (1957). Several other micro-organisms produce amylolytic enzymes especially in the postexponential phase of growth such as the yeast *Endomycopsis capsularis* (EBERTOVA, 1966a) and *Bacillus polymyxa* (GRIFFIN and FOGARTY, 1973). It has been suggested by the latter authors, that postexponential enzyme synthesis is caused by exhaustion of the carbon source of the medium.

A second explanation concerning the increased amylolytic activity of the culture filtrate in the postexponential phase of growth would be that enzymes produced and accumulated in the mycelium during the exponential phase continue to be excreted during the postexponential phase. To decide which of the two hypotheses is correct the following experiments were performed.

Starch-grown mycelium was filtered off at the time of maximum cell yield and resuspended in an acetate buffer (same volume as the culture filtrate; 0.2 M; pH 5.0) provided with cycloheximide (25 mg/l) in order to prevent enzyme synthesis. Only traces of amylolytic activities (less than 4% of the activities in the

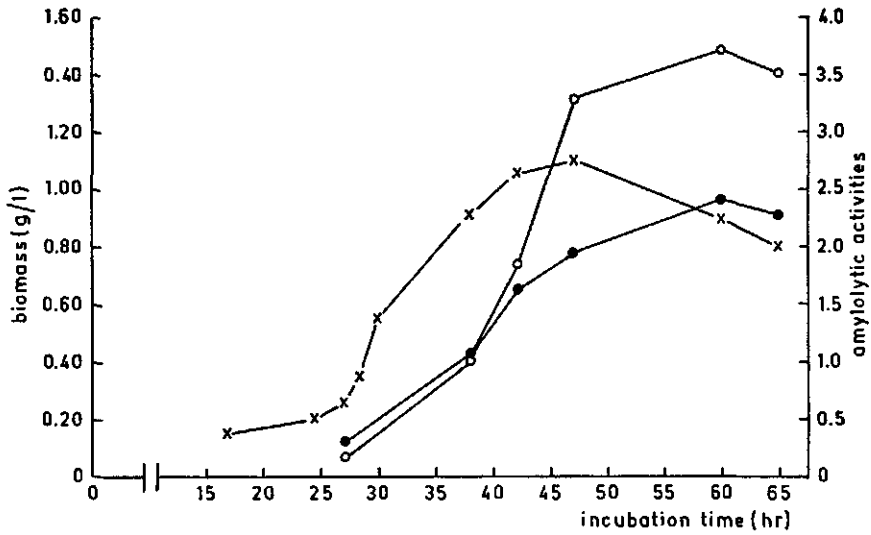


FIG. 7.1. Production of biomass and excretion of amylolytic enzymes by *T. viride* in a starch urea medium (C/N ratio 10; COD ca. 2.5 g/l) as function of the incubation time. The medium was inoculated with a standard spore suspension (1%; v/v). The pH was controlled at 4.0. × biomass (g/l); • dextrinizing activity (DA); ○ saccharifying activity (SA).

original culture filtrate) were detected, even after more than 10 hours of incubation. Similar results were found when mycelia were grown at other pH values than 4.0 (3.5, 5.0 and 7.0) and incubated after harvesting as above in buffers with pH values varying from 3.0 up to 7.0. Even grinding the mycelia in a mortar with subsequent extraction in buffer solution did not result in higher amylolytic enzyme activities.

From these experiments it can be concluded that the increase of amylolytic activity of the culture filtrate in de postexponential phase does not depend on excretion of enzymes produced during the exponential phase but is due to enzyme synthesis in the postexponential phase. Thus, the enzymes are practically completely excreted into the medium immediately after production. Any adsorption of amylolytic enzymes on the mycelial surface of *T. viride* appears to be absent, irrespective of pH. This in contrast to the observation of TONOMURA et al. (1963), that α-amylase is fixed onto the surface of *Aspergillus oryzae* at low pH, but in accordance with the findings of LINEBACK et al. (1966), who could not detect glucoamylase on the mycelial surface of *A. niger*. More details on the synthesis of amylolytic enzymes during the postexponential growth phase will be given in section 7.3.2.

### 7.2.2. *End product of starch degradation*

In order to characterize the amylolytic enzymes of *T. viride*, the end products of starch hydrolysis were identified.

A filtrate of a culture grown in starch urea medium was used for exhaustive

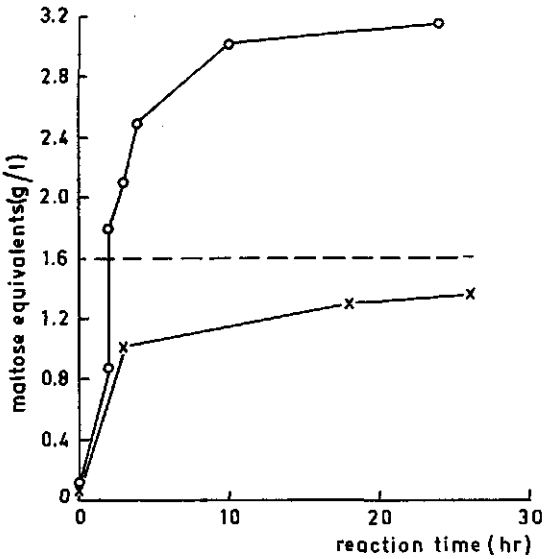


FIG. 7.2. Production of reducing compounds from soluble starch at pH 5 by the amylolytic enzymes of *T. viride* and by a commercial α-amylase preparation (Fungamyl 1600). Reducing compounds are expressed in maltose equivalents (g/l) as function of the reaction time. The broken line indicates the theoretically maximum maltose production; × Fungamyl 1600 (10 mg/l); ○ culture filtrate of *T. viride*.

degradation (at 40°C and during 26 hr) of soluble starch. Reducing sugars were estimated as maltose (mg/l) and at the end of the procedure identified by thin layer chromatography. Similar experiments were carried out with the commercial α-amylase preparation Fungamyl 1600 (see section 2.5.9). From the estimation of the reducing compounds (Fig. 7.2), it may be concluded, that glucose is the predominant final reaction product of the amylolytic action of *T. viride*, whereas maltose is the main end product of Fungamyl action. Thin layer chromatography revealed that glucose was the only detectable product of *T. viride* amylase action. Even directly after initial hydrolysis glucose could be detected by thin layer chromatography as the sole low-molecular reaction product. After Fungamyl action only maltose could be detected. In an enzyme assay with a Fungamyl solution, a SA/DA ratio of about 0.15 was found for this α-amylase, whereas a ratio of about 1.6 was found with the culture filtrate of *T. viride*. These results demonstrate that starch degradation by *T. viride* culture filtrates is mainly catalysed by one or more enzymes of the glucoamylase type, whereas α-amylase seems to play only a minor role, if at all.

With respect to the results just mentioned, it would have been more appropriate to express the saccharifying activity of the culture filtrates in glucose equivalents rather than in maltose equivalents. However, it is usual to define saccharifying amylolytic activity in maltose equivalents and this practice will be adhered to in this study.

7.2.3. *Substrate specificity of the saccharifying enzyme*

The filtrate of a culture grown in starch urea medium hydrolysed pullulan and low molecular dextrins, although at lower rates than with starch as the enzyme substrate (Table 7.1). As can be seen, maltose is hardly attacked by *T. viride* culture filtrates from starch media. As appears from experiments reported on in detail in section 7.3.2, each of the substrates could serve as the sole C source for fungal growth, except pullulan, although only low yields were obtained with maltotriose and maltose.

TABLE 7.1. Rates of hydrolysis of several substrates, expressed in % of the rate on soluble starch, by a culture filtrate of *T. viride* grown in a starch urea medium.

| Substrate | Saccharifying activity (SA on soluble starch = 100) |
|---|---|
| Soluble starch | 100 |
| Dextrin DE-20 | 80 |
| Dextrin DE-30 | 79 |
| Maltotriose | 46 |
| Pullulan | 17 |
| Maltose | traces |

7.2.4. *Influence of pH on activity and stability of the enzymes*

a. Effect of pH on enzyme activity

Cultures were grown in starch urea media at controlled pH values 3.5, 5.0 and 7.0, respectively, and mycelia were harvested at the time of maximum cell yield, as was indicated by the disappearance of the iodine blue reaction of the culture filtrate. The saccharifying and dextrinizing activities of the culture filtrate were determined as a function of the pH. The results (Fig. 7.3) did not show any in-

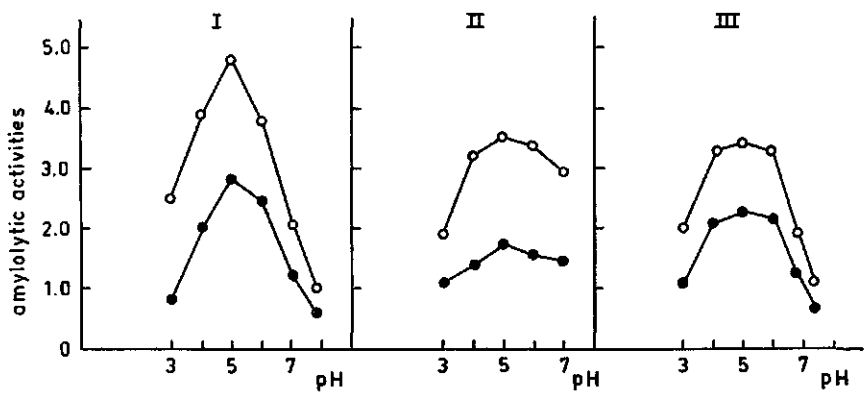


FIG. 7.3. Effect of pH on the activity of the amylolytic enzymes of *T. viride* at 30 °C with soluble starch as the enzyme substrate. The pH during fungal growth was controlled at 3.5 (I); 5.0 (II) and 7.0 (III), respectively. Dextrinizing activity • (DA); saccharifying activity ○ (SA).

fluence of the pH during growth at the optimum pH of the enzymes, and the same optimum was found for dextrinizing and saccharifying activities (pH 5.0–5.5). Lower optimum pH values than for *T. viride* amylases have been reported for glucoamylases from other fungi by OHGA et al. (1966); EBERTOVA (1966b) and LINEBACK and BAUMANN (1970). A low pH during growth which is unfavourable to the enzyme activity, results in a somewhat higher enzyme production. This is in accordance with the observation of BARTON et al. (1972), that the maximum production of glucoamylases in *A. niger* occurs at very low pH.

b. Effect of pH on enzyme stability

The residual activities after a storage of 2.5 weeks at 2°C and at various pH values are shown in Fig. 7.4. After a storage period longer than one month at 2°C, the residual activity of the enzymes was still more than 70% of the initial one at storage pH values between 4 and 7.

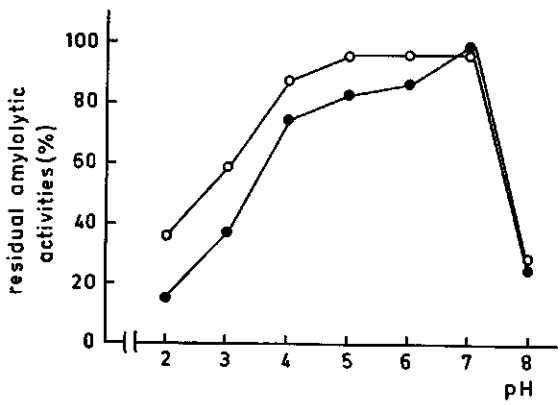


FIG. 7.4. Stability of the amylolytic enzymes of *T. viride* as function of pH. Residual activities of the enzymes determined after keeping the culture filtrates at 2°C for 2.5 weeks at various pH values. Dextrinizing activity • (DA); saccharifying activity ○ (SA).

7.2.5. *Influence of temperature on activity and stability of the enzymes*

a. Effect of temperature on enzyme activity

The amylolytic activities in the culture filtrate as a function of temperature are given in Fig. 7.5. Above 40°C the heat inactivation (see below) will have influenced the results considerably, as indicated by the broken lines.

b. Heat stability of the enzymes

The influence of heat treatments on the amylolytic activities in a filtrate of a culture grown at pH 5.0 is shown in Table 7.2. The heat stability of the saccharifying activity seems to be somewhat higher than that of the dextrinizing activity. The results presented in Table 7.2 are in accordance with those obtained with amylolytic enzymes of *Aspergillus oryzae* (ADAMS, 1953; KUNDU and DAS, 1970). These enzymes decreased in stability at temperatures above 55–60°C.

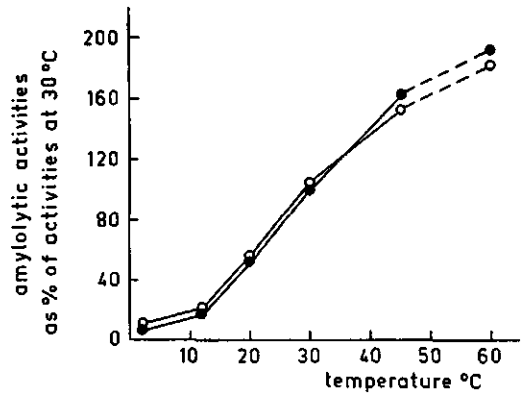


FIG. 7.5. Effect of temperature on the activity of the amylolytic enzymes of *T. viride* in 0.2 M acetate buffer at pH 5.0, with soluble starch as the enzyme substrate. The broken lines indicate a decreasing heat stability of the enzymes. Dextrinizing activity • (DA); saccharifying activity ○ (SA).

