PROCESS DEVELOPMENT FOR MICROBIAL TRANSGLUTAMINASE PRODUCTION

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STELLINGEN

1. It is much more difficult to let the world know China than to let China know the world.
2. The Chinese equivalent of the word 'crisis' is composed of two symbols: danger and opportunity. In reality, this holds for most crises.
3. Periodic evaluation of the driving ability of car drivers is likely more effective than the 'APK' in furthering traffic safety.
4. Een bus 'Buiten dienst' bereikt zijn doel sneller dan een bus 'In dienst'.
5. An international standard for name order, i.e. surname and given name, is much needed. Most people in the West have no notion of the name order of people in the East, and *vice versa*. The result can be surprising: President Bill or Mr. Zhonghua. It is even more confusing when a given name can also be used as a surname, such as Martin or Yang.
6. If one of the features characterizing the luxury life in the West is travelling and having holidays abroad, the lack of this luxury in the Orient is partly compensated by the rich culinary culture.
7. The reason why some Chinese people have a good memory is that they have to learn a very long history and thousands of characters.
8. The wisdom 'Modesty helps one go forward, whereas conceit makes one lag behind' does not apply to modern society.
9. R&D in novel protein foods would have been less important in the Netherlands if efforts were made to promote the production and consumption of aquatic products.
10. The appellations in China that clearly describe family relations are one of the unique advantages of the Chinese language.
11. Stoichiometric medium design (rational) has a particular advantage over conventional methods which are either too empirical (trial and error) or too mathematical (uniform design or fractional factorial design).

12. Using an integrated fed-batch technique is an ideal strategy in fermentations subject to both substrate limitation and product inhibition.
13. Metabolic pathway engineering will become more attractive when more examples of successful applications become available.
14. Solid-state fermentation on inert carriers could be an alternative for the production of microbial cellulose.
15. Een goede filter is zeer belangrijk voor ieder die TV kijkt.

Stellingen behorende bij het proefschrift: 'Process development for microbial transglutaminase production'.

Zhu Yang
Zeist, oktober 1997

Zhu Yang

PROCESS DEVELOPMENT FOR MICROBIAL TRANSGLUTAMINASE PRODUCTION

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Voor mijn ouders,
voor Yihua en Xiaoye

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* Part of this chapter was used in chapter 6 to make a scientific publication possible.

CHAPTER 1

INTRODUCTION TO NOVEL PROTEIN FOODS AND MICROBIAL TRANSGLUTAMINASE*

INTRODUCTION

In this thesis the development of a fermentation process for the production of microbial transglutaminase (EC 2.3.2.13) by *Streptoverticillium mobaraense* is described. The motivation for this work is the commercial importance of this enzyme for developing novel protein foods. In this chapter, both novel protein foods and transglutaminase will be briefly described followed by an outline of the thesis.

NOVEL PROTEIN FOODS

There is a world-wide increasing demand for novel protein foods. In developed countries, there is an increasing awareness that the high consumption of animal proteins can have serious consequences for both environment and health. In contrast, in developing countries, there are malnutrition problems due to protein deficiency. Mankind has to survive on earth with a rather limited amount of tillable land, while the world population is ever expanding with the consequence of a further decrease of tillable land. Therefore, for the whole world, there is an urgent need to convert plant protein to animal protein in a more efficient way and to consume

directly proteins that are conventionally not used in the daily diet. To make novel protein foods acceptable to the consumer, several criteria have to be met with respect to nutritional value, texture, flavour, safety, appearance and shelf-life. Traditional physical and chemical treatment can be used for the processing of novel protein foods. However, enzymatic treatment is a more attractive and promising alternative due to its mild process conditions and a smaller chance of leading to undesired by-products. Among the enzymes applied for protein modification, transglutaminase has recently shown its potential for accomplishing the criteria mentioned.

TRANSGLUTAMINASE

Transglutaminase catalyses *in vitro* cross-linking reactions in various proteins. This enzyme has been used in attempts to improve the functional properties of foods. Up to now, commercial transglutaminase has been merely obtained from animal tissues. The complicated separation and purification procedure results in an extremely high price of the enzyme, which hampers a wide application in food processing. Recently, studies on the production of microbial transglutaminase have been launched. The enzyme obtained from microbial fermentation has been applied in the treatment of various food proteins. Food treated with microbial transglutaminase appeared not only to meet better the desired criteria for acceptance, but also to reduce the allergenicity of certain foods. However, much work has to be done before this interesting enzyme can be produced on an industrial scale in a commercially attractive way. This thesis focuses on the development of an economic and practical process for microbial transglutaminase production.

OUTLINE OF THE THESIS

Starting a new study by making an overview of the state of the art is a good start and a quick introduction to a new field. Chapter 2 gives such an overview for transglutaminase, its sources

and production methods with an emphasis on microbial transglutaminase and its application in food processing.

To realize an economic and practical process, development of a fermentation process with a high volumetric productivity and a high product concentration is essential. Medium optimization is generally a basic step in such a development. Chapter 3 describes a new approach in medium design using a stoichiometric model based on the method developed by Xie and Wang for animal-cell culture. The results of using a medium designed on this basis show that this method is simple and effective. However, because it is a so-called "black-box" model, details of intracellular metabolic flows are not elucidated. Chapter 4 focuses more on this aspect by means of mass balances, in particular of amino acids that are important in the synthesis of cells and transglutaminase. Based on the results of this study, a fed-batch strategy is developed to further improve the fermentation. This is described in chapter 5.

During our study, progress has been made by other scientists in the same field. Chapter 6 signals the trends in the development and application of transglutaminase on the basis of this recent progress.

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CHAPTER 2

MICROBIAL TRANSGLUTAMINASE - A REVIEW OF ITS PRODUCTION AND APPLICATION IN FOOD PROCESSING

ABSTRACT

Transglutaminase (EC 2.3.2.13) catalyses an acyl-transfer reaction in which the γ-carboxamide groups of peptide-bound glutaminyl residues are the acyl donors. The enzyme catalyses in vitro cross-linking in whey proteins, soya proteins, wheat proteins, beef myosin, casein, and crude actomyosin refined from mechanically deboned poultry meat. In recent years, on the basis of the enzyme's reaction to gelatinize various food proteins through the formation of cross-links, this enzyme has been used in attempts to improve the functional properties of foods.

Up to now, commercial transglutaminase has been merely obtained from animal tissues. The complicated separation and purification procedure results in an extremely high price of the enzyme, which hampers a wide application in food processing.

Recently, studies on the production of transglutaminase by microorganisms have been started. The enzyme obtained from microbial fermentation has been applied in the treatment of food of different origins. Food treated with microbial transglutaminase appeared to have an improved flavour, appearance and texture. In addition, this enzyme can increase shelf-life and

reduce allergenicity of certain foods.

This paper gives an overview of the development of microbial transglutaminase production, including fermentation and down-stream processing, as well as examples of how to use this valuable enzyme in processing foods of meat, fish and plant origin.

INTRODUCTION

Transglutaminase (protein-glutamine γ-glutamyltransferase, EC 2. 3. 2. 13) is an enzyme capable of catalysing acyl transfer reactions introducing covalent cross-links between proteins (Nonaka *et al.* 1989) as well as peptides and various primary amines. When the ε-amino groups of lysine residues in proteins act as an acyl acceptor, ε-(γ-Glu)-Lys bonds are formed both intra- and inter-molecularly. Without primary amines in the reaction system, water becomes the acyl acceptor and the γ-carboxyamide groups of glutamine residues are deamidated, becoming glutamic acid residues (Ando *et al.* 1989). The transglutaminase-catalysed reactions are schematically shown in Figure 1 (Folk 1980; Ikura 1988; Motoki and Seguro 1994).

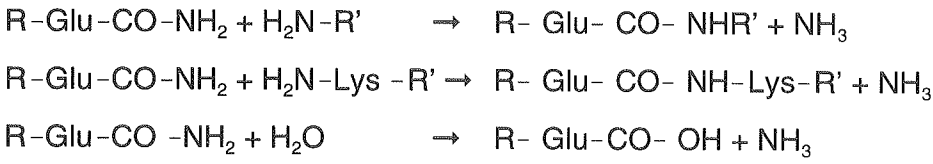

Figure 1. Transglutaminase catalysed reactions

These transglutaminase-catalysed reactions can be used to modify the functional properties of food proteins. Transglutaminase has been used to catalyse the cross-linking of a number of proteins, such as whey proteins, soya proteins, gluten, myosin and actomyosin. The modification of food proteins by transglutaminase may lead to textured products, help to protect lysine in food proteins from various chemical reactions, encapsulate lipids and/or lipid-soluble materials, form heat- and water-resistant films, avoid heat treatment for gelation, improve

elasticity and water-holding capacity, modify solubility and functional properties, and produce food proteins of higher nutritive value through cross-linking of different proteins containing complementary limiting essential amino acids (Mateis and Whitaker 1987; Kitabatake and Doi 1993; Motoki and Seguro 1994).

Transglutaminase has been found in animal and plant tissues (Folk 1980; Falcone *et al.* 1993; Yasueda *et al.* 1994) and microorganisms (Ando *et al.* 1989). Since the 1960s, the purification, characterization and application of Ca^{2+}-dependent transglutaminase of animal origin, mainly guinea-pig liver, have been intensively studied (Folk and Cole 1965, 1966; Connellan *et al.* 1971; Folk 1980; Brookhart *et al.* 1983; Matheis and Whitaker 1987; Ikura 1988; Miwa 1989; Singh 1991; Larre *et al.* 1992, 1993a, b). A process chart of transglutaminase production from different sources is shown in Figure 2.

Guinea-pig liver has been the sole source of commercial transglutaminase for decades. The scarce source and the complicated separation and purification procedure for obtaining tissue transglutaminase have resulted in an extremely high price of the enzyme, about USD 80 for one unit. It is thus not possible to apply such tissue transglutaminase in food processing on an industrial scale. Separation and purification of transglutaminase from fish tissue and plant tissue are still in their infancy. Recently efforts have been made to search for transglutaminase derived from microorganisms. Transglutaminases were found in cultures of *Streptoverticillium* sp. and *Streptomyces* sp. (Motoki *et al.* 1989; Ando *et al.* 1989, 1992). Microbial fermentation makes it possible to achieve mass production of transglutaminase from cheap substrate. A number of examples of the application of microbial transglutaminase in food processing have been announced. However, the potential of using microbial transglutaminase in food processing, as well as in cosmetics, pharmaceutical products and medical treatment, remains uncertain for commercial reasons and because of communication difficulties. This paper gives an overview of the production of microbial transglutaminase and its application in food processing.

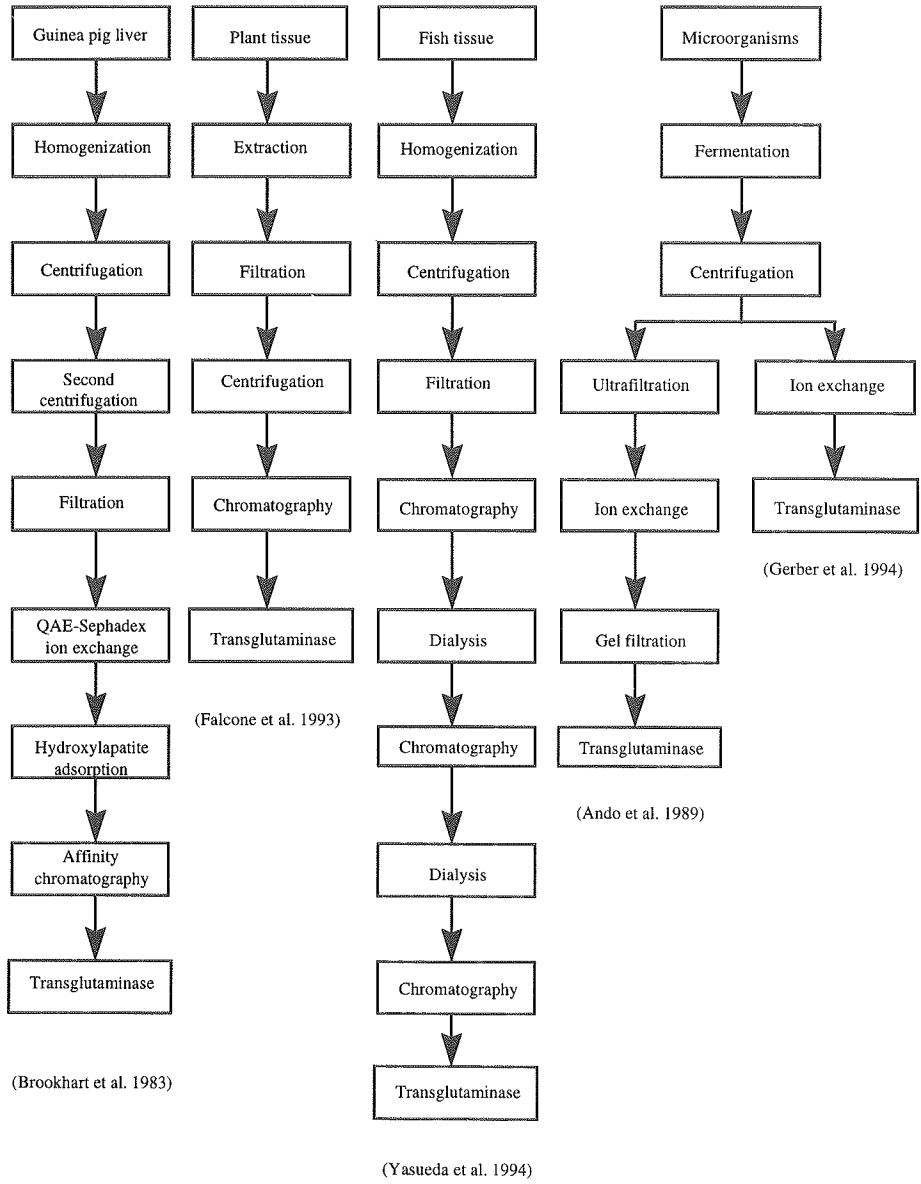

Figure 2. Process chart of transglutaminase production from different sources

PRODUCTION OF MICROBIAL TRANSGLUTAMINASE

The production of transglutaminase derived from microorganisms has not been reported until the late 1980s. Motoki *et al.* (1989) and Ando *et al.* (1989) explored the possibility of producing transglutaminase by microorganisms. Ando *et al.* (1989) screened about 5000 strains isolated from soil collected from a variety of locations. Among these strains, *Streptoverticillium* S-8112 was found to have the capability of producing transglutaminase. Motoki *et al.* (1989) reported that other *Streptoverticillium* strains, such as *S. griseocarneum*, *S. cinnamoneum* subsp. *cinnanoneum* and *S. mobaraense*, also have the ability to produce transglutaminase. Transglutaminase activity has also been found in a culture of *Streptomyces* sp. (Ando *et al.* 1992).