TABLE 7.2. Residual activities of the amylolytic enzymes of *T. viride*, determined after a heat treatment of 10 minutes at pH 5.0.

| Treatment | Residual activities (%) | |
|---|---|---|
| | SA | DA |
| Control | 100 | 100 |
| 60 °C | 98 | 88 |
| 70 °C | 63 | 30 |
| 80 °C | traces | traces |

### 7.2.6. *Enzyme inhibition by cyclomaltoheptaose*

BARTON et al. (1972) found no induction of glucoamylases of *Aspergillus niger* by Schardinger dextrins, which are cyclic oligosaccharides consisting of 6 or 7 1.4-bound α-glucose units.

In the present study no growth of *T. viride* was observed with β-cyclodextrin (cyclomaltoheptaose) as the sole C source.

MARSHALL (1973) described the inhibition of several amylolytic enzymes of different origin (especially pullulanase) by Schardinger dextrins, and he suggested the inhibition to be of a competitive type. The corresponding linear oligosaccharides (maltodextrins) did not inhibit pullulanase. The same author found an inhibition of 15% for glucoamylases of *A. niger* by α-cyclodextrin (cyclomaltohexaose).

In the present study an inhibition of about 50% of the amylolytic enzyme activities was found by β-cyclodextrin (10 mg/ml), both when starch and when dextrin DE-30 had served as the C source in the growth medium (Table 7.3).

Inhibition of fungal growth in the presence of β-cyclodextrin was observed with starch but not with glucose as the sole C source in the medium.

TABLE 7.3. Inhibition of the amylolytic enzymes of *T. viride* by β-cyclodextrin (10 mg/l). Starch was used as the substrate in the enzyme assay.

| C source in the growth medium | Inhibition (%) | |
|---|---|---|
| | SA | DA |
| Starch | 48 | 54 |
| Dextrin DE-30 | 48 | 36 |

## 7.3. REGULATION OF AMYLOLYTIC ENZYME PRODUCTION

### 7.3.1. *Influence of pH on enzyme synthesis*

Several authors have reported an effect of the pH on the production of amylolytic enzymes (e.g. EBERTOVA, 1966a; LINEBACK et al., 1966; BARTON et al., 1972). In a previous section (7.2.4) an influence of the pH was suggested on amylolytic enzyme production by *T. viride*. This effect was investigated more extensively in the following experiment. Cultures were grown in a mineral medium with starch as the C source and urea as the N source (C/N ratio 10) at different initial pH values. Only small differences in biomass concentration were observed at the end of the incubation (48 hr); the amylolytic acitivities of the culture filtrates are given in Table 7.4. It appears, that the lowest enzyme production occurs at pH values at which the enzyme activity is optimum (pH 5.0–5.5). It is noteworthy that the maximum specific growth rate occurs at pH 4.0–4.5, where neither the production, nor the activities of the enzymes have their optimum value.

TABLE 7.4. Specific amylolytic activities of filtrates of *T. viride* cultures, grown during 48 hr at various pH values.

| pH of the culture | | Amylolytic activities | |
|---|---|---|---|
| Initial | Final | SA spec. | DA spec. |
| 3.0 | 3.7 | 5.3 | 3.1 |
| 4.0 | 5.7 | 2.7 | 1.7 |
| 4.5 | 5.9 | 1.7 | 0.9 |
| 5.0 | 5.9 | 1.6 | 1.0 |
| 6.0 | 6.0 | 2.3 | 1.2 |

### 7.3.2. *Influence of carbohydrates*

In order to investigate whether synthesis of amylolytic enzymes in *Trichoderma* is constitutive or not, the enzyme activities were determined in filtrates of cultures grown in mineral urea media with starch, amylose, amylopectin, various dextrins, maltotriose, maltose, lactose or glucose as the sole source of carbon and energy. Several of these carbon sources might induce or repress production of amylolytic enzymes.

When amylose or amylopectin were used as the carbon source for growth or as the substrate in the enzyme assay, about equal amylolytic activities were

TABLE 7.5. Amylolytic activities of culture filtrates of *T. viride*, grown during 48 hr on soluble starch, amylose or amylopectin, expressed as % of activity if the organism had been grown on soluble starch.

| Substrate in growth medium | Substrate in enzyme assay | Amylolytic activities | | | |
|---|---|---|---|---|---|
| | | SA | DA | | |
| | | | 623 nm | 660 nm | 532 nm |
| Soluble starch | soluble starch | 100 | 100 | | |
| Amylose | soluble starch | 72 | 95 | | |
| Amylose | amylose | 67 | 104 | 114 | |
| Amylose | amylopectin | 67 | 102 | | |
| Amylopectin | soluble starch | 114 | 122 | | |
| Amylopectin | amylose | 90 | 120 | | |
| Amylopectin | amylopectin | 115 | 114 | | 95 |

detected as with soluble starch (Table 7.5); only the saccharifying activities were somewhat lower with amylose. The SA/DA ratio generally varies from 1.1 during initial growth to 1.9 in the postexponential phase of growth.

When maltotriose was used as the carbon source a rather low yield was obtained (Table 7.6). To enable a better comparison of the amylolytic activities of cultures with different yield, these activities were expressed as specific activities (cf. 2.5.9). The results suggest that only one glucose unit is utilized per molecule of maltotriose, leaving maltose in the culture filtrate. This was confirmed by thin layer chromatography. The specific activities on soluble starch after growth on maltotriose were about half the activities as found after growth on soluble starch (cf. Fig. 7.1).

Maltose was utilized even more slowly than maltotriose, and only a trace of amylolytic and maltase (α-glucosidase) activity could be detected in the culture filtrate of some maltose-containing cultures after 50 hr incubation. Initial growth probably occurred on dextrin contaminations in the maltose used. Growth on maltose could be improved by inoculation with a mycelium suspension instead of a spore suspension or after addition of a trace of glucose or soluble starch. A lag phase of 1–2 days or more was usually observed, and growth on maltose was always accompanied with the production of a slime which hampered filtration of the mycelium and determination of the yield. Adapted mycelium could be used as an inoculum in subsequent cultures with maltose as the sole C source, resulting again in low growth rates and cell yields. When soluble starch was

TABLE 7.6. Yield and enzyme activity after growth on maltotriose (incubation time 50 hr).

| | |
|---|---|
| Initial concentration of maltotriose (g/l) | 2.5 |
| Residual reducing compounds in glucose equivalents (g/l) | 0.65 |
| Residual total carbohydrate in glucose equivalents (g/l) | 1.64 |
| Yield of biomass (g/l) | 0.46 |
| Specific saccharifying activity on maltotriose | 1.5 |
| Specific saccharifying activity on soluble starch | 2.0 |
| Specific dextrinizing activity on soluble starch | 1.3 |

hydrolysed by Fungamyl 1600 which degrades starch mainly to maltose, and the resulting mixture was used as the sole C source in the growth medium, similar results, i.e. low growth rates and cell yields, were found. In filtrates of cultures on maltose, the specific saccharifying and dextrinizing activities were found to be much lower than with soluble starch as the C source. The maltase activity was also very low, but about the same low maltase activities were found in filtrates of cultures grown on soluble starch as the C source. In addition, this maltase activity was much less stable than the amylase activities, even when the culture filtrate was stored at 0 °C and pH 5. It may be concluded that a high production of α-amylase by the present strain would be very inefficient, because of its inability to consume maltose. Glucoamylases are incapable of hydrolysing maltose, and a stable maltase does not seem to be synthesized by the present strain.

Other *Trichoderma* strains have also been reported to utilize maltose slowly (AUBE and GAGNON, 1969; DANIELSON and DAVEY, 1973b). If maltose is utilized, however, it may be an inducer of amylolytic enzymes in *T. viride* (MANDELS and REESE, 1957) and also in other fungi such as *Aspergillus niger* (BARTON et al., 1972).

When starch was only partially hydrolysed by Fungamyl 1600 (purple colour with iodine reagent), the specific growth rate on the produced dextrins was as high as that on starch (the control) and the same or somewhat higher amylolytic activities were found in the culture filtrates of these dextrin cultures. When the products resulting from hydrolysis of starch by a culture filtrate were added as the sole carbon source to a growth medium, they were rapidly utilized by the fungus. The more the starch was hydrolysed before being used in the growth medium, the lower the amylolytic activities in the culture filtrates after 48 hr incubation (Table 7.7).

TABLE 7.7. Specific amylolytic activities of culture filtrates of *T. viride* grown on different C sources. Except dextrin DE-30, the dextrins were prepared from soluble starch (2.5 g/l) by the amylolytic enzymes of the fungus itself. The nitrogen source in the medium was urea; C/N ratio 10; initial pH 4.0; incubation time 48 hr.

| C source | Initially present reducing compounds in glucose equivalents (mg/l) | Specific amylolytic activities | |
|---|---|---|---|
| | | SA spec. | DA spec. |
| Glucose | 2500 | 0.3 | 0.2 |
| Lactose | 1280 | < 0.2 | < 0.2 |
| Soluble starch | 205 | 3.6 | 1.6 |
| Dextrin DE-30 | 670 | 4.7 | 2.3 |
| Maltotriose | 805 | 2.0 | 1.3 |
| Partially hydrolysed starch obtained by *T. viride* amylolysis | 850 | 5.6 | 3.5 |
| | 1820 | 3.4 | 2.0 |
| | 2090 | 1.3 | 1.0 |

In accompanying experiments the fungus was grown in media containing soluble starch, dextrin DE-30, maltotriose, lactose or glucose as the sole C source and urea as the N source. The amylolytic activities in the culture filtrates after 48 hr incubation are given in Table 7.7. The results show that all of the starch-derived oligosaccharides used in this experiment induce amylolytic enzyme activity. Starch fragments obtained by *T. viride* amylases on soluble starch and dextrin DE-30 induced a higher amylolytic activity than starch itself.

No growth and enzyme synthesis occurred when α-methyl-D glucoside, β-cyclodextrin or saccharose were used as the sole C source.

It may be concluded that starch as well as its degradation products can serve as inducers of amylolytic enzyme synthesis. Enzyme production in media with dextrin DE-30 or with starch partially hydrolysed by *Trichoderma* amylolytic enzymes as sole sources of carbon and energy is higher than that in media with starch, amylose or amylopectin (Table 7.7). These results are in accordance with those of WELKER and CAMPBELL (1963b), who found maltotetraose to be the best inducer of α-amylase in *Bacillus stearothermophilus*. Maltodextrins may be produced from starch by α-amylase or glucosyltransferase activity (BARTON et al., 1972).

While after exhaustion of the medium the total carbohydrate concentration was only about 1 % of the initial value when glucose served as the sole C source, higher final concentrations were observed with starch (3 %) and dextrin DE-30 (up to 5 %) as the sole C source. This suggests that (limit) dextrins and maltose remain in the medium.

In order to study the involvement of catabolite repression in the regulation of amylolytic enzyme synthesis, cultures were grown on mixtures of carbohydrates with urea as the N source (C/N ratio 10) at pH 4. The total carbohydrate concentration in each of the experiments was 2.5 g/l. The amylolytic activities were compared with those found when starch, dextrin DE-30 or glucose served as the sole C source for growth. The results (Fig. 7.6) show a higher enzyme production with dextrin DE-30 than with starch (see also Table 7.7) Both substrates induce amylolytic enzymes during growth and in the postexponential growth phase. In starch glucose medium (Fig. 7.6, III) no amylolytic enzyme synthesis is observed and starch degradation is prevented, until glucose has been consumed. Apparently, the induction of amylolytic enzymes by starch or its degradation products is counteracted by catabolite repression. The same phenomenon is observed when the mould is grown in a medium containing glucose and dextrin DE-30 (Fig. 7.6, V); enzyme synthesis does not occur in the young culture. Amylolytic enzyme synthesis is repressed by lactose (Fig. 7.6, II) to a smaller extent than by glucose. Starch degradation is not inhibited completely. Catabolite repression of amylolytic enzymes by glucose, other monosaccharides and some disaccharides has frequently been observed by several authors (e.g. TONOMURA et al., 1961; WELKER and CAMPBELL, 1963a; BARTON et al., 1969; 1972).

After growth in glucose medium, low amylolytic enzyme activities are observed (Table 7.7). Fig. 7.6 VI shows that in such a medium this enzyme is

excreted exclusively in the postexponential growth phase. Since synthesis of amylolytic enzymes was never observed in cultures with lactose as the sole C source, this postexponential enzyme synthesis in glucose cultures is probably due to the presence of inducing maltodextrin contaminations in the glucose used and does not necessarily point to a derepression of enzyme synthesis provoked by carbon limitation. Induction of amylolytic enzymes by contaminating maltodextrins has also been suggested by other authors (WELKER and CAMPBELL, 1963b; BARTON et al., 1972). Inducing dextrins are supposed to be required for postexponential enzyme synthesis as washed mycelia resuspended in buffer or in fresh medium without carbon source have never been shown to excrete amylolytic enzymes.

The difference between the SA/DA ratios in young cultures with either starch or dextrin DE-30 as the sole carbon source (Figs. 7.6, I and 7.6, IV) is noteworthy. In the former cultures it is low (0.1 to 0.7), in the latter remarkably higher (up to 3.2). In starch cultures (Fig. 7.6, I) initially dextrinizing enzymes are produced and subsequently saccharifying enzymes. These data indicate a separate regulation of the dextrinizing and saccharifying enzymes and at least two types of enzymes to be involved in the hydrolysis of starch, the α-amylase type being preferentially induced during initial growth on starch and the glucoamylase type preferentially induced by low-molecular dextrins. Since the differences in the SA/DA ratio are generally negligible at the end of the growth, an aspecific induction is suggested in the postexponential growth phase.

If glucoamylases are capable to hydrolyse both α-D(1 → 4) and (slowly) α-D(1 → 6) glucosidic linkages (PAZUR and ANDO, 1960), starch can be degraded almost completely by such enzymes. The excretion of oligo and α-1,6-glucosidases by some fungi seems to have been demonstrated (MEYRATH, 1966; KOBAYASHI, 1971), but it is difficult to identify these enzymes.

### 7.3.3. *Influence of the C/N ratio of the medium on synthesis of amylolytic enzymes*

The effect of the C/N ratio of the medium upon growth and enzyme synthesis was studied by varying the concentration of the N source (urea). Lower specific growth rates were observed at higher C/N ratios and also an important influence of this ratio was found on amylolytic activities in the culture filtrates after 48 hr incubation, when the yield of biomass was about the same for each of the cultures (Table 7.8). Lower urea concentrations (higher C/N ratios) resulted in

TABLE 7.8. Influence of the C/N ratio of the medium on final pH, nitrogen content of the biomass and amylolytic activity of the culture filtrate, after 48 hr incubation. The N source was urea; C source starch (2.5 g/l); initial pH 4.0.

| C/N ratio of the medium | Final pH of the medium | N content of the biomass (%) | Specific amylolytic activities SA spec. | DA spec. |
|---|---|---|---|---|
| 10 | 6.2 | 6.9 | 3.2 | 2.5 |
| 12.5 | 5.2 | 6.4 | 2.0 | 1.9 |
| 14.3 | 4.9 | 5.8 | 1.9 | 1.8 |
| 20 | 4.6 | 4.0 | 0.6 | 0.9 |

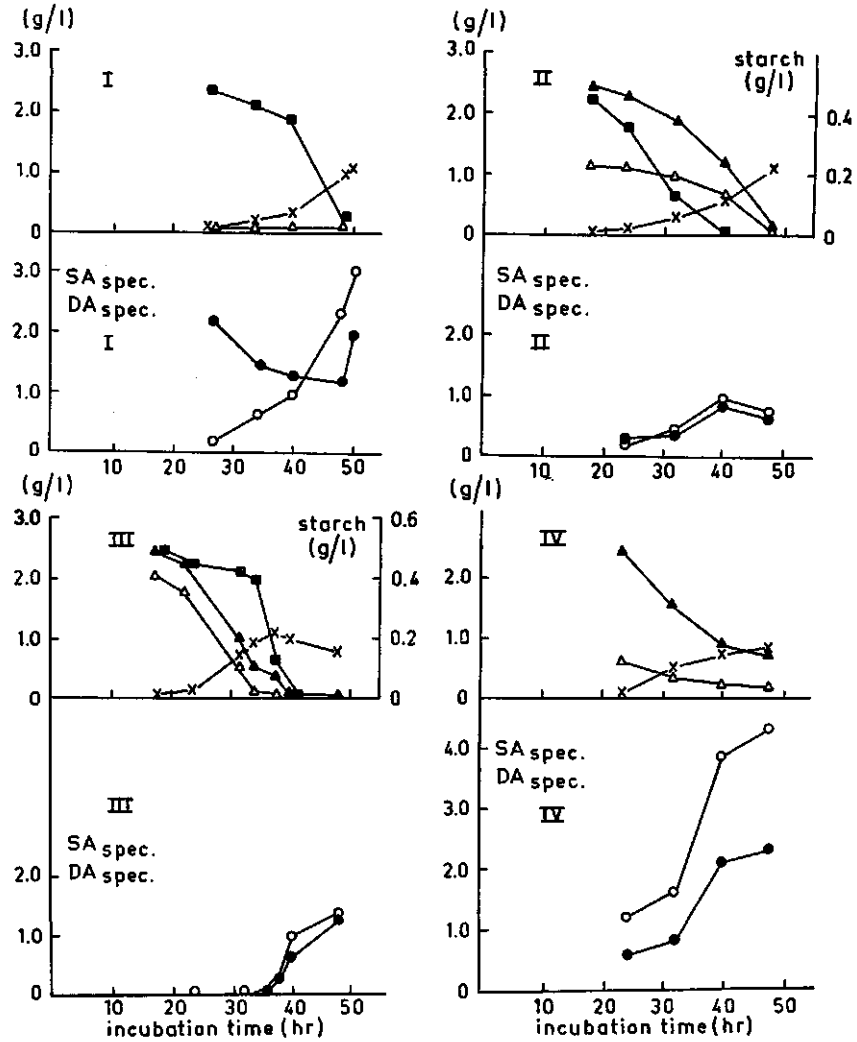


FIG. 7.6. Amylolytic activities of culture filtrates of *T. viride* during growth in media with different carbohydrates. The N source was urea; C/N ratio 10; initial pH 4.0. The media were inoculated with a standard spore suspension (1%; v/v). The carbohydrate substrates were: I starch (2.5 g/l); II lactose (2 g/l) and starch (0.5 g/l); III glucose (2 g/l) and starch (0.5 g/l); IV dextrin DE-30 (2.5 g/l); V glucose (2 g/l) and dextrin DE-30 (0.5 g/l); VI glucose (2.5 g/l); × biomass (g/l); ▲ total carbohydrates in glucose equivalents (g/l); △ reducing compounds in glucose equivalents (g/l); ■ starch (g/l); ● $DA_{spec.}$; ○ $SA_{spec.}$

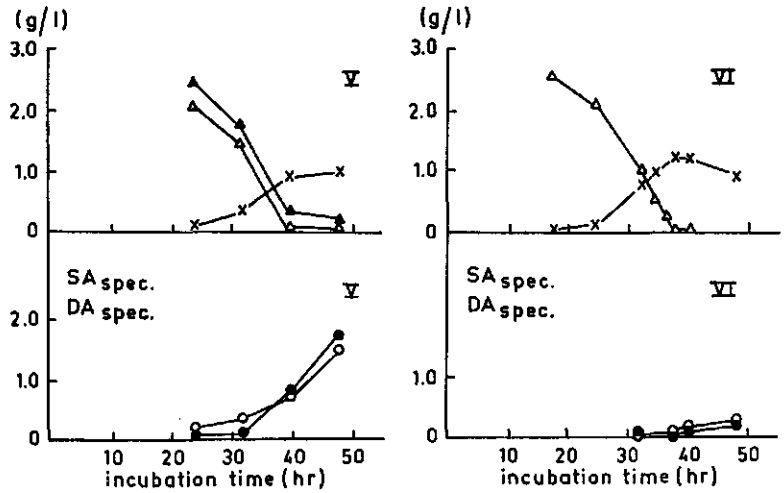


Fig. 7.6. (Continued)

lower amylolytic activities in spite of the lower final pH of the culture (cf. section 7.3.1). A similar effect of the C/N ratio on postexponential amylolytic enzyme activities was observed with other N sources, both inorganic and organic ones. The inhibiting effect of a high C/N ratio upon amylolytic enzyme synthesis can be explained by assuming catabolite repression exerted by starch degradation products. The excess of carbon nutrients is reflected by the lower N content of the biomass produced. The effect of the C/N ratio upon amylolytic enzyme synthesis found in this study is in accordance with observations of BARTON et al. (1969) in cultures of *A. niger*.

7.3.4. *Influence of the nitrogen source on synthesis of amylolytic enzymes*

In order to study the influence of the nitrogen source on the synthesis of amylolytic enzymes, cultures were grown in Biotec fermentors at a controlled pH of 4.0. Organic N sources (corn steep liquor and peptone) and inorganic N sources (ammonia, urea and nitrate) were compared with regard to growth rate and enzyme synthesis in media containing equal substrate concentrations (on the basis of COD = 2.5 g/l) and equal C/N ratios (10). In either case the Kjeldahl nitrogen concentration of the medium was adjusted to 94 mg N/l. When adjusting the C/N ratio of complex media to 10, it was assumed that the Kjeldahl N in the complex nitrogen sources originated from a protein with a carbon content of 53% (see also section 2.2.1).

The yields and amylolytic activities as a function of the incubation time are shown in Fig. 7.7. In each of the media postexponential enzyme synthesis had taken place. The lowest specific growth rate and amylolytic activities were found with $KNO_3$ as the N source, which indicates slow utilization of nitrate by the fungus. The highest postexponential amylolytic activities were found with ammonia and urea; the differences in enzyme synthesis between these latter N

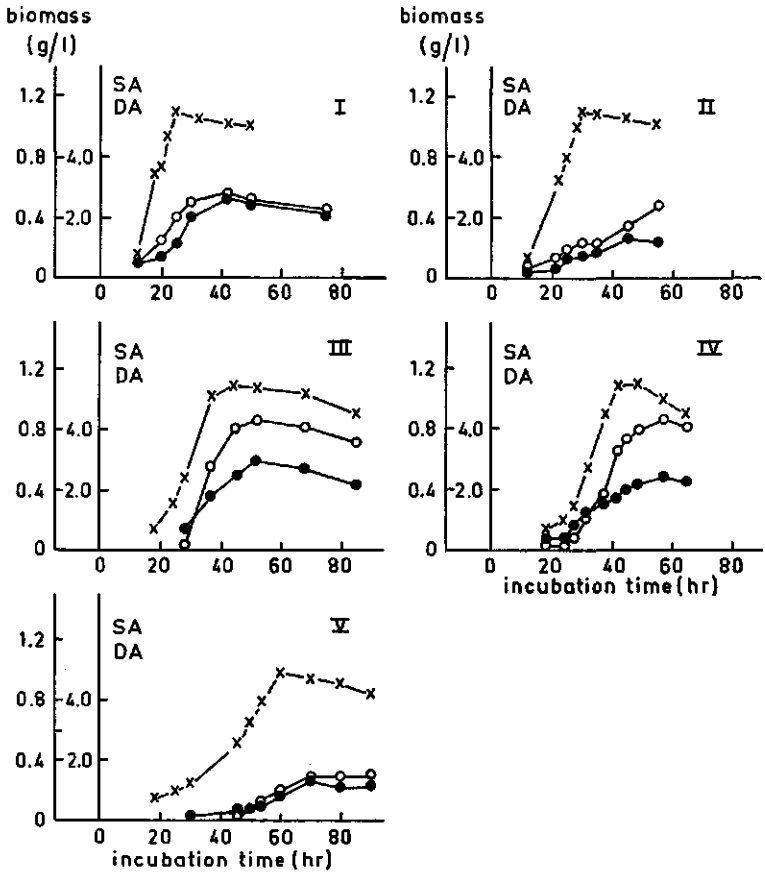


FIG. 7.7. Amylolytic activities in culture filtrates of *T. viride* during growth in media with starch and different N sources. The C/N ratio was 10 and the COD ca. 2.5 g/l. The pH was controlled at 4.0. The media were inoculated with a standard spore suspension (1%; v/v). Nitrogen sources: I CSL; II peptone; III ammonia; IV urea; V nitrate. × biomass (g/l); • DA; ○ SA.

sources are negligible. The highest specific growth rates were observed in media with organic N sources. Repression of amylolytic enzymes, in the exponential growth phase, occurred in particular with peptone. This repression was less apparent in media with corn steep liquor, probably due to the presence of inducing dextrins in this N source. Several authors have found lower amylolytic activities in cultures with organic N sources (e.g. BARTON et al., 1969, 1972) than with inorganic ones, and they suggested a catabolite repression by deamination products of amino acids. Alanine and glutamic acid e.g. can be deaminated to pyruvate and α-ketoglutarate, respectively, and the intermediates of the citric acid cycle may serve as repressors of amylolytic enzymes. An additional explanation for the lower amylolytic activities in cultures with complex media may be the lower inducer (starch) concentration in these media.