The fermentation procedure for the production of transglutaminase is in principle the same for the various microorganisms mentioned (Ando *et al.* 1989, 1992; Motoki *et al.* 1989). Glucose, sucrose, starch, glycerine and dextrin can be used as carbon source. Inorganic as well as organic nitrogen sources can be used, for instance NH_4NO_3, $(NH_4)_2SO_4$, urea, $NaNO_3$, NH_4Cl, soya, rice, maize, wheat or wheat flour, bran, defatted soya bean, maize-steep liquid, peptone, meat extract, casein, amino acids and yeast extract. Necessary minerals and trace elements are phosphate, magnesium, potassium, iron, cooper, zinc and vitamins. Non-ion surfactant and antifoam can be added if necessary. The culture is an aerobic fermentation so that aeration and agitation are necessary. The temperature for growth and product formation is between 25°C and 35°C, and the fermentation time is dependent on the culture conditions and determined by the highest transglutaminase activity that can be achieved, normally 2-4 days. Microbial transglutaminase is an extracellular enzyme dissolved in the fermentation broth so that it can be recovered through separation of the solid material from the broth. The methods normally used in enzyme purification can be used for microbial transglutaminase. For instance, ethanol, acetone, isopropyl alcohol and other organic solvents can be used in down-stream processing. Salting out with ammonium sulphate and sodium chloride, dialysis, ultra-filtration, ion-exchange chromatography, absorption chromatography, gel filtration, absorption and isoelectronic point methods can all be used to purify the enzyme. A good combination of the methods can increase efficiency and recovery. The enzyme obtained can then be mixed with

enzyme stabilizers such as various salts, sugars, proteins, lipids and surfactant (Sakamoto *et al.* 1992).

Examples of fermentation and purification have been described by Ando *et al.* (1989, 1992) and Motoki *et al.* (1989). The microorganism was pre-cultured in a medium composed of 0.2% peptone, 0.5% glucose, 0.2% K_2HPO_4, 0.1% $MgSO_4$, at pH 7.0. The strain was inoculated in 100 ml medium in a 500 ml Sagakuchi flask and cultured at 30°C for 48 h. The culture broth obtained was added to 20 l fresh medium (pH 7.0) composed of 2.0% polypeptone, 2.0% soluble starch, 0.2% K_2HSO_4, 0.1% $MgSO_4$, 0.2% yeast extract and 0.05% antifoam, and then cultured for 3 days at 30°C under aeration (10 L/min) and agitation (250 rpm). The fermentation broth had an enzyme activity ranging from 0.28 U/ml to 2.5 U/ml, dependent upon the strain used. The microorganism was separated by centrifugation at 3000 rpm. The clear liquid thus obtained was concentrated with an ultrafiltration membrane and then treated on a column of Amberlite CG-50 that had been equilibrated with 0.05 M sodium phosphate buffer (pH 6.5). The column was washed with the same buffer and then the active fractions were pooled. The pooled fraction was treated again on the Amberlite CG-50 column under the same conditions. Then the active fraction was diluted to reduce the conductivity and passed through a Blue Sepharose column (Pharmacia). After this treatment, the enzyme was purified 174 times. The total recovery of transglutaminase activity was about 42%.

Recently, a modified down-stream process for purifying microbial transglutaminase was described by Gerber *et al.* (1994). After the fermentation broth had been centrifuged and filtered, the enzyme was separated directly with a strong acid ion exchanger in a single step. According to the authors, this method is simple, rapid and has a transglutaminase recovery of 40%.

APPLICATION OF TRANSGLUTAMINASE

The production of transglutaminase by microorganisms makes it possible to apply this enzyme in a variety of food processes.

An overview of the application possibilities for microbial transglutaminase in food processing is given in Table 1. A few of these examples will be described in some detail below, in order to show the simplicity of the treatment with microbial transglutaminase and the positive effects that can be obtained.

In meat processing it is of great interest to maximize the yield of marketable products. This includes development of methods for re-structuring low-value cuts and trimmings to improve their appearance, flavour and texture and to enhance market value. Re-structuring treatment usually involves size reduction, reforming and binding (Kim 1993). In such a treatment, transglutaminase can have a very important function. Sakamoto and Soeda (1991) developed a method for producing minced-meat products containing transglutaminase. Minced meat and other food ingredients are mixed with transglutaminase, shaped, packed in pressure-resistant containers and retorted to manufacture meat products such as hamburgers, meatballs, stuffed dumplings and shao-mai (a typical Chinese food). The foods show improved elasticity, texture, taste and flavour. Minced beef and pork, flour, onion, skim-milk powder and condiments were mixed with water and microbial transglutaminase, packed with sauce in bags and retorted to make raw hamburgers. Similar methods for meat and meat products treated with transglutaminase can be found in the literature (Seguro and Motoki 1991; Soeda 1992; Takagaki and Narukawa 1990; Muguruma *et al.* 1990; Miwa 1989).

Table 1. Overview of application of microbial transglutaminase in food processing

Source	Product	Effect	Reference
Meat	Hamburger, meatballs, stuffed dumplings, shao-mai	Improved elasticity, texture, taste and flavour	Sakamoto *et al.* (1991)
	Canned meat	Good texture and appearance	Seguro and Motoki (1991)
	Frozen meat	Improved texture and reduced cost	Takagaki and Narukawa (1990)
	Moulded meat	Restructuring of meat	Matsui *et al.* (1990)

Fish	Fish-meat paste	Improved texture and appearance	Ichihara *et al.* (1990), Wakameda *et al.* (1990a)
Krill	Krill-meat paste	Improved texture	Wakameda *et al.* (1990b)
Collagens	Shark-fin imitation	Imitation of delicious food	Tani *et al.* (1990)
Wheat	Baked foods	Improved texture and high volume	Ashikawa *et al.* (1990)
Soya bean	Mapuo Doufu	Improved shelf-life	Kato *et al.* (1991)
	Fried Tofu (Aburaage)	Improved texture	Soeda *et al.* (1990)
	Tofu	Improved shelf-life	Nonaka *et al.* (1990)
Vegetables and fruits	Celery	Food preservation	Takagaki *et al.* (1991)
Casein	Mineral absorption promoters	Improved mineral absorption in intestine	Noguchi *et al.* (1992)
	Cross-linked protein	Allergenicity reduction	Yamauchi *et al.* (1991)
Gelatin	Sweet foods	Low-calorie foods with good texture, firmness and elasticity	Yamanaka and Sakai (1992)
Fat, oil and proteins	Solid fats	Pork-fat substitute with good taste, texture and flavour	Takagaki *et al.* (1990b)
Plant proteins	Protein powders	Gel formation with good texture and taste	Soeda *et al.* (1992b)
Seasonings	Seasonings	Improve taste and flavour	Kobata *et al.* (1990)

(Continued)

Ichihara *et al.* (1990) and Wakameda *et al.* (1990a) reported the methods for manufacturing fish-meat paste containing transglutaminase. Fish-meat paste products are manufactured from material containing fish meat as main ingredient and 0.1-700 unit transglutaminase per gram fish meat protein. A mixture of 100 parts of dehydrated walleye pollack (*Theragra chalcogramma)*, with 3 parts NaCl, 5 parts of potato starch, 10 parts of water, 0.5 part of monosodium glutamate and 0.01 part of transglutaminase was packed in a film, heated at

60°C for 30 min and at 90°C for 20 min, and cooled to manufacture kamaboko (Japanese fish-meat paste) with good texture and whiteness. Another processing method, reported by Tani *et al.* (1990), was the manufacture of shark-fin imitation food with transglutaminase. Shark-fin is considered as a delicious and healthy (functional) food in Southeast Asia. An imitation of shark-fin is prepared by cross-linking gelatins, collagens or a mixture thereof with transglutaminase and making a gel from the product. The collagen ingredient may be collagen fibres, collagen fibrils, collagen solutions or mixtures thereof. The molecular mass of collagens ranges from 500 Da to 50 000 Da. A shark-fin imitation food was prepared by treating a gelatin (jelly strength 244, m.p. 30°C, isoelectric point 9.1) in water at pH 7 with transglutaminase, extruding the solution through holes, forming fibrous gel and, finally, drying the product.

Kato *et al.* (1991) developed a method for manufacturing storage-stable retort mapuo-doufu (doufu is tofu in Chinese). Mapuo-doufu, braised tofu with minced beef and chili pepper, is one of the most typical hot-spiced dishes in Sichuan Province, China. In this method, retort mapuo-doufu that can be preserved at room temperature for a long time, is manufactured by treating soya bean milk solutions with coagulating agents and transglutaminase at temperatures up to 80°C to manufacture tofu (soya bean curd), optionally cutting the tofu into pieces, putting it in heat-resistant containers with minced beef and seasonings, and retort sterilization. Soya bean milk was mixed with 3 g/l glucono-σ-lactone and transglutaminase at 50°C for 1 h to manufacture tofu, which was sealed in a pouch with sauce and sterilized at 110°C. The mapuo-doufu showed good texture, taste and appearance after 6 months storage at 25°C and 60% relative humidity. Other methods for improving the taste, texture, appearance and shelf-life of tofu were reported by Nonaka *et al.* (1990) and Soeda *et al.* (1990).

Takagaki *et al.* reported (1991) a method for coating vegetables and fruits with transglutaminase and proteins for preservation. Freshness of vegetables and fruits is maintained by coating with a membrane containing transglutaminase and proteins. Cut celery was treated with an aqueous solution containing transglutaminase, proteins, gelatins and Partner-S (natural bactericide from spices) and then heated at 50°C for 5 min to form coating membranes. The coated celery was kept at 20°C for 3 days showing up to 300 bacterial

cells/g, compared to $2x10^6$ without treatment.

Yamauchi *et al.* (1991) developed a method for reducing the allergenicity of some food proteins and/or peptides. α_{s1}-Casein (23 kDa) was treated with transglutaminase at 25 °C for 20 h in water to manufacture cross-linked casein (approx. 90 kDa), which was less allergenic.

A promoting material for absorption of minerals in the human body was developed by Noguchi *et al.* (1992). It is prepared by deaminating of casein through treatment with transglutaminase. The resulting material promotes absorption of minerals in intestine and can be used in the food industry and for medicines, for instance mineral supplement formulations for adults, children and infants. The casein is soluble in neutral and slightly acid conditions and can keep minerals solubilized in the intestine.

PERSPECTIVES

It is of paramount interest to search world-wide for new protein sources and to broaden the application potentials of existing proteins for human consumption. In developing countries, many people are still suffering from starvation and efforts are being focused on producing acceptable protein foods from non-animal proteins, to solve the problem of protein deficiencies (Steinkraus 1994). On the other hand, in addition to the awareness of health problems caused by obesity, people in developed countries are increasingly aware of the environmental burden caused by the surplus of livestock (Bol and Paardekooper 1994). Facing a novel food product, consumers are very sensitive to the properties such as flavour, nutritional value, appearance, shelf life and palatability. In this respect, protein modification by enzymes, especially by microbial transglutaminase whose mass production can be achieved by fermentation from cheap substrates, is one of the most promising alternatives in developing novel protein foods.

With respect to the production of microbial transglutaminase, the microbial process has no doubt its advantage of independence of regional and climatic conditions, in addition to its

reasonable cost. But it is still of great interest to improve fermentation and down-stream processing to further reduce production cost and waste. Modification of strains by genetic engineering is one of the alternatives. However, in Western countries, there is an increasing tendency among consumers to disapprove the application of genetically engineered organisms to food and food ingredients (Jank 1995). In this respect, improvements in process technology, adoption of new fermentation technology and/or a combination thereof will offer promising perspectives.

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CHAPTER 3

MEDIUM DESIGN BASED ON STOICHIOMETRIC ANALYSIS OF MICROBIAL TRANSGLUTAMINASE PRODUCTION BY *Streptoverticillium mobaraense*

ABSTRACT

A stoichiometric model was developed for the application of medium design in microbial transglutaminase production by *Streptoverticillium mobaraense*. The model avoids dealing with all the metabolic reactions involved by simply lumping them into a single reaction. With the help of measurement results, an analysis of the nutrients' roles and biochemical knowledge of the microorganism, all stoichiometric coefficients in the model were calculated. These coefficients were used for medium design. With this designed medium, microbial transglutaminase activity was increased 4-fold, compared to that in the basal medium.

Key words: Stoichiometric model, medium design, microbial transglutaminase

INTRODUCTION

Microbial transglutaminase (MTG; protein-glutamine γ-glutamyltransferase, EC 2. 3. 2. 13) has recently acquired interest due to its attractive application potential in food industries (Motoki and Seguro 1994; Zhu *et al.* 1995). Several strains have been available (Ando *et al.*

1989) for the production of MTG. However, it is always of interest to improve the enzyme productivity of the strains on hand by means of medium optimization. The conventional medium optimization method based on trial and error is often ineffective, expensive, labourious and time-consuming (Zhang *et al.* 1993). Many experiments have usually to be done before satisfactory results are obtained.

One of the powerful methods for medium design is the analysis of the metabolic-fluxes distribution (Varma and Palsson 1994). However, analysis of metabolic fluxes involves in general the establishment of the reaction network, a mathematical matrix and equations to calculate all the fluxes. A rather effective and simple method described by Xie and Wang (1994) avoids dealing with all the reactions involved in the metabolic pathway by lumping them into one singular reaction. With this method, the intricate cellular metabolism will be lumped into a "black box", thereby avoiding the details to be considered in the stoichiometric model. The stoichiometric coefficient for each nutrient in the equation can be determined by an analysis of the roles of nutrients in the synthesis of cell mass and product. As a consequence, the stoichiometric equation simplifies the complicated metabolism into a simple but useful single equation which is helpful in understanding cell growth, designing culture medium and controlling the nutritional environment in cell cultivation.

For microbial cultivation, the "black box" method can be rather easily applied because the biochemical and physiological knowledge of most microorganisms is well established. In our study, the basic principle described by Xie and Wang (1994) is applied to medium design for MTG production by *Streptoverticillium mobaraense*, with some further modification and simplification.

STOICHIOMETRIC MODEL

The growth of *Streptoverticillium mobaraense* and the synthesis of MTG require nutrients such as carbon, nitrogen and mineral sources, among many others. Assuming that vitamins and minerals are sufficiently provided in the medium, the conversion of substrate into cell

mass, product and energy can be simply expressed as the conversion of carbon and nitrogen source, as shown in Figure 1. Stoichiometrically, it can be expressed in the following equation:

$$\theta_{glc}\ [glucose] + \sum_{i=1}^{20} \theta_{a,i}\ [amino\ acid]_i = [cell\ mass] + \theta_{MTG}\ [product] + \theta_{ATP}\ [ATP] \qquad (1)$$

here θ_{glc} is the stoichiometric coefficient for glucose (mmol/mg biomass),

$\theta_{a,i}$ is the stoichiometric coefficient for the i th amino acid (mmol/mg biomass),

θ_{MTG} is the stoichiometric coefficient for product (mg MTG/mg biomass) and

θ_{ATP} is the stoichiometric coefficient for ATP (mmol/mg biomass).

In equation (1), for the sake of simplification, all possible by-products are not taken into consideration by assuming that their amounts are negligible. Obviously, the stoichiometric coefficient of biomass is defined as 1.

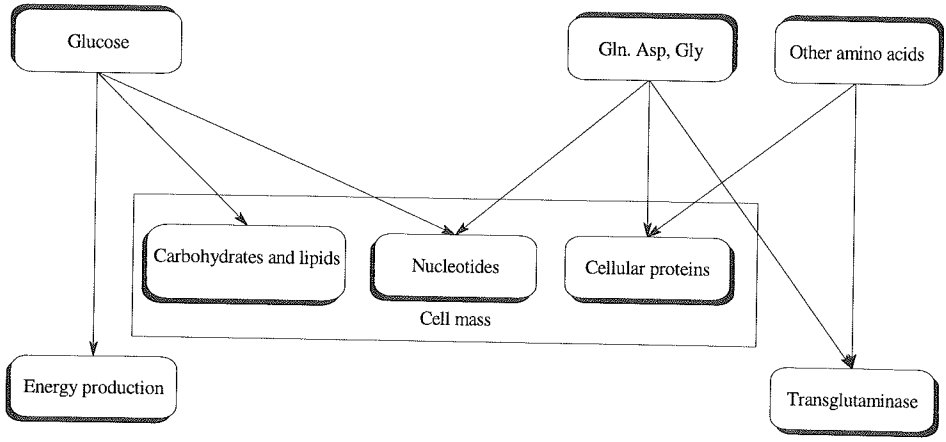

Figure 1. Schematic pathways from substrate to cell mass, MTG and energy (adapted from Xie and Wang, 1994)

Although equation (1) seems very simple, there are 23 unknown parameters which have to be determined. With the help of measurement results and a theoretical analysis of the components' roles in biomass and product synthesis, as shown in Figure 1, a stoichiometric

model can be established to determine all the coefficients. Glucose is needed for energy production and for the synthesis of carbohydrates, lipids and sugars in nucleotides. All 20 amino acids are required for the synthesis of cellular protein and product. Glutamine, aspartic acid and glycine are also consumed for the synthesis of RNA and DNA. The detailed theoretical analysis for the determination of all stoichiometric coefficients, as discussed below, follows the line of reasoning described by Xie and Wang (1994).

Energy production

Here glucose is assumed to be the sole energy source. Stoichiometrically, it can be expressed as:

$$C_6H_{12}O_6 \rightarrow 36\ ATP \tag{2}$$

Synthesis of carbohydrates

Carbohydrates have a similar overall chemical composition and can be expressed as $(CH_2O)_n$. Based on a carbon balance, one glucose can provide 6 carbons for the synthesis of carbohydrates, as given in equation (3):

$$C_6H_{12}O_6 \rightarrow 6\text{-}(CH_2O)\text{-} \tag{3}$$

Synthesis of lipids

Here sources other than glucose for the synthesis of cell lipids are not considered. An empirical formula of the lipids mixture is assumed to be $(CH_2O_xP_y)_m$. Since the total fraction of oxygen and phosphate in cellular lipids is small, it is assumed to be negligible compared to the fractions of carbon and hydrogen. Therefore, the empirical formula can be further simplified as $(CH_2)_m$. Stoichiometrically, one glucose can provide 6 carbons in the synthesis of cellular lipids, as given in equation (4):

$$C_6H_{12}O_6 \rightarrow 6\text{-}(CH_2)\text{-} \tag{4}$$

Synthesis of nucleotides

DNA and RNA are synthesized from the pentosephosphate cycle. The substrate source for these pentoses is glucose. Stoichiometrically, one glucose can provide 6 carbons in the synthesis of these pentoses, as given in equation (5) and equation (6), respectively:

$$C_6H_{12}O_6 \rightarrow \frac{6}{5}\ -(X\text{-}C_5H_7O\text{-}PO_4)- \qquad (for\ \ DNA\ \ synthesis) \tag{5}$$

$$C_6H_{12}O_6 \rightarrow \frac{6}{5}\ -(X\text{-}C_5H_7O_2\text{-}PO_4)- \qquad (for\ \ RNA\ \ synthesis) \tag{6}$$

where *X* means DNA and RNA bases (guanine, adenine, thymine, cytosine and uracil).

Three amino acids, glutamine, aspartic acid and glycine, are involved in the synthesis of nucleotides. Equations (7) to (11) are based on the nitrogen balance of microbial synthesis of these nucleotides (Ingraham *et al.* 1983; Stanier *et al.* 1976):

$$2\ Gln + Asp + Gly \rightarrow guanine + 2\ Glu \tag{7}$$

$$2\ Gln + 2\ Asp + Gly \rightarrow adenine + 2\ Glu \tag{8}$$

$$2\ Gln + Asp \rightarrow cytosine + 2\ Glu \tag{9}$$

$$Gln + Asp \rightarrow uracil + Glu \tag{10}$$

$$Gln + Asp \rightarrow thymine + Glu \tag{11}$$

From equations (7) to (11), it can be seen that the amount of glutamate formed is equal to the amount of glutamine consumed.

Synthesis of cell proteins and product

The synthesis of cell proteins is expressed in a simplified form:

$$\sum_{i=1}^{20} \theta_{a,i}^{prot}\ [amino\ acid]_i = [cell\ proteins] \tag{12}$$

where $\theta_{a,\,i}^{prot}$ means the stoichiometric coefficients for the i th amino acid in the synthesis of cellular protein (mmol of the i th amino acid per mg cell protein).

For MTG, it can be expressed exactly, because its amino acid sequence and molecular weight are known (Kanaji *et al.* 1993):

$$\sum_{i=1}^{20} \theta_{a,\,i}^{MTG}\,[amino\ acid]_i = \frac{1}{37{,}869} \times [23\ Glu + 10\ Gln + 30\ Arg + 21\ Pro + 26\ Asp + 21\ Asn + 6\ Met + 14\ Thr + 5\ Ile + 18\ Lys + 9\ Trp + 16\ Phe + 15\ Tyr + 27\ Ser + 26\ Gly + 1\ Cys + 26\ Ala + 17\ Val + 12\ Leu + 8\ His] = MTG \quad (13)$$

As soon as all stoichiometric coefficients for glucose and amino acids in equation (1) have been determined, a rational medium can be designed (see Results and Discussion).

EXPERIMENTAL

Microorganism

Streptoverticillium mobaraense (CBS 20778) was obtained from the Nederlands Centraalbureau voor Schimmelcultures (CBS, Baarn, Netherlands). The microorganism was cultured on oat-meal agar at 30 °C for 7 days. Spore suspension was prepared in sterile water and used for inoculation.

Fermentation

Batch culture

Batch culture was done as described by Ando *et al.* (1989) with slight modifications. The basal medium was composed of (g/L) soluble starch 20, peptonized milk 20, $MgSO_4$ 2, KH_2PO_4 2, K_2HPO_4 2 and yeast extract 2. Fermentation was done in a 2.5 L Bioflo II fermentor (New Brunswick, NJ) with a working volume of 2.0 L. Aeration was maintained at 2.0 L/min. Agitation was kept at 400 rpm for the first 18 hours and turned to 500 rpm

thereafter. Sterile polypropylene glycol (PPG) in a concentration of 0.5 g/L was used for automatic foam control. pH was set at 6.5 and controlled automatically using 1.0 M HCl and 1.0 M NaOH.

Continuous culture

The medium for the continuous culture was the same as in the batch culture. The fermentor was additionally equipped with a carbon dioxide and oxygen analyzer. The fermentation was switched to continuous at 24 h and run at a dilution rate of 0.08 h^{-1}. Steady state was assumed when CO_2 evolution rate, biomass concentration, residual sugar and transglutaminase activity in the effluent were constant.

Assays

Biomass dry weight

Biomass dry weight was measured by filtering samples through a pre-weighed filter paper (Whatman, GF/C). The residue on the filter was washed with distilled water and dried at 105 °C until constant weight.

Residual sugar

Residual sugar was measured according to the method described by Miller (1960). Glucose was used for preparing the calibration curve.

MTG activity

The colorimetric procedure described by Grossowicz *et al.* (1950) was used for transglutaminase activity measurement. N-α-CBZ-gln-gly (Sigma) was used as substrate. A calibration curve was made using L-glutamic acid γ-monohydroxamate. One unit of transglutaminase is defined as the formation of 1 μmol L-glutamic acid γ-monohydroxamate per minute at 37 °C.