7.3.5. *Repression by glutamate and other non-carbohydrates*

In the previous section repression of amylolytic enzymes by organic N sources was reported. In the present section experiments are described in which the ability of glutamate to repress the synthesis of amylolytic enzymes in *T. viride* was tested. This amino acid served as a rapidly assimilated nitrogen source for *Trichoderma sp.* in the study of DANIELSON and DAVEY (1973c).

The fungus was grown in a medium containing starch (1.6 g/l) and glutamate (0.98 g/l) at a C/N ratio 10 and at pH 4.0. The yield of biomass, concentration of starch, and amylolytic activities as a function of the incubation time are given in Fig. 7.8. In the initial phase of growth a higher specific growth rate, a lower rate of starch hydrolysis (iodine blue reaction) and lower amylolytic activities were observed than in media with urea as the N source (cf. Fig. 7.6, I). These data point to a preferential utilization of glutamate as the C-N source and to catabolite repression of the amylolytic enzymes by degradation products of glutamate.

Because amino acids may repress the synthesis of amylolytic enzymes, a number of other non-carbohydrates were tested for their ability to repress those enzymes when urea served as the nitrogen source in the medium. No amylolytic activities were detected in media containing acetate, malate or citrate as the sole C source while the yield on these C sources was much lower than on starch. Traces of saccharifying activities were found in the postexponential phase when ethanol or glycerol served as C source; the yield on these substrates was higher than on starch. Each of the C sources mentioned above could partly repress the postexponential enzyme synthesis when added in a concentration of 1 g per litre to starch cultures at the end of the exponential phase of growth. As regards acetate, besides a possible catabolite repression, the toxic effect of this acid has almost certainly contributed to the decreased enzyme activity (cf. chapter 4).

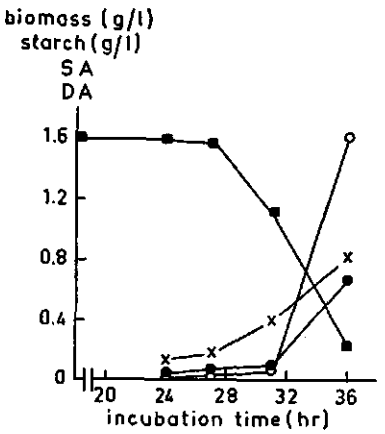


FIG. 7.8. Yield of biomass and amylolytic activities during growth of *T. viride* in a medium containing starch (1.6 g/l) as the C source and glutamate (0.98 g/l) as the N source (C/N ratio 10). The initial pH was 4.0. The medium was inoculated with a standard spore suspension (0.5%; v/v). × biomass (g/l); ■ starch (g/l); ● DA; ○ SA.

## 7.4. Summary and conclusions

Since starch is one of the predominant carbon compounds in corn waste effluents and little information is available on the amylolytic enzymes of *T. viride*, the mechanism of starch hydrolysis by this fungus was studied.

The strain used consumed starch almost as readily as glucose but maltose and the end product resulting from hydrolysis of starch by a fungal α-amylase preparation were hardly assimilated.

It was shown that the amylolytic enzyme system is completely extracellular and consists mainly of one or more enzymes of the glucoamylase type, yielding glucose as the main product of starch hydrolysis; α-amylase seems to play only a minor role because no maltose could be detected. The saccharifying activity of filtrates from starch cultures on dextrin DE-30 and maltotriose was somewhat lower than that on soluble starch; the maltase activity was negligible.

The optimum pH value for both the saccharifying and the dextrinizing activity was between 5.0 and 5.5; the enzymes were rather stable within a pH range from 4 up to 7. The heat stability of the enzymes was about similar to that reported for the amylolytic enzymes of other fungi. β-Cyclodextrin inhibited the enzymes to some extent when added in fairly high concentrations (10 mg/ml).

The synthesis of amylolytic enzymes required the presence of starch or dextrins; in the absence of these compounds no enzyme synthesis was observed. The enzyme system was equally well induced by starch, amylose and amylopectin. Several readily utilizable carbon sources such as glucose, glutamate and other organic N compounds were shown to exert catabolite repression which inhibited enzyme induction by starch or dextrins. Enzyme synthesis occurred both in the exponential and in the stationary growth phase. In the latter the ratio between saccharifying and dextrinizing enzyme activities was high. In the exponential growth phase this ratio depended on the nature of the inducing compound. Growth on starch resulted in an initially high production of dextrinizing activity; subsequently, the saccharifying activity increased and became predominant in the course of exponential growth. In dextrin DE-30 cultures the saccharifying activity was predominant from the very beginning.

It may be concluded that the amylolytic enzyme system of *T. viride* consists of at least two different types of enzymes, the synthesis of each being regulated specifically. The importance of α-amylase in starch degradation by the strain used in this study is questionable, since the main low-molecular product of this enzyme, maltose, is hardly utilizable by the fungus and is not detected after hydrolysis of starch by culture filtrates. The production of the optimum amount of α-amylase is the result of a careful regulation. An excess of α-amylase would liberate maltose from starch resulting in a decreased yield of biomass.

# SUMMARY

The purpose of this investigation was to study the microbiological aspects of the production of microbial protein ('single cell protein'; SCP) from corn waste effluents with simultaneous reduction of the COD of these effluents.

For practical reasons the corn waste water itself was not used in the experiments but a model was chosen, consisting of tap water to which corn steep liquor (CSL) and carbon sources (as a rule glucose) were added.

A fungus was chosen as a model organism because of the composition of the waste stream (high content of starch) and the low COD level of such a stream (ca. 6000 mg/l). The costs of separation of the biomass increase at decreasing concentrations of micro-organisms. Fungi have an advantage over bacteria and yeasts in that fungal mycelium can be separated by relatively simple and cheap filtration techniques.

Chapter 1 gives a survey of literature on SCP and on the production of such proteins from wastes. In particular, attention was given to the use of fungi for this purpose. The production of algae and the use of hydrocarbons as substrate were mentioned only incidentally.

The fungus imperfectus *Trichoderma viride* was chosen as the model organism in the investigations. This choice was based on the following data from literature: a. relatively high specific growth rate; b. high (crude) protein content; c. low optimum pH; d. ability to utilize many (macromolecular) compounds as carbon source; e. no toxic properties of the biomass observed in feed trials.

In chapter 2 a summary is presented of the materials and methods used in the investigations.

Chapter 3 deals with batch experiments in which factors were examined affecting growth rate, yield constant, crude protein content, exhaustion of the medium (COD reduction and nitrogen uptake) etc. As a rule the media contained a COD level of ca. 2500 mg/l in order to ensure a sufficient oxygen supply.

The highest COD reduction rate in CSL glucose media was observed at an initial C/N ratio of 12.6 and an initial pH between 3.5 and 5.5 (Fig. 3.1). The highest COD reduction observed was about 95% (Fig. 3.4). The yield constant $Y$ (mg biomass produced per mg COD reduced), decreased from about 0.5 after initial growth to about 0.4 at exhaustion of the medium (Fig. 3.5). The maximum specific growth rate ($\mu_{max}$) observed was 0.28 $hr^{-1}$.

The crude protein content of the fungus varied from about 20 to 60% (Fig. 3.8.). This content decreased with increasing initial C/N ratio of the medium and with increasing incubation time (Figs. 3.8 and 3.9). More than 80% of the nitrogen present in CSL was taken up from the medium; this figure appeared to be independent of the pH and also of the C/N ratio of the medium if C/N > 9

(Fig. 3.10). At lower C/N ratios a higher percentage of the nitrogen remained in the medium. The nitrogen content of the culture filtrate increased after exhaustion of the medium, but more rapidly at low C/N ratios of the fresh medium than at high C/N ratios.

Decrease of the C/N ratio in the waste stream to an optimum value can better be achieved by the addition of urea or (and) ammonium salts than by addition of CSL, in order to avoid increase of the COD remaining in the stream (Fig. 3.12 and Table 3.2).

The maximum observed specific respiration rate of the fungus was ca. 7 mmol $O_2$/g dry biomass.hr (Fig. 3.11).

The morphology of the fungus depended on a large number of factors such as composition of the growth medium, nature and size of the inoculum, growth conditions and age of the culture.

In chapter 4 the utilization is described of ethanol, lactic acid and acetic acid as carbon sources for *T. viride*. These compounds are present in the waste stream besides glucose and starch.

The highest yield constant ($Y$) was found with ethanol (higher than with glucose) and the lowest with lactic acid (Table 4.1). The $Y$ values found with *T. viride* in the present study were about the same as those reported for *Candida utilis* by HERNANDEZ and JOHNSON in 1967 (Table 4.3). At an initial pH of 4.5, acetic acid appeared to be taken up simultaneously with glucose but before ethanol, while lactic acid was utilized after ethanol (Table 4.2). Lactic acid was consumed very slowly; the remaining COD in culture filtrates from CSL media must probably be attributed to a large extent to lactic acid originating from CSL. Acetic acid caused a strong growth rate inhibiting effect even at very low concentrations (Fig. 4.3). In the presence of acetic acid in concentrations above 7.5 mM and at pH values below 4.5, growth of the fungus in the pellet form was observed. Concentrations of ethanol above ca. 80 mM (0.5%; v/v) had also a toxic effect (Fig. 4.1). A growth rate inhibiting effect of lactic acid could hardly be found, at least at concentrations below 25 mM (Fig. 4.2).

Considering the concentrations of ethanol, acetic acid and lactic acid in corn waste effluents, the growth rate inhibiting effects of these carbon sources can be expected to be nihil, in practice.

Chapter 5 describes the continuous production of mycelium from CSL media, both in a single-stage system and in a single-stream dual-stage system. Culture vessels were used with a capacity of 3 litres.

Although it was impossible to determine the maximum specific growth rate ($\mu_{max}$) exactly because of the effect of wall growth, the value of $\mu_{max}$ was probably not markedly different from that found with batch cultures. The biomass concentrations were calculated from the nitrogen balances because of the badly reproducible sampling of the fermentor contents. At an equal COD level of the fresh medium, the maximum yield of biomass in continuous cultures was lower than that obtained in batch cultures; also the maximum COD reduction ob-

served was somewhat lower than in batch cultures (Fig. 5.1[a–c]). At dilution rates lower than 0.05 $hr^{-1}$ autolysis of mycelium was observed, accompanied by an increased COD of the culture filtrate. At C/N ratios between 10 and 15 the yield constant ($Y$) varied from about 0.51 at $D = 0.24\ hr^{-1}$ to about 0.36 at $D = 0.05\ hr^{-1}$; the crude protein content varied from 40 to 60%. The maximum nitrogen recovery from media with a ratio C/N > 10 was about the same as observed in batch culture. The maintenance coefficient calculated with the equation of PIRT (1965) was 25–30 mg glucose/g biomass.hr (Fig. 5.2). Higher COD levels of the fresh medium did not result in a higher percentage of COD reduction at a dilution rate of 0.05 $hr^{-1}$.

Single-stream dual-stage cultures (Fig. 5.3) were not markedly advantageous to a single-stage system (Table 5.1). In a numerical approach it was shown theoretically that indeed such a dual-stage system is not necessarily advantageous to a single-stage system, and if so, that the second stage must not necessarily have a larger volume than the first one (Tables 5.3; 5.4; 5.5).

The results obtained experimentally in this study may have been highly affected by the small process scale used, especially because of the considerable wall growth.

Chapter 6 deals with the amino acid composition of the fungal biomass and with the nature of the nitrogen compounds in the culture filtrate.

About 80% of the nitrogen present in the biomass was found to be amino acid nitrogen (Table 6.2). In each of the biomass analyses, the proteins were deficient in the sulphur-containing amino acids (methionine and cysteine), independently whether CSL media or mineral media had been used.

Approximately 90% of the nitrogen present in the culture filtrates of CSL media was amino acid nitrogen (Table 6.2) which was for more than 95% present as peptides, and for less than 5% as free amino acids. These peptides were partly present already in the CSL media and were partly excreted by the fungus. Various amino acids, in particular proline, cysteine and histidine, are present in CSL-containing media in much larger amounts than those utilized by the fungus (cf. Tables 2.2 and 6.2). Amino acid nitrogen, largely present as peptides, was also excreted when the fungus was grown on a mineral medium with glucose and urea (Table 6.2).

The last chapter describes the regulation of the synthesis of starch-hydrolysing enzymes by *T. viride*. The specific growth rate on starch (one of the predominant carbon compounds in corn waste effluents) was only slightly lower than that on glucose; maltose was consumed very slowly, accompanied by formation of a slime.

The amylolytic enzyme system appeared to be completely extracellular; it consists mainly of one or more enzymes of the glucoamylase type. Because no maltose was detected by thin layer chromatography, α-amylase seems to play only a minor role. The high ratio between saccharifying and dextrinizing activity of culture filtrates (SA/DA about 1.6), compared to that of a relatively pure α-

amylase preparation obtained from *Aspergillus oryzae* (SA/DA = 0.15), points also to the presence of mainly glucoamylases (see also Fig. 7.2).