RESULTS AND DISCUSSION

1. Determination of stoichiometric coefficients

Stoichiometric coefficient for product

According to equation (1), the stoichiometric coefficient for product, θ_{MTG}, can be defined by:

$$\theta_{MTG} = \frac{amount\ (mg)\ of\ MTG\ secreted}{amount\ (mg)\ of\ biomass\ produced} \tag{14}$$

It is assumed that the cell death rate is relatively small compared to the growth rate and it is therefore neglected. In a continuous stirred-tank reactor (CSTR) in steady state, the mass balances for biomass B and product P are given by:

$$F \times B_{feed} - F \times B_{out} + r_B = 0 \tag{15}$$

$$F \times P_{feed} - F \times P_{out} + r_p = 0 \tag{16}$$

The concentration of biomass B_{feed} and product P_{feed} in the feeding medium are zero, thus:

$$\theta_{MTG} = \frac{r_p}{r_B} = \frac{P_{out}}{B_{out}} \tag{17}$$

where F is the flow rate in continuous culture (ml/h);

r_B is the production rate of biomass (mg/h);

r_p is the production rate of MTG (mg/h);

B_{out} is the biomass concentration (mg/ml) in the reactor and outlet and

P_{out} is MTG concentration (mg/ml) in the reactor and outlet.

For the determination of θ_{MTG} (mg/mg), data were obtained from an average level of biomass dry weight and an average MTG activity measured in samples from the CSTR in steady state. The specific activity of MTG, 1 unit/mg, reported by Sakamoto *et al.* (1994) is used for calculation. Substituting these data into equation (17) gives $\theta_{MTG} = 4.16 \times 10^{-2}$ (mg/mg).

Stoichiometric coefficient for ATP

Considering that ATP has to meet the requirement for both growth and maintenance (Stouthamer 1973), the stoichiometric coefficient for ATP can be expressed in a simple form as:

$$\theta_{ATP} = \frac{q_{ATP}}{\mu} = \frac{1}{Y_{ATP}} + \frac{m_{ATP}}{\mu} \qquad (18)$$

with μ is the specific growth rate (h^{-1}),

q_{ATP} is the specific ATP consumption rate [mmol ATP/(mg biomass · h)],

Y_{ATP} is the cellular yield on ATP (mg biomass/mmol ATP) and

m_{ATP} is maintenance ATP consumption rate [mmol ATP/(mg biomass · h)].

Data on ATP yield for *Streptoverticillium* are not available in the literature. Therefore, the data for *Escherichia coli* are used here (Roels 1983). The ATP yield for cell synthesis, Y_{ATP}, is 0.33 mol dry biomass per mol ATP. The molecular weight of microbial biomass can be determined by assuming that the biomass molecular formula (van 't Riet and Tramper 1991) has the form of $CH_{1.8}O_{0.5}N_{0.16}S_{0.0045}P_{0.0055}$. The molecular weight of biomass thus obtained is 25. Then ATP yield is 8.25 mg dry biomass per mmol ATP. For maintenance, the coefficient m_{ATP} is 0.175 mol ATP/(moles biomass· h), or 7×10^{-3} mmol ATP/(mg biomass· h). The specific growth rate μ is the same as the dilution rate in steady state in a chemostat. It was set at 0.08 h^{-1} in the experiment. Substituting these data into equation (18) gives $\theta_{ATP} = 0.21$ (mmol/mg)

Stoichiometric coefficient for glucose

It is necessary to know the cell composition for the determination of the stoichiometric coefficient for glucose. Here it is assumed that the cell is composed (dry-weight fraction) of protein (Z_p), DNA (Z_{DNA}), RNA (Z_{RNA}), lipids (Z_{Lip}) and carbohydrate (Z_{Car}). Because there is no large difference in composition of microbial cells, data were taken from the literature (Stouthamer 1973). The weight fraction of carbohydrate (Z_{Car}), protein (Z_p), lipid (Z_{Lip}) RNA (Z_{RNA}) and DNA (Z_{DNA}) is 0.156, 0.524, 0.094, 0.157 and 0.032, respectively.

In the system, glucose is consumed for the synthesis of lipids, carbohydrates, pentoses for DNA and RNA and for energy (ATP) production. Therefore, the stoichiometric coefficient fo glucose should be the sum of these components. By using equations (2) to (6), the stoichiometric coefficient for glucose can be expressed as:

$$\theta_{glc} = \theta_{glc}^{Lip} + \theta_{glc}^{Car} + \theta_{glc}^{DNA} + \theta_{glc}^{RNA} + \theta_{glc}^{ATP} =$$

$$\frac{Z_{Lip}}{M_{CH_2} \times 6} + \frac{Z_{Car}}{M_{CH_2O} \times 6} + \frac{Z_{DNA}}{M_{DNA} \times \frac{6}{5}} + \frac{Z_{RNA}}{M_{RNA} \times \frac{6}{5}} + \frac{\theta_{ATP}}{36} \qquad (19)$$

where M_{CH_2} is the molecular weight of the cellular lipid unit, M_{CH_2O} is the molecular weigh

of the cellular carbohydrate unit, M_{DNA} is the average molecular weight of DNA and M_{RNA} is the average molecular weight of RNA.

For the calculation of the stoichiometric coefficient for glucose in RNA and DNA synthesis, the base composition (molar fraction) of adenine (D_A), guanine (D_G), cytosine (D_C) and thymine (D_T) for DNA and adenine (R_A), guanine (R_G), cytosine (R_C) and uracil (R_U) for RNA are taken from the literature (Ingraham *et al.* 1983). The molar fraction of each base in DNA is about 0.25. In RNA, R_A, R_G, R_C and R_U are 0.26, 0.32, 0.2 and 0.22, respectively. The average molecular weights of the basic unit of DNA, X-C_5H_7O-PO_4 (M_{DNA}), and RNA, X-$C_5H_7O_2$-PO_4 (M_{RNA}), can be derived from the base compositions of DNA and RNA, as given in the following equations:

$$M_{DNA} = M_{PO_4} + M_{C_5H_7O} + D_A M_{C_5H_4N_5} + D_G M_{C_5H_4ON_5} + D_C M_{C_4H_4ON_3} + D_T M_{C_5H_5O_2N_2} \qquad (20)$$

$$M_{RNA} = M_{PO_4} + M_{C_5H_7O_2} + R_A M_{C_5H_4N_5} + R_G M_{C_5H_4ON_5} + R_C M_{C_4H_4ON_3} + R_U M_{C_4H_3O_2N_2} \qquad (21)$$

By inserting the data from the literature and the result of equations (18), (20) and (21) into equation (19), the stoichiometric coefficient for glucose thus becomes $\theta_{glc} = 8.34 \times 10^{-3}$ (mmol/mg).

Stoichiometric coefficients for amino acids

Based on equations (7) to (11), the stoichiometric coefficients for amino acids (Gln, Gly, Asp and Glu) in RNA and DNA synthesis are:

$$\theta_{gln}^{nuc} = 2\,[N_G + N_A + N_C] + N_T + N_U \tag{22}$$

$$\theta_{gly}^{nuc} = N_G + N_A \tag{23}$$

$$\theta_{asp}^{nuc} = N_G + 2\,N_A + N_C + N_T + N_U \tag{24}$$

$$\theta_{glu}^{nuc} = -\,\theta_{gln}^{nuc} \tag{25}$$

where N_A, N_G, N_C, N_T and N_U are the total moles of adenine, guanine, cytosine, thymine and uracil per mg biomass, respectively (mmol/mg biomass). The calculation was done on the basis of equations (26) to (32) and the data of cell composition (Stouthamer 1973):

$$N_{DNA} = \frac{Z_{DNA}}{M_{DNA}} \tag{26}$$

$$N_{RNA} = \frac{Z_{RNA}}{M_{RNA}} \tag{27}$$

and thus:

$$N_A = N_{DNA}\,D_A + N_{RNA}\,R_A \tag{28}$$

$$N_G = N_{DNA}\,D_G + N_{RNA}\,R_G \tag{29}$$

$$N_C = N_{DNA}\,D_C + N_{RNA}\,R_C \tag{30}$$

$$N_T = N_{DNA}\,D_T \tag{31}$$

32

$$N_U = N_{RNA} R_U \tag{32}$$

Substituting the results of equations (26) to (32) into equations (22) to (25), stoichiometric coefficients for Asp, Gln, Glu and Gly in the synthesis of DNA and RNA can be determined (column 4 of Table I).

The stoichiometric coefficients for amino acids can be obtained based on equations (1), (12), (13) and (22) through (25):

$$\theta_{a,i} = \theta_{a,i}^{prot} \times Z_p + \theta_{a,i}^{MTG} \times \theta_{MTG} + \theta_{a,i}^{nuc} \tag{33}$$

Note that the term $\theta_{a,i}^{nuc} = 0$, except for Gln, Gly, Asp and Glu.

According to Figure 1, equations (12), (13) and (33), the amino-acid composition data of the microorganism and MTG are needed. Since the amino-acid composition of *Streptoverticillium* sp. is not available, general data for microbial cells (Morowitz 1968) are used in the calculation. The results of the term $\theta_{a,i}^{prot} \times Z_p$ in equation (33) thus determined are given in column 2 of Table I.

Substituting the results of equations (13) and (17) into equation (33), the term $\theta_{a,i}^{MTG}$ is calculated for each amino acid (Table I, column 3). Note that the specific activity of crude MTG (Sakamoto *et al.* 1994) has been used in the calculation of θ_{MTG}, which implies that extracellular proteins other than MTG have been included in the analysis. On the other hand, the amino acid composition and molecular weight of purified MTG (Kanaji *et al.* 1993) have been used to calculate $\theta_{a,i}^{MTG}$. This causes an uncertainty in the estimated amino acids requirements for MTG synthesis $(\theta_{a,i}^{MTG} \times \theta_{MTG})$ in Table I. However, these requirements are

generally smaller that 10% of the total amino acid requirement of $\theta_{a,i}$ calculated from equation (33). Therefore, the estimation of amino acids requirement for the synthesis of cellular protein and MTG will not cause significant errors in the medium evaluation.

Table I. Stoichiometric coefficients for amino acids in synthesis of cell protein, MTG, nucleotides and biomass in equation (33)

Amino acid	$\theta_{a,i}^{prot} \times Z_p$	$\theta_{a,i}^{MTG} \times \theta_{MTG}$	$\theta_{a,i}^{nuc}$	$\theta_{a,i}$
Alanine	4.54×10^{-4}	0.29×10^{-4}		4.83×10^{-4}
Arginine	2.52×10^{-4}	0.32×10^{-4}		2.84×10^{-4}
Aspartate	2.01×10^{-4}	0.28×10^{-4}	7.65×10^{-4}	9.94×10^{-4}
Asparagine	1.01×10^{-4}	0.23×10^{-4}		1.24×10^{-4}
Cysteine	1.01×10^{-4}	0.011×10^{-4}		1.02×10^{-4}
Glutamate	3.53×10^{-4}	0.25×10^{-4}	$- 10.96 \times 10^{-4}$	-7.18×10^{-4}
Glutamine	2.01×10^{-4}	0.11×10^{-4}	10.96×10^{-4}	13.1×10^{-4}
Glycine	4.03×10^{-4}	0.29×10^{-4}	3.57×10^{-4}	7.89×10^{-4}
Histidine	0.50×10^{-4}	0.087×10^{-4}		0.59×10^{-4}
Isoleucine	2.52×10^{-4}	0.055×10^{-4}		2.57×10^{-4}
Leucine	4.03×10^{-4}	0.13×10^{-4}		4.16×10^{-4}
Lysine	4.03×10^{-4}	0.20×10^{-4}		4.23×10^{-4}
Methionine	2.01×10^{-4}	0.066×10^{-4}		2.07×10^{-4}
Phenylalanine	1.51×10^{-4}	0.17×10^{-4}		1.68×10^{-4}
Proline	2.52×10^{-4}	0.23×10^{-4}		2.75×10^{-4}
Serine	3.02×10^{-4}	0.29×10^{-4}		3.31×10^{-4}
Threonine	2.52×10^{-4}	0.15×10^{-4}		2.67×10^{-4}
Tryptophan	0.50×10^{-4}	0.099×10^{-4}		0.60×10^{-4}
Tyrosine	1.01×10^{-4}	0.16×10^{-4}		1.17×10^{-4}
Valine	3.02×10^{-4}	0.18×10^{-4}		3.20×10^{-4}

2. Evaluation of the basal medium

Once the stoichiometric equation governing growth and production has been determined, the

concentration of each nutrient needed can be determined in a rational manner. According to the definition of stoichiometric coefficients in equation (1), for the k th nutrient in the form of glucose and amino acid, the stoichiometric coefficient θ_k can be expressed as:

$$\theta_k = \frac{mmoles\ of\ nutrient\ consumed}{mg\ biomass\ produced} \tag{34}$$

According to equation (19), at least 8.34×10^{-3} mmol glucose is required for the synthesis of 1 mg biomass. Based on an average biomass concentration of $B_{out} = 6$ (mg/ml), the minimum amount of glucose required in the initial medium should be:

$$\theta_{glc} \times B_{out} \times M_{glc} = 8.34 \times 10^{-3} \times 6 \times M_{C_6H_{12}O_6} = 9.0\ (g/L) \tag{35}$$

Similarly, the amount of each amino acid required in the initial medium should at least be:

$$C_{a,i}^{\min} = \theta_{a,i} \times B_{out} \times M_{a,i}\ (mg/ml) \tag{36}$$

where $C_{a,i}^{\min}$ is the minimum requirement of the i th amino acid in the initial medium (mg/ml); $M_{a,i}$ is the molecular weight of the i th amino acid. The minimal requirement of all amino acids thus calculated is given in Table II.

The concentration of residual sugar measured in the fermentation broth was 13.5 mg/ml. This implies that the amount of sugar is far beyond the minimum requirement for the growth of the microorganism and energy supply. Therefore, the initial concentration of soluble starch, 2%, in the medium will not bring about the limitation of carbon source during fermentation. In our study, soluble starch was used as sole carbon source instead of glucose because the latter was not suitable for MTG production (results not shown). In general, microorganisms are capable of hydrolysing starch into monosaccharide. The amount of monosaccharide formed will be at least the same as that of starch because water is introduced into the molecule. Therefore, the amount of soluble starch, 2%, used in the medium will be sufficient to meet the requirement listed in Table II.

Table II. Amino acid in medium with 2% peptone, minimal requirement according to stoichiometric model[a] and supplementation in designed medium[d]

Nutrients	C_k^{med} (g/L)	C_k^{min} (g/L)	C_k^{med} / C_k^{min}	AA_{sup} (g/L)
Alanine	0.396	0.258	1.53	-
Arginine	0.150	0.297	0.505	0.30
Aspartate	0.542[b]	0.794	0.683	0.649
Asparagine	-	0.103	0	0.155
Cystine	0.088	0.074	1.19	0.023
Glutamate	1.93[c]	-	1.68[c]	-
Glutamine	-	1.15		-
Glycine	0.388	0.355	1.09	0.145
Histidine	-	0.055	0	0.083
Isoleucine	0.240	0.202	1.19	0.063
Leucine	0.700	0.327	2.14	-
Lysine	0.524	0.371	1.41	-
Methionine	0.186	0.185	1.01	0.092
Phenylalanine	0.356	0.167	2.13	-
Proline	0.802	0.190	4.22	-
Serine	0.306	0.209	1.46	-
Threonine	0.274	0.191	1.44	-
Tryptophan	0.106	0.074	1.43	-
Tyrosine	0.242	0.127	1.90	-
Valine	0.578	0.225	2.56	-
Starch	20	9	2.47	-

a Assuming $B_{out} = 6$ (mg/ml)
b total amount of Asp and Agn;
c total amount of Glu and Gln;
d expecting a 50% increase of biomass.

Since neither inorganic nitrogen nor free amino acids could provide good growth and product synthesis (results not shown here), peptone was used throughout the experiment. Here an assumption is made that the peptone can be completely hydrolysed by the microorganism. The amount of amino acids in peptone is also given in Table II.

In Table II, C_k^{med} is the amount of the k th nutrient existing in the medium, $C_k^{\min}$ is the minimum concentration of the k th nutrient in the medium to meet the stoichiometric model, $C_k^{med} / C_k^{\min}$ is the ratio between existing amount and minimum requirement of amino acids or glucose, and AA_{sup} is the supplement of the i th amino acid in the designed medium. As can be seen from Table II, by comparing the required amount of amino acids and that existing in the medium before design, there is a shortage of certain amino acids in the medium, in particular arginine, aspartate, asparagine and histidine. This implies that these amino acids might have been the limiting factor in fermentation. In measuring glutamate and glutamine, the results given are always expressed as a total. Therefore, both glutamate and glutamine can be considered to be non-limiting.

3. Designed medium

The new medium is designed to give amino acids a rational proportion according to the stoichiometric model. It can be seen in Table II that Arg, Asp, Agn, and His are in shortage while only Pro is available in excess by a factor 4.22. Most other amino acids, except for Leu, Phe, Tyr and Val, are in surplus by a factor ranging from 1.1 to 1.6. Considering that a 50% increase of biomass will come close to the critical point above which other limitations such as oxygen supply might occur, the new medium is therefore designed based on the stoichiometric model (equation 36) assuming that a 50% increase of biomass will be achieved. The supplementation of certain amino acids is listed in Table II. The composition of the new designed medium has thus the following composition (g/L): starch 20, peptone 20, $MgSO_4$ 2, KH_2PO_4 2, K_2HPO_4 2, yeast extract 2, PPG 0.5, Arg 0.30, Asp 0.649, Asn 0.155, Cys 0.023, Gly 0.145, His 0.083, Ile 0.063 and Met 0.092. The existing concentrations of Lys, Ser, Thr

and Try in peptone are very close to the theoretical values so that their shortage can be compensated by the yeast extract.

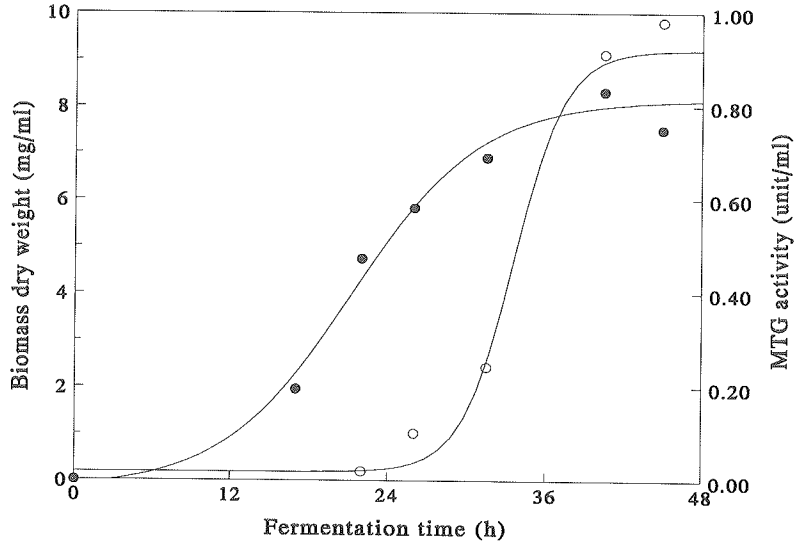

Figure 2. Course of growth and MTG activity with designed medium in batch fermentation. ● Biomass, ○ MTG.

The batch fermentation using stoichiometrically designed medium was done under the same conditions as described in Experimental. The course of biomass dry weight and MTG production is shown in Figure 2. Although biomass was less increased than expected, i.e. 130% instead of 150%, MTG production is significantly improved. The reason of less increase of biomass might be some other limitations such as oxygen transfer, because high viscosity was observed during the fermentation. For the product, as can be seen by comparing these results with those when unmodified medium is used, as shown in Figure 3, MTG production is increased 4-fold. It implies that amino acids play an important function in the synthesis of MTG. Although the exact mechanism of MTG synthesis needs to be uncovered, the results imply that certain peptides and some amino acids in peptone are necessary in the metabolic pathway of MTG synthesis or the formation of its important precursors which are impossible to be synthesized by the microorganism itself. This can also explain why culture

medium without organic nitrogen source could hardly produce any MTG (results not shown here). Using unmodified medium, these peptides and/or amino acids probably became limiting factors which inhibited the synthesis of MTG.

In Figure 2, the time needed to reach the maximum MTG activity is about 40 h which is considerably less than 65 h needed with unmodified medium. This indicates that a more effective and economic fermentation process is achieved by using stoichiometrically designed medium.

The results prove that this "black box" method adapted after Xie and Wang (1994) is a powerful tool in medium design for microbial fermentation as well as for animal-cell culture (Xie and Wang 1994). The stoichiometric model, together with data taken from the literature for MTG composition (Kanaji *et al.* 1994) and activity (Sakamoto *et al.* 1994), cell

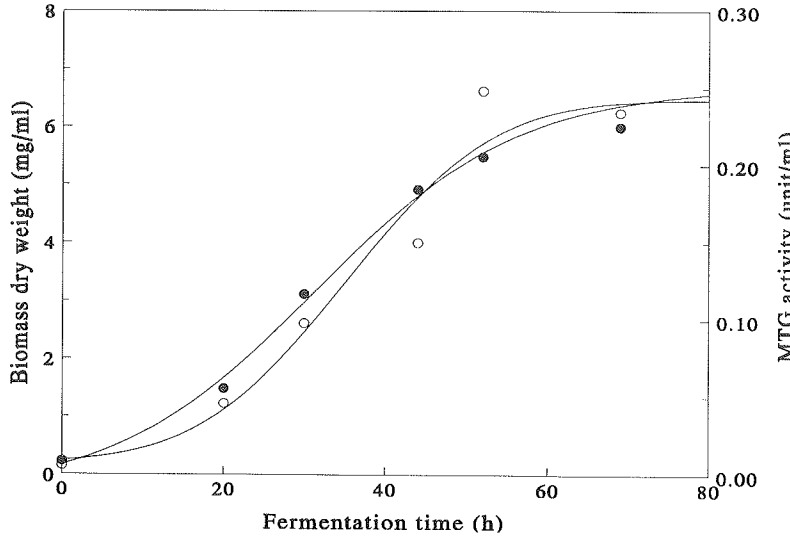

Figure 3. Course of growth and MTG activity before optimization in batch fermentation. ● biomass, ○ MTG.

composition (Morowitz 1968, Stouthamer 1973; van 't Riet and Tramper 1991) and ATP requirements (Roels 1983; Stouthamer 1973) and the experimentally determined value for

θ_{MTG}, gives a reasonable estimation of the minimum amount of amino acid requirement in the medium, despite of several simplifications and assumptions. In principle, this strategy can also be applied to other fermentation processes where such a stoichiometric model can be developed. However, in order to understand the biochemical mechanism of MTG synthesis in more detail, tools other than the "black box" must be adopted, for instance studies of the kinetics, biochemistry of MTG synthesis and the analysis of the metabolic-fluxes distribution.

NOMENCLATURE

B	biomass concentration (mg/ml)
D_A, D_G, D_C and D_T	DNA base composition of adenine, guanine, cytosine and thymine respectively (mol fraction)
F	flow rate of continuous culture (ml/h)
$M_{a,i}$	molecular weight of the *i* th amino acid
m_{ATP}	maintenance ATP consumption rate [mmol ATP/(mg biomass · h)]
M_{CH_2}, M_{CH_2O}	molecular weight of cellular carbohydrate and lipid unit
M_{RNA}, M_{DNA}	average molecular weight of RNA and DNA
N_A, N_G, N_C, N_T, N_U	total moles of adenine, guanine cytosine, thymine and uracil per mg biomass (mmol/mg)
P	MTG concentration (mg/ml)
q_{ATP}	specific ATP consumption rate [mmol ATP/(mg biomass · h)]
r_B	production rate of biomass (mg/h)
r_p	production rate of MTG (mg/h)
R_A, R_G, R_C and R_U	RNA base composition of adenine, guanine, cytosine and uracil respectively (mole fraction)
X	stands for DNA and RNA bases, adenine, guanine, cytosine, thymine, and

uracil

Y_{ATP} cellular yield on ATP (mg biomass/mmol ATP)

Z_p, Z_{DNA}, Z_{RNA}, Z_{Lip} and Z_{Car}

dry weight percentage of cellular protein, DNA, RNA, lipids and carbohydrates

Greeks

θ_{glc} stoichiometric coefficient for glucose (mmol/mg biomass)

$\theta_{a,i}$ stoichiometric coefficient for the *i* th amino acid (mmol/mg biomass)

θ_{MTG} stoichiometric coefficient for product (mg MTG/mg biomass)

θ_{ATP} stoichiometric coefficient for ATP (mmol/mg biomass)

$\theta_{a,\,i}^{prot}$ stoichiometric coefficient for the *i* th amino acid in synthesis of cell protein (mmol/mg cellular protein)

$\theta_{a,\,i}^{MTG}$ stoichiometric coefficient for the *i* th amino acid in synthesis of product MTG (mmol/mg MTG)

$\theta_{a,\,Gln}^{nuc}$, $\theta_{a,\,Gly}^{nuc}$, $\theta_{a,\,Asp}^{nuc}$, $\theta_{a,\,Glu}^{nuc}$

stoichiometric coefficient for glutamine, glycine, aspartate and glutamate in synthesis of cellular nucleotides

θ_{glc}^{Car} , θ_{glc}^{Lip} , θ_{glc}^{DNA} , θ_{glc}^{RNA}

stoichiometric coefficient for glucose in synthesis of cellular carbohydrate, lipids , DNA and RNA

θ_k stoichiometric coefficient of the *k* th nutrient (glucose or amino acid)

μ specific growth rate (h^{-1})

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CHAPTER 4

MICROBIAL TRANSGLUTAMINASE PRODUCTION BY *Streptoverticillium mobaraense* - ANALYSIS OF AMINO-ACID METABOLISM USING MASS BALANCES

ABSTRACT

Metabolic flows, especially those of amino acids, were determined and analysed at different stages of a batch fermentation for transglutaminase production by *Streptoverticillium mobaraense*. The method is mainly based on mass balances and measurements of amino acids and other metabolites. The measurements included consumption rate of glucose, intake rates of all amino acids and production rates of carbon dioxide, cell mass and transglutaminase. Three groups of metabolic flows were determined by three different methods. Those in the first group are determined by solely using measurement results. The second group deals with the synthesis of most amino acids. The metabolic flows were determined by using a mass-balancing method considering the contribution of these amino acids to the synthesis of cells and product, i.e. transglutaminase. The third group includes the reactions covering all other important intermediates. The metabolic flows in this group were calculated by a metabolite-balancing method. Metabolic flows during different fermentation phases were thus determined. The distribution of metabolic flows of amino acids implies that growth and transglutaminase production are active as long as there are free amino acids available in the

medium. An important factor which limits further growth and production is probably the cross-linking action of transglutaminase on the nitrogen source in the medium. The results suggest that a nitrogen source other than peptone and/or amino acids might improve growth and production.