The optimum pH and the heat stability of the amylolytic enzymes of *T. viride* were about similar to those of amylolytic enzymes of other fungi (Table 7.2; Figs. 7.3; 7.4; 7.5).

The synthesis of amylolytic enzymes required the presence of starch or dextrins as inducer (Table 7.7; Fig. 7.6). Several readily utilizable carbon sources such as glucose, glutamate and other organic nitrogen compounds were shown to exert catabolite repression (Figs. 7.6; 7.7; 7.8).

Enzyme synthesis occurred both in the exponential and in the stationary growth phase (Fig. 7.1). Growth on starch resulted in an initially high dextrinizing activity; subsequently, the saccharifying activity increased and became predominant in the course of exponential growth (Fig. 7.6.1). In dextrin DE-30 cultures the saccharifying activity was predominant from the very beginning (Fig. 7.6 IV).

It may be concluded that the amylolytic enzyme system of *T. viride* consists of at least two different types of enzymes (the one more saccharifying, the other more dextrinizing), the synthesis of each being regulated specifically.

Since maltose, the main final low-molecular product of α-amylase activity, is hardly utilized by the strain of *T. viride* used in this study, the production of α-amylase has to be carefully regulated by the fungus.

## ACKNOWLEDGEMENTS

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# SAMENVATTING

Het doel van dit onderzoek was de microbiologische aspekten te bestuderen van de produktie van microbieel eiwit ('single cell protein'; SCP) uit afvalstromen van de maiszetmeelindustrie, onder gelijktijdige verlaging van de chemische zuurstofbehoefte ('chemical oxygen demand'; COD) van dit afvalwater.

Om praktische redenen werd in de experimenten niet het afvalwater zelf gebruikt, maar werd een model daarvoor gekozen bestaande uit leidingwater waaraan ingedikt maisweekwater (corn steep liquor; CSL) en koolstofbronnen (in het algemeen glucose) werden toegevoegd.

Als organisme werd een schimmel gekozen in verband met de samenstelling van het afvalwater (relatief hoog zetmeelgehalte) en het lage COD-niveau van een dergelijke afvalstroom (ca. 6000 mg/l). De kosten voor afscheiding van de biomassa nemen toe bij afnemende concentratie van microörganismen. Schimmels hebben het voordeel boven bakteriën en gisten dat mycelium kan worden afgescheiden door middel van relatief eenvoudige en goedkope filtratietechnieken.

Hoofdstuk 1 geeft een literatuuroverzicht over SCP en de produktie ervan op afvalstoffen. In het bijzonder wordt aandacht besteed aan het gebruik van schimmels voor dit doel. De produktie van algen en het gebruik van koolwaterstoffen als substraat worden slechts terloops genoemd.

De schimmel *Trichoderma viride* werd gekozen als modelorganisme voor het onderzoek. Deze keuze is gebaseerd op de volgende literatuurgegevens:
a. relatief hoge specifieke groeisnelheid; b. hoog (ruw) eiwitgehalte; c. lage optimale pH; d. vermogen om vele (macromoleculaire) verbindingen als koolstofbron te benutten; e. geen toxische eigenschappen van de biomassa bekend uit voederproeven.

In hoofdstuk 2 wordt een samenvatting gepresenteerd van de in het onderzoek gebruikte materialen en methoden.

Hoofdstuk 3 beschrijft experimenten met ladingsgewijze cultures ('batch cultures') waarin faktoren werden onderzocht die de groeisnelheid, opbrengstcoëfficient, ruw eiwitgehalte, uitputting van het medium (COD-afname en stikstofafname) etc. beïnvloeden. Gewoonlijk hadden de media een COD-niveau van niet meer dan 2500 mg/l teneinde verzekerd te zijn van voldoende zuurstofvoorziening.

De hoogste snelheid van COD-afname in CSL-glucose-media werd gevonden bij een initiële C/N-verhouding van 12,6 en een initiële pH tussen 3,5 en 5,5 (Fig. 3.1). De hoogst waargenomen COD-afname was ongeveer 95% (Fig. 3.4). De opbrengstcoëfficiënt $Y$ (mg geproduceerde biomassa per mg afgenomen COD) daalde van ongeveer 0,5 in het begin van de groei tot ongeveer 0,4 bij uit-

putting van het medium (Fig. 3.5). De hoogst waargenomen specifieke groeisnelheid ($\mu_{max}$) was 0,28 per uur.

Het ruw-eiwitgehalte van de schimmel varieerde van ongeveer 20% tot 60% (Fig. 3.8). Dit gehalte nam af met toenemende C/N-verhouding van het medium en met toenemende incubatietijd (Figuren 3.8 en 3.9). Meer dan 80% van de in CSL aanwezige stikstof werd opgenomen; dit cijfer bleek onafhankelijk te zijn van de pH en ook van de C/N-verhouding van het medium indien deze verhouding groter was dan 9 (Fig. 3.10). Bij lagere C/N-verhoudingen bleef een hoger percentage stikstof in het medium achter. Het stikstofgehalte van het cultuurfiltraat nam weer toe na uitputting van het medium, en wel des te sneller naarmate de C/N-verhouding van het verse medium lager was.

Verlaging van de C/N-verhouding in het afvalwater tot een optimale waarde kan beter tot stand worden gebracht door toevoeging van ureum of (en) ammoniumzouten dan door toevoeging van CSL, teneinde verhoging van de rest-COD in het afvalwater te voorkomen (Fig. 3.12 en Tabel 3.2).

De hoogste specifieke ademhalingssnelheid van de schimmel was ca. 7 mmol $O_2$/g droge biomassa.uur (Fig. 3.11).

De morfologie van de schimmel was afhankelijk van een groot aantal faktoren, zoals samenstelling van het kweekmedium, aard en omvang van het entmateriaal, kweekomstandigheden en ouderdom van de cultuur.

In hoofdstuk 4 wordt het gebruik beschreven door *T. viride* van ethanol, melkzuur en azijnzuur als koolstofbronnen. Deze verbindingen zijn aanwezig in de afvalstroom naast glucose en zetmeel.

De hoogste opbrengstcoëfficient ($Y$) werd gevonden met ethanol (hoger dan met glucose) en de laagste met melkzuur (Tabel 4.1). De $Y$-waarden die gevonden werden met *T. viride* kwamen ongeveer overeen met die welke werden bepaald voor *Candida utilis* door HERNANDEZ en JOHNSON in 1967 (Tabel 4.3). Uit voedingsoplossingen met verschillende koolstofbronnen bleek bij een begin-pH van 4,5 azijnzuur tegelijk met glucose, doch eerder dan ethanol, te worden opgenomen, terwijl melkzuur het laatst werd benut (Tabel 4.2). Het laatstgenoemde zuur werd zeer langzaam geconsumeerd; de overblijvende COD in cultuurfiltraten van CSL-media moet voor een belangrijk deel worden toegeschreven aan melkzuur dat afkomstig is van CSL. Azijnzuur veroorzaakte reeds in zeer lage concentratie een sterk remmend effect op de groeisnelheid (Fig. 4.3). Bij aanwezigheid van azijnzuur in concentraties hoger dan 7,5 mM werd groei in de 'pellet'-vorm waargenomen, indien de pH lager was dan 4,5. Concentraties ethanol hoger dan 80 mM (0,5%; v/v) hadden eveneens een toxisch effect (Fig. 4.1). Melkzuur had geen duidelijk toxisch effect in concentraties lager dan 25 mM (Fig. 4.2).

De concentraties van ethanol, azijnzuur en melkzuur in aanmerking genomen, zullen de groeiremmende effecten van deze verbindingen in het afvalwater van de maiszetmeelindustrie nihil zijn.

Hoofdstuk 5 beschrijft de continue produktie van mycelium op CSL-media,

zowel in een ééntrapssysteem als in een tweetrapssysteem zonder extra substraattoevoeging in de tweede trap. Er werden cultuurvaten gebruikt met een inhoud van 3 liter.

Hoewel het onmogelijk was de maximale specifieke groeisnelheid exact te bepalen vanwege de invloed van wandgroei, was deze waarde waarschijnlijk niet aanmerkelijk verschillend van die gevonden met ladingsgewijze cultures. De geproduceerde hoeveelheden biomassa werden berekend met behulp van de stikstofbalans vanwege de slecht reproduceerbare monsterneming uit het cultuurvat. Bij een gelijk COD-niveau van het verse medium, was de maximale opbrengst aan biomassa in continu-cultuur lager dan die verkregen met ladingsgewijze cultures; ook de maximaal waargenomen COD-verlaging was iets kleiner dan met ladingsgewijze cultures (Fig. 5.1$^{a-c}$). Bij verdunningssnelheden ($D$) lager dan 0,05 per uur werd autolyse van mycelium waargenomen en een verhoogde COD van het cultuurfiltraat. Bij C/N-verhoudingen tussen 10 en 15 varieerde de opbrengstcoëfficient ($Y$) van ongeveer 0,51 bij $D = 0{,}24$ per uur tot ongeveer 0,36 bij $D = 0{,}05$ per uur; het ruw-eiwitgehalte varieerde van 40 tot 60%. De maximale stikstofopname uit media met een C/N-verhouding groter dan 10 kwam ongeveer overeen met die gevonden in de ladingsgewijze cultuur. De onderhoudscoëfficient, berekend uit de vergelijking van PIRT (1965), was 25–30 mg glucose/g biomassa.uur (Fig. 5.2).

Hogere COD-niveaus van het medium resulteerden niet in hogere percentages COD-afname bij een verdunningssnelheid van 0.05 per uur.

Tweetrapscultures zonder extra substraattoevoer in de tweede trap (Fig. 5.3) hadden geen duidelijk voordeel boven een ééntrapssysteem (Tabel 5.1). In een numerieke benadering werd theoretisch aangetoond dat een dergelijk tweetrapssysteem inderdaad niet noodzakelijk voordeliger is dan een ééntrapssysteem en, als het dat wel is, dat de tweede trap dan niet noodzakelijk een groter volume dient te hebben dan de éérste trap (Tabellen 5.3; 5,4; 5.5).

De in dit onderzoek experimenteel verkregen resultaten kunnen sterk beïnvloed zijn door de kleine schaal waarop de proeven zijn uitgevoerd, in het bijzonder door aanzienlijke wandgroei.

Hoofdstuk 6 beschrijft de aminozuursamenstelling van het mycelium en de aard van de stikstofverbindingen in het cultuurfiltraat.

Ongeveer 80% van de in de biomassa aanwezige stikstof bleek aminozuurstikstof te zijn (Tabel 6.2). In elk van de biomassa-analyses was het eiwit deficiënt met betrekking tot de zwavelhoudende aminozuren (methionine en cysteïne), onafhankelijk ervan of een CSL-medium of een mineraal medium werd gebruikt.

Na zure hydrolyse bleek ongeveer 90% van de in de cultuurfiltraten van CSL-media aanwezige stikstof aminozuurstikstof te zijn (Tabel 6.2). Meer dan 95% van die aminozuurstikstof in de cultuurfiltraten moet worden toegeschreven aan peptiden, minder dan 5% aan vrije aminozuren. De peptiden zullen gedeeltelijk afkomstig zijn geweest van CSL en gedeeltelijk zijn uitgescheiden door de schimmel. Verschillende aminozuren, in het bijzonder proline, cysteïne en histidine zijn

in CSL-bevattende media in veel grotere hoeveelheden aanwezig dan worden benut door de schimmel (Vgl. de tabellen 2.2 en 6.2). Wanneer de schimmel werd gekweekt in een mineraal medium met glucose en ureum werd eveneens aminozuurstikstof uitgescheiden, grotendeels in de vorm van peptiden (Tabel 6.2).

Het laatste hoofdstuk beschrijft de regulatie van de synthese van zetmeelafbrekende enzymen door *T. viride*. De specifieke groeisnelheid op zetmeel (een van de voornaamste koolstofverbindingen in het afvalwater) was slechts weinig lager dan op glucose; maltose werd zeer langzaam benut onder slijmvorming.

Het amylolytisch enzymsysteem bleek volledig extracellulair te zijn; het bestaat in hoofdzaak uit één of meerdere enzymen van het glucoamylase-type. Omdat geen maltose als afbraakprodukt van zetmeel kon worden aangetoond m.b.v. dunnelaagchromatografie, lijkt α-amylase slechts een ondergeschikte rol te spelen. De hoge verhouding tussen saccharogene en dextrinogene activiteit van het cultuurfiltraat (SA/DA ongeveer 1,6), vergeleken met die van een relatief zuivere α-amylase van *Aspergillus oryzae* (SA/DA = 0,15), wijst eveneens op de aanwezigheid van voornamelijk glucoamylases (zie ook Fig. 7.2).

De optimum-pH en de hittestabiliteit van de amylolytische enzymen van *T. viride* waren ongeveer gelijk aan die van amylolytische enzymen van andere schimmels (Tabel 7.2; Figuren 7.3; 7.4; 7.5).