Keywords: Metabolite balancing, metabolic flow, microbial transglutaminase, *Streptoverticillium mobaraense*, mass balancing.

INTRODUCTION

Microbial transglutaminase (MTG; protein-glutamine γ-glutamyltransferase, EC 2.3.2.13) is a recently developed enzyme that has acquired increasing interest because of its attractive application potential in food industries (Zhu *et al.* 1995, Motoki & Seguro 1994). Efforts have been made since the late 1980's in screening for new strains (Ando *et al.* 1989), modifying the downstream processing (Gerber *et al.* 1994) and improving fermentation by stoichiometric analysis for medium design (Zhu *et al.* 1996). The latter method of medium design on the basis of a stoichiometric analysis proved to be effective and easy to do. Using medium rationally optimized on the basis of the nutrient requirements for cell synthesis and MTG production, MTG production by *Streptoverticillium mobaraense* was increased four times. By comparing the MTG production using the optimized medium to that using the basal medium, it was found that the shortage of certain amino acids limited the synthesis of MTG. However, it was not possible with the results obtained from this "black-box" model to find out the metabolic pathway details of certain amino acids which are important for the synthesis of cellular proteins and MTG, because all the reactions involved in the metabolic pathway were lumped into one reaction. One alternative for the analysis of metabolic pathway details is to set up metabolite balances for the determination of all important metabolic flows. The results of such a metabolic-flow analysis can be helpful in understanding metabolic regulations and patterns. More information about metabolic-flow distribution may allow further optimization of MTG production. Compared to the "black-box" method, the analysis of the metabolic flow distribution is in general also based on the stoichiometry of the organism but deals in much more detail with important reactions and branch points within the

metabolic pathway of growth and product formation. Since two decades, metabolic-flow distribution analysis has been intensively studied for several microbial processes (Erickson 1978, Aiba & Matsuoka 1979, Papoutsakis 1984, Papoutsakis & Meyer 1985, Holms 1986, Goel *et al.* 1993, Stephanopoulos & Vallino 1991, Vallino & Stephanopoulos 1990, 1993, Bailey 1991, Varma & Palsson 1993, 1994, Jørgensen *et al.* 1995, van Gulik & Heijnen 1995, Marx *et al.* 1996, Nielsen & Villadsen 1994) as well as for processes in which insect cells and animal cells are used (Ferrance *et al.* 1993, Bonarius *et al.* 1996, Savinell & Palsson 1992 a, b).

In general, by analysing metabolic-flow distribution of a given system, one can (1) expect to find out bottlenecks in cell growth and product synthesis, and (2) determine the nutrient requirements for cell culture processes (Bonarius *et al.* 1996). According to Varma and Palsson (1994), two items of metabolic information are required for establishing the mathematical formulation of a metabolic-flow balance model. First, metabolic stoichiometry is required to write down all the important chemical reactions that take place in the metabolic network of interest. In most cases this includes all intermediary metabolic reactions. In the establishment of all important reactions involved in the system, there are general reactions in primary metabolisms which can be found in the literature. There are also special reactions which exist in the metabolic pathway of the pertinent strain, normally the production of a target metabolite, in our case the synthesis of MTG. Second, information is needed about the demands that are placed on the metabolic system. These demands include biomass synthesis, maintenance requirements and in some cases the secretion of an important product. For this purpose, it is essential to get the information about the composition of the cell and the product, and the nutrients requirement for their synthesis. Normally these data can be obtained from the literature or determined by measurements.

This paper presents the work done in finding out the metabolic-flow distribution, mainly that of amino acids, at different fermentation stages of a batch process, in the metabolic pathway of *S. mobaraense* for MTG production. The method used for the determination of this metabolic-flow distribution is based on mass balances (Ferrance *et al.* 1993, Bonarius *et al.* 1996).

THEORY

1. Mass balances over amino acids

From our previous results it is known that all 20 amino acids play an important role in the synthesis of cell mass and MTG. Therefore, the analysis of the metabolic-flow distribution focuses on the metabolism of amino acids. For a batch fermentation process, the consumption rate of a given amino acid between any two sampling intervals can be determined by mass balancing.

For the *i* th amino acid, its excretion rate from the cell into the medium (or its intake from the medium into the cell), can be determined by measuring its concentration change in the medium:

$$r_{a,i}^{c \to m} = \frac{C_{a,i}^{t_2} - C_{a,i}^{t_1}}{t_2 - t_1} \qquad [mmol \cdot L^{-1} \cdot h^{-1}] \tag{1}$$

where $r_{a,i}^{c \to m}$ is the excretion rate of the *i* th amino acid from the cell into the medium. If the result of Equation (1) is negative, an intake of the *i* th amino acid from the medium into the cell occurred.

For the mass balances of the *i* th amino acid, the following metabolic flows around this amino acid pool must be considered, as shown in Figure 1:

- its excretion from the cell to the medium (or intake from the medium to the cell), as can be determined by Equation (1);
- its flow to the synthesis of cell mass $(\theta_{a,i}^{cell} \times \Delta C_{cell})$, with $\theta_{a,i}^{cell}$ is the stoichiometric coefficient of the *i* th amino acid for the synthesis of 1 g cell mass and Δc_{cell} is the increase of cell mass;

- its flow to the synthesis of product $(\theta_{a,i}^{MTG} \times \Delta C_{MTG})$ with $\theta_{a,i}^{MTG}$ is the stoichiometric coefficient of the *i* th amino acid for the synthesis of MTG and ΔC_{MTG} is the increase of MTG;
- its flows from and/or to other intermediate metabolites, i.e. the synthesis of the amino acid itself $(r_{a,i}^{\Delta t})$ and/or its consumption for the synthesis of other amino acids (Note that the latter is only valid for Asp, Glu and Ser, see also Table 1 and Figure 2).

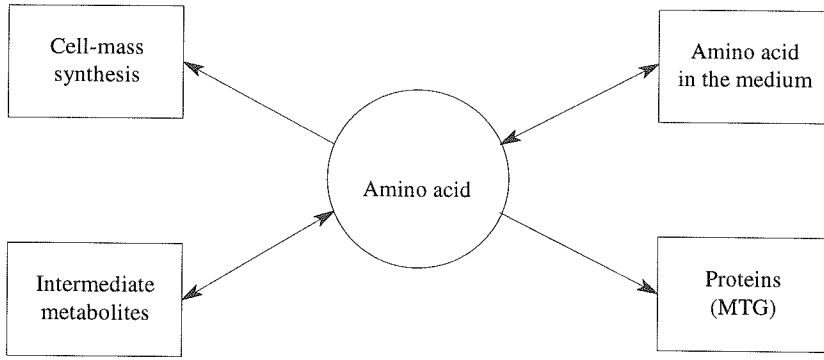

Figure 1. Schematic pathways for the *i* th amino acid; for all amino acids, except Asp, Glu and Ser, only one reaction exists between the *i* th amino acid and the intermediate metabolite.

The total mass balance for the *i* th amino acid, as shown in Figure 1, can thus be expressed as:

$$\frac{\Delta C_{a,i}}{\Delta t} = \frac{C_{a,i}^{t_2} - C_{a,i}^{t_1}}{t_2 - t_1} = r_{a,i}^{\Delta t} - \frac{\Delta C_{cell}}{\Delta t} \times \theta_{a,i}^{cell} - \frac{\Delta C_{MTG}}{\Delta t} \times \theta_{a,i}^{MTG} \qquad (2)$$

or:

$$r_{a,i}^{\Delta t} = \frac{\Delta C_{cell} \times \theta_{a,i}^{cell} + \Delta C_{MTG} \times \theta_{a,i}^{MTG} + \Delta C_{a,i}}{\Delta t} \quad [mmol \cdot L^{-1} \cdot h^{-1}] \qquad (3)$$

where $r_{a,i}^{\Delta t}$ is the average production rate of the *i* th amino acid between the two sampling points t_2 and t_1; ΔC_{cell} is the concentration difference of cell mass (dry weight) between t_2 and t_1 ($g \cdot L^{-1}$); ΔC_{MTG} is the concentration difference of MTG between t_2 and t_1 ($g \cdot L^{-1}$); $\Delta C_{a,i}$ is the concentration difference of the *i* th amino acid between t_2 and t_1 ($mmol \cdot L^{-1}$); $\theta_{a,i}^{cell}$ is the stoichiometric coefficient of the *i* th amino acid for the synthesis of cell mass ($mmol \cdot g^{-1}$) and $\theta_{a,i}^{MTG}$ is the stoichiometric coefficient of the *i* th amino acid for the synthesis of MTG ($mmol \cdot g^{-1}$). The two stoichiometric coefficients in Equations (2) and (3) give a quantitative relationship between the *i* th amino acid and the synthesis of cell mass and MTG (Zhu *et al.* 1996).

Note that this equation is not valid for Asp, Glu and Ser, which are involved in some other reactions as shown in Table 1 and Figure 2. For example, Glu is produced from AKG through reaction (53) and consumed for the production of Lys, Ile, Val, Ala, Pro, Arg and Gln, in addition to its contribution to the synthesis of cell and product (see schematic description in Figure 4.d and Table 1 and 2). Therefore, the determination of the production rate of Glu cannot be simply determined by Equation (3). This is also valid for Ser and Asp. The metabolic flows of these three amino acids must be determined by metabolite balancing, as described later in this paper.

2. Biochemistry

In order to obtain all metabolic flows of amino acids, including their transfer from medium to cell (or the other way around), their production and for some of them (Asp, Glu and Ser) their contribution to the synthesis of other amino acids, the model must cover all the reactions which are related to amino acids. Because all reactions that involve amino acids are only a part of the primary metabolic pathway of the strain concerned, the stoichiometric network must include other reactions in the primary metabolism of the organism. Since detailed biochemistry describing the primary metabolic pathway of *S. mobaraense* is not available, it is