De synthese van amylolytische enzymen door *T. viride* vereist de aanwezigheid van zetmeel of dextrinen als induktor; in afwezigheid van deze verbindingen werd geen enzymsynthese waargenomen (Tabel 7.7; Fig. 7.6). Verschillende gemakkelijk benutbare koolstofbronnen zoals glucose, glutamaat en andere organische stikstofverbindingen bleken katabolietrepressie uit te oefenen (Figuren 7.6; 7.7; 7.8).

Enzymsynthese vond plaats zowel in de exponentiële als in de stationaire groeifase (Fig. 7.1). Groei op zetmeel resulteerde in een initieel hoge dextrinogene activiteit; daarna steeg de saccharogene activiteit en deze werd overheersend in het verloop van de exponentiële groei (Fig. 7.6 I). In cultures met dextrine DE-30 als koolstofbron was de saccharogene activiteit van het begin af aan overheersend (Fig. 7.6 IV).

Geconcludeerd mag worden dat het amylolytische enzymsysteem van *T. viride* uit tenminste twee verschillende typen enzymen bestaat, waarvan de synthese afzonderlijk wordt gereguleerd.

Omdat maltose, het belangrijkste laagmolekulaire eindprodukt van de α-amylase activiteit, bijna niet kan worden benut door de in dit onderzoek gebruikte stam van *T. viride*, moet men aannemen dat de produktie van α-amylase nauwkeurig door de schimmel wordt gereguleerd.

# REFERENCES


ABBOTT, B. J. and A. CLAMEN (1973). The relationship of substrate, growth rate, and maintenance coefficient to single cell protein production. Biotechnol.Bioeng., **15**, 117–127.

ADAMS, M. (1953). Amylases; their kinds and properties and factors which influence their activity. Food Technol., **7**, 35–38.

AIBA, S., M. SHODA and M. NAGATANI (1968). Kinetics of product inhibition in alcohol fermentation. Biotechnol.Bioeng., **10**, 845–864.

ALEXANDER, M. (1965). Introduction to soil microbiology, John Wiley & Sons Inc., New York-London-Sydney.

ALLPORT, N. L., J. W. KEYSER (1957) Colorimetric analysis. Vol. I: Determinations of clinical and biochemical significance. Second edition, London, Chapman & Hall Ltd.

ANDERSON, R. F., R. A. RHODES, G. E. N. NELSON, M. C. SHEKLETON, A. BARRETS and M. ARNOLD (1958). Lysine, methionine and tryptophan content of micro-organisms. I. Bacteria. J. Bacteriol., **76**, 131–135.

ANONYMUS (1960). Standard methods for the examination of water and waste water, 399, Amer.Pub.Health Assoc., New York.

AUBE, C., C. GAGNON (1969). Effect of carbon and nitrogen nutrition on growth and sporulation of *Trichoderma viride* Pers. ex Fries. Can.J.Microbiol., **15**, 703–706.

BARKER, S. B. and W. H. SUMMERSON (1941). The colorimetric determination of lactic acid in biological material. J.Biol.Chem., **138**, 535–554.

BARTON, L. L., D. R. LINEBACK and C. E. GEORGI (1969). The influence of nitrogen and carbon sources on the production of glucoamylase by *Aspergilli*. J.Gen.Appl.Microbiol., **15**, 327–344.

BARTON, L. L., C. E. GEORGI and D. R. LINEBACK (1972). Effect of maltose on glucoamylase formation by *Aspergillus niger*. J.Bacteriol., **111**, 771–777.

BHATTACHARJEE, J. K. (1970). Microorganisms as potential sources of food. Adv.Appl. Microbiol., **13**, 139–161.

BILAI, V. I. (1963). Antibiotic-producing microscopic fungi. Elsevier Publ. Company, Amsterdam-London-New York.

BISBY, G. R. (1939). *Trichoderma viride* Pers. ex Fries, and notes on *Hypocrea*. Trans.Brit. Mycol.Soc., **23**, 149–168.

BLUMENTHAL, H. J. and S. ROSEMAN (1957). Quantitative estimation of chitin in fungi. J. Bacteriol., **74**, 222–224.

BUNKER, H. J. (1963). Microbial food. In 'Biochemistry of industrial microorganisms', pp. 34–67. Eds.: C. Rainbow and A. H. Rose, Acad. Press, New York-London.

CARTER, B. L. A. and A. T. BULL (1969). Studies of fungal growth and intermediary carbon metabolism under steady and non-steady state conditions. Biotechnol.Bioeng., **11**, 785–804.

CASTRO, A. C., A. J. SINSKEY and S. R. TANNENBAUM (1971). Reduction of nucleic acid content in *Candida* yeast cells by bovine pancreatine ribonuclease, a treatment. Appl. Microbiol., **22**, 422–427.

CHOUDHARY, A. Q. and S. J. PIRT (1965). Metal-complexing agents as metal buffers in media for growth of *Aspergillus niger*. J.Gen.Microbiol., **41**, 99–107.

CHURCH, B. D., H. A. NASH and W. BROSZ (1972). Use of fungi imperfecti in treating food processing wastes. Dev.Ind.Microbiol., **13**, 30–46.

COCHRANE, V. W. (1958). 'Physiology of fungi'. New York – John Wiley & Sons, Inc. London – Chapman & Hall Ltd.

COLLINS, R. P. and A. F. HALIM (1972). Characterization of the major aroma constituent of the fungus *Trichoderma viride*. J.Agr.Food Chem., **20**, 437–438.

COOPER, C. M., G. A. FERNSTROM and S. A. MILLER (1944). Performance of agitated gas-liquid contactors. Ind.Eng.Chem., **36**, 504–509.

DANIELSON, R. M. and C. B. DAVEY (1973a). The abundance of *Trichoderma* propagules and the distribution of species in forest soils. Soil Biol.Biochem., **5**, 485–494.

DANIELSON, R. M. and C. B. DAVEY (1973b). Non-nutritional factors affecting the growth of *Trichoderma* in culture. Soil Biol.Biochem., **5**, 495–504.
DANIELSON, R. M. and C. B. DAVEY (1973c). Carbon and nitrogen nutrition of *Trichoderma*. Soil Biol.Biochem., **5**, 505–515.
DAWES, E. A. and D. W. RIBBONS (1964). Some aspects of the endogenous metabolism of bacteria. Bacteriol.Rev., **28**, 126–149.
DELANEY, R. A. M. and J. T. WORGAN (1970). The production of protein foods by the mycelial growth of fungi in culture media prepared from plant waste products. Int.Congr. Food Sci.Technol., **3**, Washington, D. C. Abstr. p. 113.
DEMAIN, A. L. (1968). Production of purine nucleotides by fermentation. Progr.Ind.Microbiol., **8**, 35–72.
DOCTOR, V. M. and L. KERUR (1968). *Penicillium* mycelium waste as protein supplement in animals. Appl.Microbiol., **16**, 1723–1726.
EBERTOVA, H. (1966a). Amylolytic enzymes of *Endomycopsis capsularis*. I. Formation of the amylolytic system in cultures of *Endomycopsis capsularis*. Folia Microbiol., **11**, 14–20.
EBERTOVA, H. (1966b). Amylolytic enzymes of *Endomycopsis capsularis*. II. A study of the properties of isolated α-amylase, amyloglucosidase and maltase-transglucosidase. Folia Microbiol., **11**, 422–438.
EDWARDS, V. H. (1970). The influence of high substrate concentrations on microbial kinetics. Biotechnol.Bioeng., **12**, 679–712.
EDWARDS, V. H., M. J. GOTTSCHALK, A. Y. NOOJIN, III, L. B. TUTHILL and A. L. TANNAHILL (1970). Extended culture: The growth of *Candida utilis* at controlled acetate concentrations. Biotechnol.Bioeng., **12**, 975–999.
EGERAAT, A. W. S. M. VAN (1972). Pea-root exudates and their effect upon root-nodule bacteria. Thesis Agr. University, Wageningen, The Netherlands.
ELSWORTH, R., G. A. MILLER, A. R. WHITAKER, D. KITCHING and P. D. SAYER (1968). Production of *Escherichia coli* as a source of nucleic acids. J.Appl.Chem., **17**, 157–166.
EMERSON, S. (1950). The growth phase in *Neurospora* corresponding to the logarithmic phase in unicellular organisms. J.Bacteriol., **60**, 221–223.
EVANS, E. (1955). Survival and recolonization by fungi in soil treated with formalin or carbon disulphide. Trans.Brit.Mycol.Soc., **38**, 335–346.
FALANGHE, H., A. K. SMITH and J. J. RACKIS (1964). Production of fungal mycelial protein in submerged culture of soybean whey. Appl.Microbiol., **12**, 330–334.
FAN, L. T., L. E. ERICKSON, P. S. SHAH and B. I. TSAI (1970). Effect of mixing on the washout and steady-state performance of continuous cultures. Biotechnol.Bioeng., **12**, 1019–1068.
F.A.O. (1957). Protein requirements. Report of the FAO Committee, Rome. Food Agr.Organ. U.N.
F.A.O. (1964). Protein. World Food Problems, number 5, p. 13. Rome. Food Agr.Organ. U.N.
F.A.O. (1968). Amino acid content of foods and biological data on proteins. Food Agr. Organ.U.N., Rome.
FENCL, Z. (1969). Production of microbial protein from carbon sources. In 'Global impacts of applied microbiology II', pp. 63–70. Biotechnol.Bioeng.Symp. I. Ed.: E. L. Gaden, Jr. Interscience Publishers.
FENCL, Z. and M. BURGER (1958). Some aspects of continuous culture of food yeast. In 'Continuous culture of micro-organisms', p. 165. 1st Intern.Symp. on the continuous culture of micro-organisms. Ed.: I. Malek, Publishing House of the Czechoslovak Academy of Science, Prague.
FENCL, Z. and J. LEOPOLD (1959). The inhibition of spore germination of the mould *Aspergillus niger* by acetic acid. Folia Microbiol., **4**, 7–15.
FENCL, Z., J. ŘIČICA and J. KODEŠOVÁ (1972). The use of the multi-stage chemostat for microbial product formation. J.Appl.Chem.Biotechnol., **22**, 405–416.
FODA, M. S., S. A. SALEM, S. M. HEGAZI and S. M. BADR-ELDIN (1973). Biochemical studies for utilization of corn steep liquor in production of proteins. J.Sci.Fd.Agr., **24**, 17–22.
FOSTER, J. W. (1949). Chemical activities of fungi. New York, Acad. Press.

GHOSE, T. K. (1969). Continuous enzymatic saccharification of cellulose with culture filtrates of *Trichoderma viride* QM6a. Biotechnol.Bioeng., **11**, 239–261.
GHOSE, T. K. and M. MANDELS (1970). Enzymatically hydrolyzed cellulose as a substrate for single cell protein. X Int.Congr.Microbiol., Abstracts, p. 215, Mexico, D.F., Mexico.
GRAY, W. D. (1962). Microbial protein for the space age. Dev. Ind.Microbiol., **3**, 63–71.
GRAY, W. D. (1966). Microorganisms as potential food sources. Dev. Ind.Microbiol., **7**, 203–228.
GRAY, W. D. (1970) The use of fungi as food and in food processing I. Critical Rev. Food Technol., 1 (2) The Chemical Rubber Co., London-Butterworths.
GRAY, W. D. and M. ABOU-EL-SEOUD (1966). Fungi imperfecti as sources of protein. In: 'Micro-organisms as potential food sources'. Dev.Ind.Microbiol., **7**, 221–225.
GRAY, W. D., F. F. OCH and M. ABOU-EL-SEOUD (1964). Fungi imperfecti as a potential source of edible protein. Dev.Ind.Microbiol., **5**, 384–389.
GRIFFIN, P. J. and W. M. FOGARTY (1973). Production of an amylolytic enzyme by *Bacillus polymyxa* in batch cultures. J.Appl.Chem.Biotechnol., **23**, 301–308.
HALL, H. H. (1962). Applied microbiology in animal nutrition. Adv.Appl.Microbiol., **4**, 77–99.
HANSFORD, G. S. and A. E. HUMPHREY (1966). The effect of equipment scale and degree of mixing on continuous fermentation yield at low dilution rates. Biotechnol.Bioeng., **8**, 85–96.
HARRISON, J. S. (1967). Aspects of commercial yeast production. Process Biochem., **2**, 41–45.
HASEGAWA, S. and J. H. NORDIN (1969). Enzymes that hydrolyze fungal cell wall polysaccharides. I. Purification and properties of an endo α-D-(1→3)-glucanase from *T. viride*. J. Biol.Chem., **244**, 5460–5470.
HEDÉN, C. G., N. MOLIN, U. OLSEN and A. RUPPRECHT (1971). Preliminary experiments on spinning bacterial proteins into fibres. Biotechnol.Bioeng., **13**, 147–150.
HEDENSKOG, G., H. MOGREN (1973). Some methods for processing of single cell protein. Biotechnol.Bioeng., **15**, 129–142.
HEDENSKOG, G., H. MOGREN and L. ENEBO (1970). A method for obtaining protein concentrates from microorganisms. Biotechnol.Bioeng., **12**, 947–959.
HERBERT, D., R. ELSWORTH and R. C. TELLING (1956). The continuous culture of bacteria: a theoretical and experimental study. J.Gen.Microbiol., **14**, 601–622.
HERNANDEZ, E. and M. J. JOHNSON (1967). Energy supply and cell yield in aerobically grown micro-organisms. J.Bacteriol., **94**, 996–1001.
HESSELTINE, C. W. and H. W. A. L. WANG (1967). Traditional fermented foods. Biotechnol. Bioeng., **9**, 275–288.
HODGE, J. E. and B. T. HOFREITER (1962). Determination of reducing sugars and carbohydrates. In 'Methods in carbohydrate chemistry' I [115], 380–390. Eds.: R. L. Whistler and M. L. Wolfrom, Acad.Press, New York.
HOSPODA, J. (1966). Industrial application of continuous fermentation. In: 'Continuous culture of microorganisms', p. 443. Eds.: I. Malek and Z. Fencl. Acad.Press. New York-London.
HOU, C. T., A. CIEGLER and C. W. HESSELTINE (1972). New mycotoxin, Trichotoxin A, from *T. viride*, isolated from southern leaf blight-infected corn. Appl.Microbiol., **23**, 183–185.
HOWELL, J. A., C. T. CHI and U. PAWLOWSKY (1972). Effect of wall growth on scale up problems and dynamic operating characteristics of the biological reactor. Biotechnol. Bioeng., **14**, 253–265.
HUANG, F. and C. K. RHA (1971). Rheological properties of single cell protein concentrate: dope formation and its flow behaviour. J.Food Sci., **36**, 1131–1134.
JARVIS, F. G. and M. J. JOHNSON (1947). The role of the constituents of synthetic media for penicillin production. J.Am.Chem.Soc., **69**, 3010–3017.
KAMPF, G. und U. BEHRENS (1966). Über die Assimilation der in Melasseschlempe vorkommenden organischen Säuren. Z.Allg.Mikrobiol., **6**, 237–244.