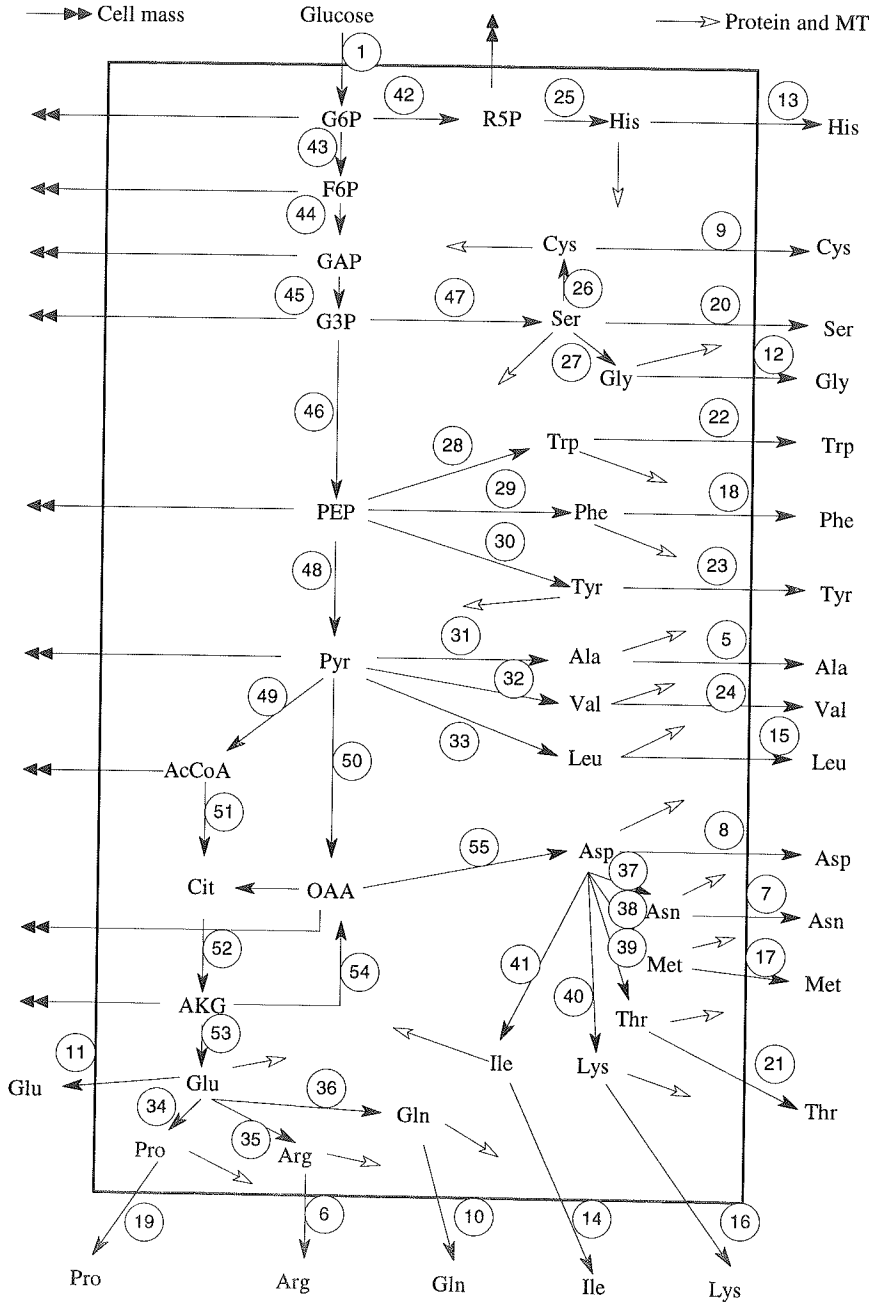

Figure 2. Reaction network

assumed that the strain obeys the primary metabolism of representative bacteria, such as *Escherichia coli*. Although the primary metabolism of an organism can involve thousands of reactions, the main metabolic pathways have the same features (Stryer 1981). Metabolic pathways such as the Embden-Meyerhof-Parnas (EMP) pathway and the tricarboxylic acid (TCA) pathway are ubiquitous (Stryer 1981). Therefore, the reaction network set up for *S. mobaraense* involves these pathways. Some precursors for the synthesis of amino acids are formed in the TCA cycle, and have to be supplied by the carboxylation of pyruvate into oxaloacetate, as shown in Table 1, reaction (50). The information on the synthesis of biomass from important intermediates is obtained from the literature (Ingraham *et al.* 1983). Except for cellular protein, the synthesis of all other cellular components (carbohydrates, lipids and RNA/DNA) is lumped into one reaction, as given in Table 1, reaction (2). The reason for the exclusion of protein synthesis from this lumped reaction is that all 20 amino acids should be dealt with separately in order to focus on their metabolic functions in growth and production. As it can be seen in Equation (3), the contribution of all amino acids to the synthesis of cell mass is quantatively expressed in their stoichiometric coefficients and is used for amino acid mass balances. The stoichiometric requirement of all twenty amino acids in the synthesis of cell mass and MTG has been calculated in our previous paper (Zhu *et al.* 1996), and is used for mass balancing of total amino acids, as shown in Reactions (3) and (4) in Table 1. In Table 1, three different kinds of reactions are listed. All related metabolites are listed in Table 2. A schematic pathway of these reactions and metabolites are shown in Figure 2.

Table 1. Stoichiometry of the reaction network

Reactions of which the metabolic flows can be directly determined by measurement	
1)	GLC + ATP = G6P
2)	2.5 ACCOA + 0.087 AKG + 0.071 F6P + 0.205 G6P + 0.129 GAP + 0.619 G3P + 0.754 R5P + 0.051 PEP + 0.028 OAA + 0.028 PYR + 0.21 ATP = CELL MASS (mmol · g^{-1} cell mass)
3)	0.454 ALA + 0.252 ARG + 0.201 ASP + 0.101 ASN + 0.101 CYS + 0.353 GLU + 0.201 GLN + 0.403 GLY + 0.05 HIS + 0.252 ILE + 0.403 LEU + 0.403 LYS + 0.201 MET + 0.151 PHE + 0.252 PRO + 0.302 SER + 0.252 THR + 0.05 TRP + 0.101 TYR + 0.302 VAL = 0.52 cellular protein (mmol · g^{-1})
4)	0.687 ALA + 0.792ARG + 0.687ASP + 0.555 ASN + 0.264 CYS + 0.607 GLU + 0.264 GLN + 0.687 GLY + 0.211 HIS + 0.132 ILE + 0.317 LEU +0 .475 LYS + 0.158 MET + 0.423 PHE + 0.555 PRO + 0.713 SER + 0.37 THR + 0.238 TRP +

0.396 TYR + 0.449 VAL = MTG (mmol · g^{-1} MTG)

5) ALAm = ALAc
6) ARGm = ARGc
7) ASNm = ASNc
8) ASPm = ASPc
9) CYSm = CYSc
10) GLNm = GLNc
11) GLUm = GLUc
12) GLYm = GLYc
13) HISm =HISc
14) ILEm = ILEc
15) LEUm = LEUc
16) LYSm = LYSc
17) METm = METc
18) PHEm = PHEc
19) PROm = PROc
20) SERm = SERc
21) THRm = THRc
22) TRPm = TRPc
23) TYRm =TRYc
24) VALm = VALc

Reactions of which the metabolic flows can be determined by Equation (3)

25) R5P + ATP + GLN = HIS + AKG + 2 NADH
26) SER + ACCOA = CYS
27) SER = GLY
28) 2 PEP + ATP + GLN + SER = TRP + GLU + PYR + CO_2
29) 2 PEP + NADPH + ATP + GLU = PHE + AKG + CO_2
30) 2 PEP + NADPH+ ATP + GLU = TYR + AKG + NADH + $2CO_2$
31) PYR + GLU = ALA + AKG
32) 2 PYR + NADPH + GLU = VAL + CO_2 + AKG
33) 2 PYR + NADPH + GLU + ACCOA = LEU + AKG + H_2O + $2CO_2$
34) GLU + 2NADPH + ATP = PRO
35) 2 GLU + ASP + 2ATP + NADPH + ACCOA = ARG + AKG
36) GLU + NH_3 + ATP = GLN
37) ASP + ATP + NH_3 = ASN
38) ASP + ATP + 2 NADPH + CYS = MET + PYR + NH_3
39) ASP + 2 ATP + 2 NADPH = THR
40) ASP + 2 NADPH + ATP + GLU + PYR = LYS + AKG + CO_2
41) ASP + 3 NADPH + 2 ATP + PYR + GLU = ILE + CO_2 + AKG + NH_3

Reactions of which the metabolic flows must be determined by metabolite balancing

42) G6P = R5P + CO_2 + 2 NADPH
43) G6P = F6P
44) F6P + ATP = 2 GAP
45) GAP = G3P + ATP + NADH

46)	G3P = PEP
47)	G3P + GLU = SER + AKG + NADH
48)	PEP = PYR + ATP
49)	PYR = ACCOA + CO_2 + NADH
50)	PYR + CO_2 + ATP = OAA
51)	ACCOA + OAA = CIT
52)	CIT = AKG + NADPH + CO_2
53)	AKG + NH3 + NAPDH = GLU
54)	AKG = OAA + ATP + CO_2 + 2 NADH + FADH
55)	OAA + GLU = ASP + AKG

(Continued)

(Note: subscripts m and c in reactions (5) through (24) indicate the amino acid in the medium and in the cell, respectively.)

3. Determination of metabolic-flow distributions

The first group of the reactions shown in Table 1 actually involves those reactions which have a corresponding extracellular metabolite that can be measured directly. This group includes the metabolic flows of the intake of all twenty amino acids (mmol $\cdot$ L^{-1} $\cdot$ h^{-1}) from the medium to cell (their intake rate), or from the cell into the medium (their excretion rate). This can be simply determined by measuring the amino acid concentration and calculated by Equation (1). In other words, they are determined by the direct measurement of the amino acid concentration at two different sampling intervals. This group also includes the metabolic flows of glucose to G6P, the synthesis of cell mass and product, which can be respectively determined by measuring the consumption rate of glucose, the production rate of cell mass and MTG. This holds for the reactions (1) through (24) in Table 1.

The second group deals with the synthesis of certain amino acids such as reactions (25) through (41). The metabolic flows can be determined by Equation (3). Note that each of these 17 reactions concerns the synthesis of an amino acid that is only involved in one reaction for its production. The metabolic flows of these 17 reactions can be determined by Equation (3). For Asp, Ser and Glu, it must be treated in a different way described below.

Table 2. Metabolites considered in the reaction network

Metabolite		Functions
[1]	ACCOA	intermediate and cell synthesis
[2]	AKG	intermediate and cell synthesis
[3]	ALA	from pyr and for protein, MTG
[4]	ARG	from Glu and for protein, MTG
[5]	ASN	from Asp and for protein, MTG
[6]	ASP	from OAA and for Arg, Asn, Met, Thr, Lys, Ile, protein and MTG
[7]	Cell mass	exclusive protein, from ACCOA, AKG, F6P, G6P, G3P, GAP, R5P, PEP, OAA and PYR)
[8]	CIT	intermediate
[9]	CYS	from Ser and for protein and MTG
[10]	F6P	intermediate and cell synthesis
[11]	GAP	intermediate and cell synthesis
[12]	G3P	intermediate and cell synthesis
[13]	G6P	intermediate and cell synthesis
[14]	GLN	from Glu and for protein and MTG
[15]	GLU	from AKG and for Phe, Ala, Val, Leu, Gln, Pro, Asn, Asp, Lys, Ile, protein and MTG
[16]	GLC	directly measurable
[17]	GLY	from Ser and for protein and MTG
[18]	HIS	from R5P and for protein and MTG
[19]	ILE	from Asp and for protein and MTG
[20]	LEU	from PYR and for protein and MTG
[21]	LYS	from Asp and for protein and MTG
[22]	MET	from Asp and for protein and MTG
[23]	MTG	directly measurable
[24]	OAA	intermediate and cell synthesis
[25]	PEP	intermediate and cell synthesis
[26]	PHE	from PEP and for protein and MTG
[27]	PRO	from Glu and for protein and MTG
[28]	PROTEIN	directly measurable (52.4% in dry cell mass)
[29]	PYR	intermediate and cell synthesis
[30]	R5P	intermediate and cell synthesis
[31]	Ser	from G3P and for Cys, Gly, protein and MTG
[32]	THR	from Asp and for protein and MTG
[33]	TRP	from PEP and for protein and MTG
[34]	TYR	from PEP and for protein and MTG
[35]	VAL	from PEP and for protein and MTG

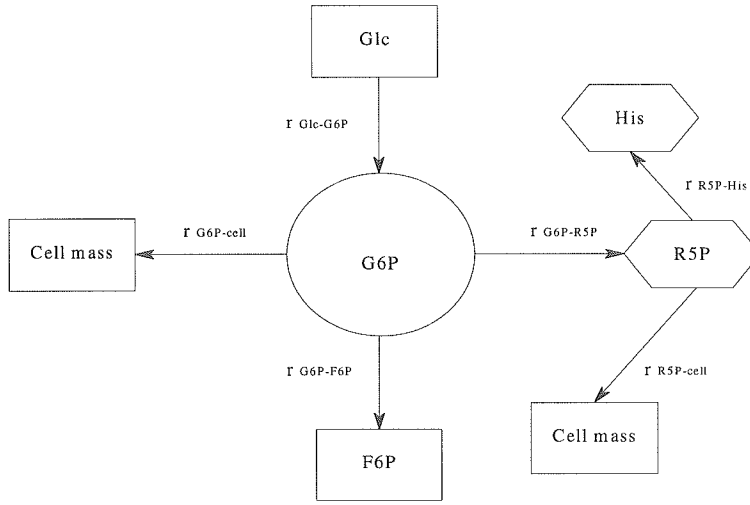

Figure 3. Schematic of metabolic balancing of G6P

For the calculation of the rest of the metabolic flows involved in the metabolic pathways, namely for reactions (42) through (55), the method of metabolite balancing is used. Although this metabolite balancing method has been described elsewhere (Erickson 1978, Aiba & Matsuoka 1979, Papoutsakis 1984, Papoutsakis & Meyer 1985, Holms 1986, Goel *et al.* 1993, Stephanopoulos & Vallino 1991, Vallino & Stephanopoulos 1990, 1993, Bailey 1991, Varma & Palsson 1993, 1994, Jørgensen *et al.* 1995, van Gulik & Heijnen 1995, Marx *et al.* 1996, Nielsen & Villadsen 1994, Ferrance *et al.* 1993, Bonarius *et al.* 1996, Savinell & Palsson 1992 a, b), a brief explanation is given here. For example, to calculate the metabolic flow from G6P to F6P, $r_{G6P\rightarrow F6P}$, a mass balance can be established over the metabolite G6P, as shown in Figure 3. The inflow is coming from glucose and outflows are going to F6P, cell mass and R5P. Around the G6P pool, $r_{glc\rightarrow G6P}$ can be determined by directly measuring the glucose consumption and $r_{G6P\rightarrow cell}$ can be determined by directly measuring the cell growth. For the determination of $r_{G6P\rightarrow R5P}$, the results of $r_{R5P\rightarrow His}$ and $r_{R5P\rightarrow cell}$ are used. Here the former is determined by Equation (3) and the latter is determined by directly measuring the cell mass production rate. By applying the pseudo-steady-state approximation here for the intracellular metabolites, i.e. there is no net accumulation of intermediate metabolites, a metabolite balance can thus be set up for the determination of the flow $r_{G6P\rightarrow F6P}$:

$$r_{G6P\rightarrow F6P} = r_{Glc\rightarrow G6P} - r_{G6P\rightarrow cell} - r_{G6P\rightarrow R5P} = r_{glc} - 0.205r_{cell} - r_{His} - 0.754r_{cell} \quad (4)$$

For the determination of the metabolic flows around certain metabolite pools which are involved in more reactions, i.e. either consumed for the synthesis of other metabolites or produced from other reactions, their balancing can be performed in a similar way. In the whole reaction system, as can be seen in Table 1, these pools include AcCoA, Pyr, AKG and Glu. A schematic representation of metabolic balancing around these pools is shown in Figure 4. Based on the same principle as Equation (4), a set of metabolite balancing equations can be established to determine the metabolic flows of reactions of (42) through (55). These equations are given in the Appendix.