KATZ, M. and E. T. REESE (1968). Production of glucose by enzymatic hydrolysis of cellulose. Appl.Microbiol., **16**, 419–420.
KIHLBERG, R. (1972). The microbe as a source of food. Ann.Rev.Microbiol., **26**, 427–466.
KINOSHITA, S., O. TERADA and K. OISHI (1961). Method of producing citric acid by fermentation. U.S.Patent office, 2, 993, 838.
KOBAYASHI, T. (1971). Hydrolysis of pullulan by a novel enzyme from *Aspergillus niger*. Agr. Biol.Chem., **35**, 971–973.
KOBAYASHI, T., G. VAN DEDEM and M. MOO-YOUNG (1973). Oxygen transfer into mycelial pellets. Biotechnol.Bioeng., **15**, 27–45.
KUNDU, A. K. and S. DAS (1970). Production of amylase in liquid culture by a strain of *Aspergillus oryzae*. Appl.Microbiol., **19**, 598–603.
LIGGET, R. W. and H. KOFFLER (1948). Corn steep liquor in microbiology. Bacteriol.Rev., **12**, 297–311
LINEBACK, D. R. and W. E. BAUMANN (1970). Properties of a glucoamylase from *Aspergillus phoenicus*. Carbohydr.Res., **14**, 341–353.
LINEBACK, D. R., C. E. GEORGI and R. L. DOTY (1966). Glucoamylase (α-1,4-glucan glucohydrolase) production by *Aspergillus niger* as influenced by medium composition. J.Gen. Appl.Microbiol., **12**, 27–38.
LIPPINSKY, E. S. and J. H. LITCHFIELD (1970). Algae, bacteria and yeasts as food or feed. Critical Rev.Food Technol., **1**, 581–618.
LITCHFIELD, J. H. (1964). The mass cultivation of *Morchella* species in submerged culture and their potential uses as sources of protein. In: 'Global impacts of applied microbiology', Ed.: M. P. Starr. Almqvist & Wiksell, Stockholm-Göteborg-Uppsala. John Wiley & Sons, Inc. New York-London-Sydney.
LITCHFIELD, J. H. (1967a). Submerged culture of mushroom mycelium. In: 'Microbial Technology', pp. 107–144. Ed.: H. J. Peppler. Reinhold Publ. Corporation, New York-Amsterdam-London.
LITCHFIELD, J. H. (1967b). Morel mushroom mycelium as a food-flavouring material. Biotechnol.Bioeng., **9**, 289–304.
LITCHFIELD, J. H. (1968) The production of fungi. In: 'Single-Cell Protein' pp. 309–329. Eds.: R.I. Mateles and S. R. Tannenbaum. M. I. T. Press, Cambridge, Massachusetts.
LITCHFIELD, J. H., R. C. OVERBEEK and R. S. DAVIDSON (1963). Mushroom culture. Factors affecting the growth of morel mushroom mycelium in submerged culture. J.Agr. Food Chem., **11**, 158–162.
MAHLOCH, J. L. (1972). Fungi present during the aerobic decomposition of selected solid-waste substrates. Dev.Ind.Microbiol., **13**, 308–316.
MANDELS, M. and E. T. REESE (1957). Induction of cellulase in *Trichoderma viride* as influenced by carbon sources and metals. J.Bacteriol., **73**, 269– 278.
MANDELS, M., J. WEBER and R. PARIZEK (1971). Enhanced cellulase production by a mutant of *Trichoderma viride*. Appl.Microbiol., **21**, 152–154.
MANDELS, M., L. HONTZ and J. NYSTROM (1974). Enzymatic hydrolysis of waste cellulose. Biotechnol.Bioeng., **16**, 1471–1493.
MARSHALL, J. J. (1973). Inhibition of pullulanase by Schardinger dextrins. FEBS Letters, **37**, 269–273.
MATELES, R. I. and S. R. TANNENBAUM (1968). Single-Cell Protein. M.I.T. Press, Cambridge, (Mass.Inst.Technol.), Massachusetts.
MAUL, S. B., A. J. SINSKEY and S. R. TANNENBAUM (1970). New process for reducing the nucleic acid content of yeast. Nature, **228**, 181.
MAXON, W. D. (1955). Microbiological Process Report. Continuous fermentation. A discussion of its principles and applications. Appl.Microbiol., **3**, 110–122.
MCINTOSH, A. F. and J. MEYRATH (1963). Size of inoculum and nitrogen metabolism in *Aspergillus oryzae*. J.Gen.Microbiol., **33**, 57–62.
MENEZES, H. C. DE, T. J. B. DE MENEZES and H. V. BOAS Jr. (1973). Fungal cultures and culture mediums for the production of cellulase. Biotechnol.Bioeng., **15**, 1123–1129.

MEYER, C. E. (1966). U-21,963, a new antibiotic. II Isolation and characterization. Appl. Microbiol., **14**, 511–512.
MEYRATH, J. (1966) Fungal amylase production. Process Biochem., **1**, 234–238.
MEYRATH, J. and A. F. MCINTOSH (1963). Size of inoculum and carbon metabolism in some *Aspergillus* species. J.Gen.Microbiol., **33**, 47–56.
MEYRATH, J. and E. ROTH (1973). Self-inhibiting extracellular proteins from *Aspergillus oryzae*. Experientia, **29**, 1168–1171.
MILLER, D. S. (1968). Results published by J. T. Worgan (1973a).
MINODA, Y. and K. YAMADA (1963). Acid-stable α-amylase of black *Aspergilli*. Part 1. Detection and purification of acid-stable dextrinizing amylase. Agr.Biol.Chem., **27**, 806–811.
MITRA, G. and C. R. WILKE (1975). Continuous cellulase production. Biotechnol.Bioeng., **17**, 1–13.
MIZUNUMA, T. (1963). Studies on the metabolism of *Aspergilli*. Part 1. Substrates of endogenous respiration. Agr.Biol.Chem., **27**, 88–98.
MONOD, J. (1942). Recherches sur la croissance des cultures bactériennes, deuxième édition, 1959, Hermann, Paris.
MONOD, J. (1950). La technique de culture continue, théorie et applications. Ann.Inst. Pasteur, **79**, 390–410.
MOORE, S. and W. H. STEIN (1954). Procedures for the chromatographic determination of amino acids on four per cent cross-linked sulfonated polystyrene resins. J.Biol.Chem., **211**, 893.
MORTON, A. G. and D. BROADBENT (1955). The formation of extracellular nitrogen compounds by fungi. J.Gen.Microbiol., **12**, 248–258.
MOUBASHER, A. H. (1963). Selective effects of fumigation with $CS_2$ on the soil fungus flora. Trans.Brit.Mycol.Soc., **46**, 338–344.
NELSON, G. E. N., R. F. ANDERSON, R. A. RHODES, M. C. SHEKLETON and H. H. HALL (1960). Lysine, methionine and tryptophan content of microorganisms. II. Yeasts. Appl.Microbiol., **8**, 179–182.
NG, A. M. L., J. E. SMITH and A. F. MCINTOSH (1973). Conidiation of *Aspergillus niger* in continuous culture. Arch.Mikrobiol., **88**, 119–126.
OHGA, M., K. SHIMIZU and Y. MORITA (1966). Studies on amylases of *Aspergillus oryzae* cultured on rice. Part II. Some properties of glucoamylases. Agr.Biol.Chem., **30**, 967–972.
OOKA, T., Y. SHIMOJIMA, T. AKIMOTO, I. TAKEDA, S. SENOH and J. ABE (1966). A new antibacterial peptide 'Suzukacillin'. Agr.Biol.Chem., **30**, 700–702.
OSER, B. L. (1968). Evaluation of the safety of new food products. In: 'Single-Cell Protein', pp. 153–162. Eds.: R. I. Mateles and S. R. Tannenbaum. M.I.T. Press, Cambridge, Massachusetts.
PALUMBO, S. A. and L. D. WITTER (1969). Influence of temperature on glucose utilization by *Pseudomonas fluorescens*. Appl.Microbiol., **18**, 137–141.
PARK, D. (1965). Survival of microorganisms in soil. In: 'Ecology of soil-borne pathogens-prelude to biological control', pp. 82–97. Eds.: K. F. Baker and W. C. Snyder, Univ. of California Press, Berkeley, California.
PATHAK, S. G. and R. SESHADRI (1965). Use of *Penicillium chrysogenum* mycelium as animal food. Appl.Microbiol., **13**, 262–266.
PAZUR, J. H. and T. ANDO (1960). The hydrolysis of glucosyl oligosaccharides with α-D-(1→4) and α-D-(1→6) bonds by fungal amyloglucosidase. J.Biol.Chem., **235**, 297–302.
PEITERSEN, N. (1975). Production of cellulase and protein from barley straw by *Trichoderma viride*. Biotechnol.Bioeng., **17**, 361–374.
PEPPLER, H. J. (1967). Yeast Technology. In: 'Microbial Technology', pp. 145–171. Ed.: H. J. Peppler. Reinhold Publ.Corporation, New York-Amsterdam-London.
PEPPLER, H. J. (1970). Food Yeasts. In: 'The Yeasts' III Yeast Technology, pp. 421–462. Eds.: A. H. Rose and J. S. Harrison. Acad.Press, London-New York.
PHILLIPS, D. H. (1966). Oxygen transfer into mycelial pellets. Biotechnol.Bioeng., **8**, 456–460.
PIRT, S. J. (1965). The maintenance energy of bacteria in growing cultures Proc.Roy.Soc. B., **163**, 224–231.

PIRT, S. J. (1967). A theory of the mode of growth of fungi in the form of pellets in submerged culture. Proc.Roy.Soc.B, **166**, 369–373.

PIRT, S. J. (1972). Introductory lecture. Prospects and problems in continuous flow culture of micro-organisms. J.Appl.Chem.Biotechnol., **22**, 55–64.

PIRT, S. J. and D. S. CALLOW (1958). The relationship between maximum oxygen solution rates in a bacterial culture and in a sodium sulphite solution under comparable aeration conditions. J.Appl.Bacteriol., **21**, 206-210.

PIRT, S. J. and D. S. CALLOW (1959). Continuous-flow culture of the filamentous mould *Penicillium chrysogenum* and the control of its morphology. Nature, **184**, 307–310.

PIRT, S. J. and D. S. CALLOW (1960). Studies of the growth of *Penicillium chrysogenum* in continuous flow culture with reference to penicillin production. J.Appl.Bacteriol., **23**, 87–98.

PÖHLAND, D., M. RINGPFEIL und U. BEHRENS (1966). Die Assimilation von Glucose, Xylose und Essigsäure durch *Candida utilis*. Z.Allg. Mikrobiol., **6**, 387–395.

PONTANEL, H. G. GOUNELLE DE (1972). Protein from hydrocarbons. Symp.Aix en Provence. Acad.Press, London.

PORGES, N., J. B. PEPINSKY, N. C. HENDLER and S. R. HOOVER (1950). Biochemical oxidation of dairy wastes. I. Methods of study. Sewage and Ind.Wastes, **22**, 318–325.

PRINGSHEIM, H. und S. LICHTENSTEIN (1920). Versuche zur Anreicherung von Kraftstroh mit Pilzeiweiss. Cellulosechemie, **1**, 29–39.

PYKE, T. R. and A. DIETZ (1966). U-21,963, a new antibiotic. I. Discovery and biological activity. Appl.Microbiol., **14**, 506–510.

RAGHEB, H. S. and F. W. FABIAN (1955). Growth and pectolytic activity of some tomato molds at different pH levels. Food Res., **20**, 614–625.

REICHELT, J. L. and H. W. DOELLE (1971). The influence of dissolved oxygen concentration on phosphofructokinase and the glucose metabolism of *Escherichia coli* K-12. Antonie van Leeuwenhoek, **37**, 497–506.

REISER, C. O. (1954). Food Yeast. *Torula* yeast from potato starch wastes. J.Agr.Food Chem., **2**, 70–74.

RHODES, A. and D. L. FLETCHER (1966). Principles of Industrial Microbiology, Pergamon Press, Oxford.

RHODES, R. A., H. H. HALL, R. F. ANDERSON, G. E. N. NELSON, M. C. SHEKLETON and R. W. JACKSON (1961). Lysine, methionine and tryptophan content of microorganisms. III Moulds. Appl.Microbiol., **9**, 181–184.

ŘIČICA, J. (1970). Multi-stage systems. In: 'Methods in Microbiology' vol. 2, pp. 329–348. Eds.: J. R. Norris and D. W. Ribbons. Acad.Press, London-New York.

RIGHELATO, R. C. and S. J. PIRT (1967). Improved control of organism concentration in continuous cultures of filamentous microorganisms. J.Appl.Bacteriol., **30**, 246–250.

RIGHELATO, R. C., A. P. J. TRINCI and S. J. PIRT (1968). The influence of maintenance energy and growth rate on the metabolic activity, morphology and conidiation of *Penicillium chrysogenum*. J.Gen.Microbiol., **50**, 399–412.

SAKSENA, S. B. (1960). Effect of carbon disulphide fumigation on *Trichoderma viride* and other soil fungi. Trans.Brit.Mycol.Soc., **43**, 111-116.

SAMSON, F. E., A. M. KATZ and D. L. HARRIS (1955). Effects of acetate and other short chain fatty acids on yeast metabolism. Arch.Biochem.Biophys., **54**, 406–423.

SCHAECHTER, M., O. MAALØE and N. O. KJELDGAARD (1958). Dependency on medium and temperature of cell size and chemical composition during balanced growth of *Salmonella typhimurium*. J.Gen.Microbiol., **19**, 592–606.

SCHLENK, F. and J. L. DAINKO (1965). Action of ribonuclease preparations on viable yeast cells and spheroplasts. J. Bacteriol., **89**, 428–436.

SCHULZE, K. L. and R. S. LIPE (1964). Relationship between substrate concentration, growth rate and respiration rate of *Escherichia coli* in continuous culture. Arch. Mikrobiol., **48**, 1–20.

SIMPSON, F. J. (1959). Microbial pentosanases. III Some factors affecting the production of pentosanases by *Aspergillus niger* and *Trichoderma viride*. Can.J.Microbiol., **5**, 99–107.

SLUMP, P. (1969). Karakterisering van de voedingswaarde van eiwitten in voedingsmiddelen door hun aminozuursamenstelling en de invloed van verhitting en loogbehandeling op de benutbaarheid van aminozuren (with a summary in English). Thesis, University of Amsterdam (V.U.).

SNYDER, H. E. (1970). Microbial sources of protein. Adv.Food Res., **18**, 85–140.

SOLOMONS, G. L. (1967). Anti-foams. Process Biochem., **2**, 47–48.

SOLOMONS, G. L. (1969). Materials and methods in fermentation. Acad.Press, London-New York.

SOLOMONS, G. L. (1973). Fungal protein, In: 'Food from fungi', Microbiology Group Symposium March 1972, J.Sci.Food Agric., **24**, 637-639.

SOMOGYI, M. (1952). Notes on sugar determination. J.Biol.Chem., **195**, 19–23.

SPAANDER, P. en R. PRINS (1965). Bepaling van de oxydeerbaarheid van afvalwater volgens de kalium dichromaatmethode. Water, **49**, 48–51.

STEEL, R. (1958). Substrates for fermentation processes. In: 'Biochemical Engineering', pp. 65–94. Ed.: R. Steel, Heywood & Company Ltd., London.

THATCHER, F. S. (1954). Foods and feeds from fungi. Ann.Rev.Microbiol., **8**, 449–472.

TONOMURA, K., H. SUZUKI, N. NAKAMURA, K. KURAYA and O. TANABE (1961). On the inducers of α-amylase formation in *Aspergillus oryzae*. Agr.Biol.Chem., **25**, 1–6.

TONOMURA, K., K. IWAMA, F. FUTAI, O. TANABE (1963). Cell bound α-amylase in *Aspergillus oryzae*. Part III. Fixation of exocellular α-amylase to mycelia. Agr.Biol.Chem., **27**, 128–132.

TVEIT, M. (1967). Industrial aspects of microbial food production with special reference to the potential of the new Symba-Yeast process. In: 'Biology and the manufacturing industries'. Inst. of biology. Symp.number 16, pp. 3–10. Ed.: M. Brook, Acad.Press, London/New York.

UPDEGRAFF, D. M. (1971). Utilization of cellulose from waste paper by *Myrothecium verrucaria*. Biotechnol.Bioeng., **13**, 77–97.

UPDEGRAFF, D. M., R. ESPINOSA, L. GRIFFIN and J. KING (1973). The production of animal feed by the growth of fungi on wastes from the coffee and rum-distilling industries. Dev. Ind.Microbiol., **14**, 317–324.

VRIES, O. M. H. DE and J. G. H. WESSELS (1973). Release of protoplasts from *Schizophyllum commune* by combined action of purified α-1,3-glucanase and chitinase derived from *Trichoderma viride*. J.Gen.Microbiol., **76**, 319–330.

WANG, D. I. C. (1968). Cell recovery. In: 'Single-Cell Protein', pp. 217–228. Eds.: R. I. Mateles and S. R. Tannenbaum, M.I.T. Press, Cambridge, Massachusetts.

WATSON, T. G. (1972). The present status and future prospects of the turbidostat. J.Appl. Chem.Biotechnol., **22**, 229–243.

WEINDLING, R. (1932). *Trichoderma lignorum* as a parasite of other soil fungi. Phytopathol., **22**, 837–845.

WEINDLING, R. (1937). Isolation of toxic substances from the culture filtrates of *Trichoderma* and *Gliocladium*. Phytopathol., **27**, 1175–1177.

WEINDLING, R. and O. H. EMERSON (1936). The isolation of a toxic substance from the culture filtrate of a *Trichoderma*. Phytopathol., **26**, 1068–1070.

WELKER, N. E. and L. L. CAMPBELL (1963a). Effect of carbon sources on formation of α-amylase by *Bacillus stearothermophilus*. J.Bacteriol., **86**, 681–686.

WELKER, N. E. and L. L. CAMPBELL (1963b). Induction of α-amylase of *Bacillus stearothermophilus* by maltodextrins. J.Bacteriol., **86**, 687–691.

WILEY, A. J. (1954). Food and feed yeast. In: 'Industrial fermentations', vol I, pp. 307–343. Eds.: L. A. Underkofler and R. J. Hickey, Chemical Publishing Company, Inc., New York.

WILKINSON, J. F. (1971). Hydrocarbons as a source of single-cell protein. In: 'Microbes and biological productivity', Symp.Soc.Gen.Microbiol., **21**, 15–46. Eds.: D. E. Hughes and A. H. Rose, Cambridge University Press.

WILLIMON, E. P. and J. F. ANDREWS (1969). Multi-stage biological processes for waste treatment. J.Water Poll.Contr.Fed., **41**, 99–112.

WOLDENDORP, J. W. (1970). Het gebruik van industrieel afvalwater voor de produktie van 'Single Cell Protein'. Rapport D.S.M., Central Laboratory, Geleen, The Netherlands, Aug. 1970.
WORGAN, J. T. (1968). Culture of the higher fungi. Progr.Ind.Microbiol., 8, 74–139.
WORGAN, J. T. (1971). The production of protein foods by the growth of fungal mycelium in submerged culture. 1st Int.Congr. of Mycology. Abstr. p. 106, Exeter, U.K., Sept. 1971.
WORGAN, J. T. (1972). Utilization of agricultural and food industry wastes by microbiological processes. In: 'Symposium on by-products of the food industry', pp. 11–26, Oct. 1972. The British food manufacturing industries research association.
WORGAN, J. T. (1973a). Protein production by micro-organisms from carbohydrate substrates. In: 'The biological efficiency of protein production', pp. 339–361. Ed.: J. G. W. Jones, Cambridge University Press.
WORGAN, J. T. (1973b). World supplies of proteins from unconventional sources. In: 'Proteins in human nutrition', pp. 47–74. Eds.: J. W. G. Porter and B. A. Rolls, Acad.Press.
WRIGHT, J. M. (1956). Biological control of a soil-borne *Pythium* infection by seed inoculation. Plant and Soil, 8, 132–140.
YAMADA, K., J. TAKAHASHI, Y. KAWABATA, T. OKADA and T. ONIHARA (1968). SCP from yeast and bacteria grown on hydrocarbons. In: 'Single-Cell Protein', pp. 192–207. Eds.: R. I. Mateles and S. R. Tannenbaum. M.I.T. Press, Cambridge, Massachusetts.
YANO, T., T. KODAMA and K. YAMADA (1961). Fundamental studies on the aerobic fermentation. Part VIII. Oxygen transfer within a mold pellet. Agr.Biol.Chem., 25, 580–584.
ZEVENHUIZEN, L. P. T. M. (1966). Function, structure and metabolism of the intracellular polysaccharide of *Arthrobacter*. Thesis, University of Amsterdam (G.U.).

# LIST OF SYMBOLS AND ABBREVIATIONS

| Symbol/ abbreviation | Expressed as | Definition |
|---|---|---|
| COD | $mg.l^{-1}$ | Chemical oxygen demand. Units mass of oxygen required for chemical oxidation of a unit volume of substrate |
| CSL | | Corn steep liquor |
| C/N ratio | | Mass ratio carbon to nitrogen |
| $D$ | $hr^{-1}$ | Dilution rate. Units volume of inflowing medium per unit volume of culture medium in the chemostat per unit time |
| DA (spec) | | (Specific) dextrinizing activity of the amylolytic enzymes. |
| DE | | Dextrose equivalents; the figure after DE indicates how many grams of glucose have the same number of reducing equivalents as 100 grams of the dextrin |
| $F$ | $l.hr^{-1}$ | Flow rate. Units volume of inflowing medium in the chemostat per unit time |
| $K_s$ | $g.l^{-1}$ | Michaelis constant of growth-limiting substrate with respect to the specific growth rate in the growth rate equation of MONOD (1942) |
| $m$ | $hr^{-1}$ | Maintenance coefficient. Units mass of energy source consumed for maintenance per unit of biomass per unit time |
| NA | | Nucleic acids |
| OD | $mmol.g^{-1}.hr^{-1}$ | Oxygen demand of the growing microorganism. Units mass of oxygen consumed per unit dry weight of biomass per unit time |
| OTR | $mmol.l^{-1}.hr^{-1}$ | Oxygen transfer rate. Units mass of oxygen dissolved per unit volume per unit time, measured by the sulphite method of COOPER et al. (1944) |
| $S$ | $g.l^{-1}$ | Concentration of growth-limiting substrate (in general or in the chemostat) |
| $S_r$ | $g.l^{-1}$ | Concentration of growth-limiting substrate in fresh medium flowing into the chemostat |

| | | |
|---|---|---|
| SA (spec) | | (Specific) saccharifying activity of the amylolytic enzymes |
| SCP | | Single cell protein |
| $t$ | hr | Time |
| $t_d$ | hr | Doubling time. Time in which the biomass has been doubled |
| $V$ | l | Working volume of a chemostat |
| $X$ | $g.l^{-1}$ | Mycelium concentration. Units of biomass per unit volume of culture |
| $Y$ | | Yield constant. Units of biomass formed per unit mass of limiting substrate (energy source or not) consumed |
| $Y_G$ | | True (maximum) growth yield constant Units of biomass theoretically formed per unit mass of energy source consumed, if no energy were required for maintenance |
| $Y_{sub}$ | | Total or observed growth yield constant. Units of biomass formed per unit mass of energy source consumed |
| $\mu$ | $hr^{-1}$ | Specific growth rate. Units of biomass formed per unit of biomass present per unit time |
| $\mu_{max}$ | $hr^{-1}$ | Maximum specific growth rate. Specific growth rate at saturating concentrations of the growth-limiting substrate |