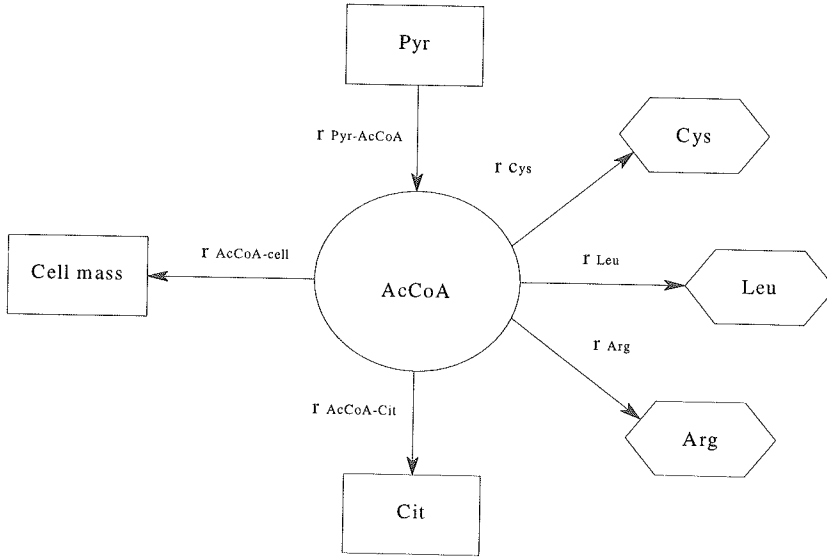

Figure 4 (a). Schematic of metabolic balancing of AcCoA. Because AcCoA is consumed in the synthesis of Cys, Leu and Arg, this must be taken into the consideration of metabolic balancing of AcCoA.

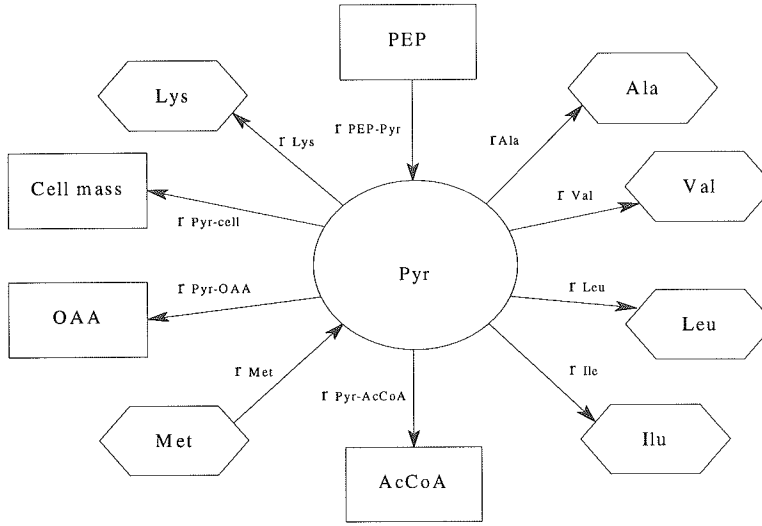

Figure 4 (b). Schematic of metabolic balancing of Pyr

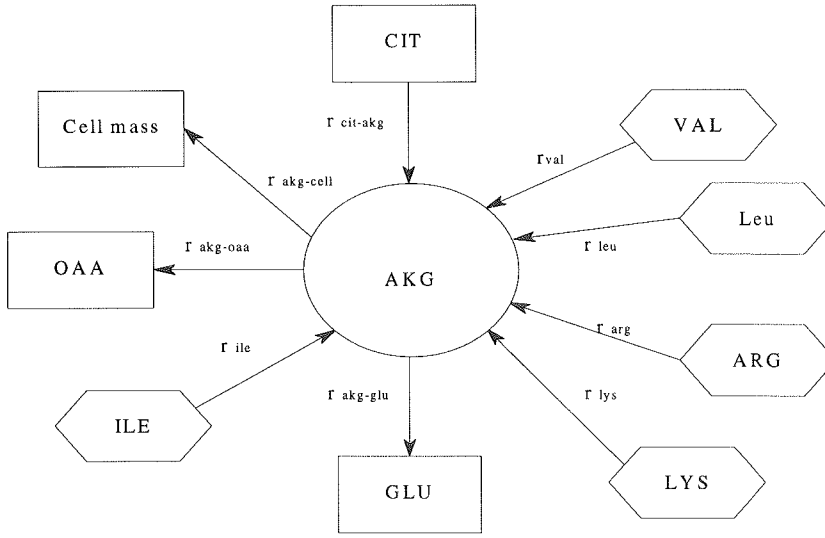

Figure 4 (c). Schematic of metabolic balancing of AKG

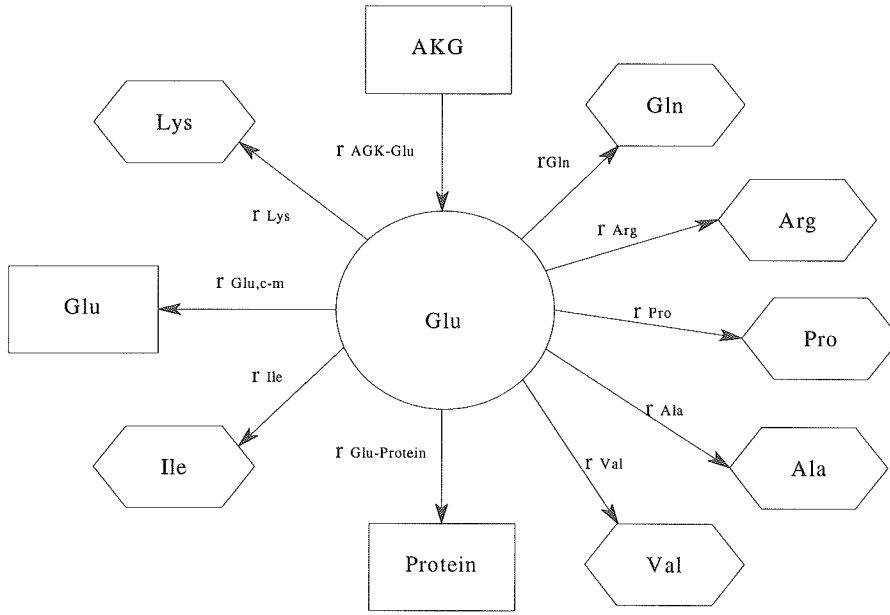

Figure 4 (d). Schematic of metaoblic balancing of Glu

MATERIALS AND METHODS

Fermentation

Microorganism

Streptoverticillium mobaraense (CBS 20778) was obtained from the Nederlands Centraalbureau voor Schimmelcultures (CBS, Baarn, Netherlands). The microorganism was pre-cultured on oat-meal agar at 30 °C for 7 days. Spore suspension was prepared in sterile water and used for inoculation.

Batch culture

Batch culture was carried out as described by Ando *et al* (1989) with modifications (Zhu *et al.* 1996). The medium was composed of (g/L) soluble starch (20), peptone (20), $MgSO_4 \cdot 7H_2O$ (2), KH_2PO_4 (2), K_2HPO_4 (2), yeast extract (2) and was supplemented with certain amino

acids (Zhu *et al.* 1996). Fermentation was done in a 2.5 L Bioflo II fermenter (New Brunswick, NJ) with a working volume of 2.0 L. Aeration was maintained at 1.0 L/min. Agitation was set at 400 rpm for the first 18 h and turned to 500 rpm afterwards. Sterile polypropylene glycerol (PPG) in a concentration of 0.5 g/L was used for automatic foam control. CO_2 evolution rate was measured and recorded automatically by a gas analyser. pH was set at 6.5 and controlled automatically using 1.0 M HCl and 1.0 M NaOH.

Assay

Biomass dry weight

Biomass dry weight was measured by filtering samples through a preweighed filter paper (Whatman, GF/C). The residue on the filter was washed with distilled water and dried at 105 °C until constant weight.

Residual sugar

Starch was completely hydrolysed before measurement. Pure glucose released was measured according to the method described by Miller (1960). Glucose was used for preparing the calibration curve.

MTG activity

The colorimetric procedure described by Grossowicz *et al.* (1950) was used for MTG activity measurement. A calibration curve was made using L-glutamic acid γ-monohydroxamate. One unit of MTG is defined as the formation of 1 μmol L-glutamic acid γ-monohydroxamate per minute at 37 °C.

Amino acids

All amino acidsin the sample were measured by an amino-acid analyser, Biotronik LC 6001 (Maintal, Germany). Since Asn and Gln are converted to Asp and Glu, respectively, due to hydrolysis, their concentration was calculated following the reported ratio (Bonarius *et al.* 1996).

RESULTS AND DISCUSSION

Fermentation profiles

Fermentation profiles of *S. mobaraense* for MTG production are illustrated in Figure 5. These profiles include growth, MTG production, CO_2 evolution, glucose consumption, dissolved oxygen (DO) and total amino acids concentration both in free and protein-bound form, during the fermentation. Because similar results were found in several fermentation batches (results not shown here), it can be concluded that these profiles are representative.

The whole fermentation course can be divided into four phases, namely 0-12 h, 12-24 h, 24-36 h and 36-48 h. The reason of dividing the whole fermentation period into four phases can be explained by Figure 5. During the first phase, from 0 to 12 h, the increase of cell mass, the consumption of glucose, evolution of CO_2, decrease of DO and amino acids in the medium, are not significant. MTG activity can hardly be detected during the first phase. During the second phase, from 12 to 24 h, cell mass, CO_2, DO, glucose and amino acids change very sharply. Within this phase, the increase of MTG activity is, however, not significant. It is therefore dominated by growth and the start of MTG production. During the third phase, from 24 to 48 h, the growth continues at a lower rate and MTG production is dominating. After 36 h, not only growth but also MTG production and glucose consumption have stopped. The growth curve in Figure 5 shows a slight increase after 36 h, but this might have been caused by measurement errors in cell mass dry weight at the end of the fermentation due to protein precipitation caused by MTG cross-linking. Also the profiles of other parameters show these four representative phases. Therefore, we investigated the metabolic flows and analysed the fermentation profiles during the first three fermentation periods.

Determination of metabolic flows

As mentioned earlier (see Table 1), all the metabolic flows are divided into three types. Table 3 gives the directly measured metabolic flows, including glucose consumption, cell synthesis (including cellular proteins although the latter are treated separately), MTG production and the excretion (or intake) of all 20 amino acids from the cell into the medium. Table 4 gives the metabolic flows that are determined by Equation (3), namely all amino acids except Asp, Glu

and Ser. The rest metabolic flows, i.e. those of reaction (42) through (55), are determined by metabolite balancing equations listed in the Appendix. The results obtained by solving these equations are given in Table 5.

Discussion of metabolic flows and amino-acid profiles

1. The first phase (0-12 h)

During this phase, as shown in Figure 5, only slight changes occurred in cell mass synthesis, MTG production, DO, glucose consumption, CO_2 evolution and total amino acids concentration. Almost all amino acids are taken up by the cells, as shown in the 0-12 h column of Table 3, all $r_{a,i}^{c\to m}$, except $r_{Met}^{c\to m}$, have a negative value. However, all amino acids, except Trp, Phe, Tyr and Gln, are also produced during this period, even though there are amino acids available in the medium; this can be seen in the corresponding column (0 - 12 h) of Table 4. The amount of amino acids required for cell synthesis is stoichiometrically determined (Zhu *et al.* 1996). If the intake of amino acids from the medium does not meet such a stoichiometric requirement, the shortage of pertinent amino acids must be produced by the cell itself. This phenomenon is also reflected by the reactions which provide important precursors of amino acid synthesis. These flows include $r_{Pyr\to OAA}$, $r_{AKG\to Glu}$ and $r_{OAA\to Asp}$, as shown in the corresponding column (0 - 12 h) of Table 5.

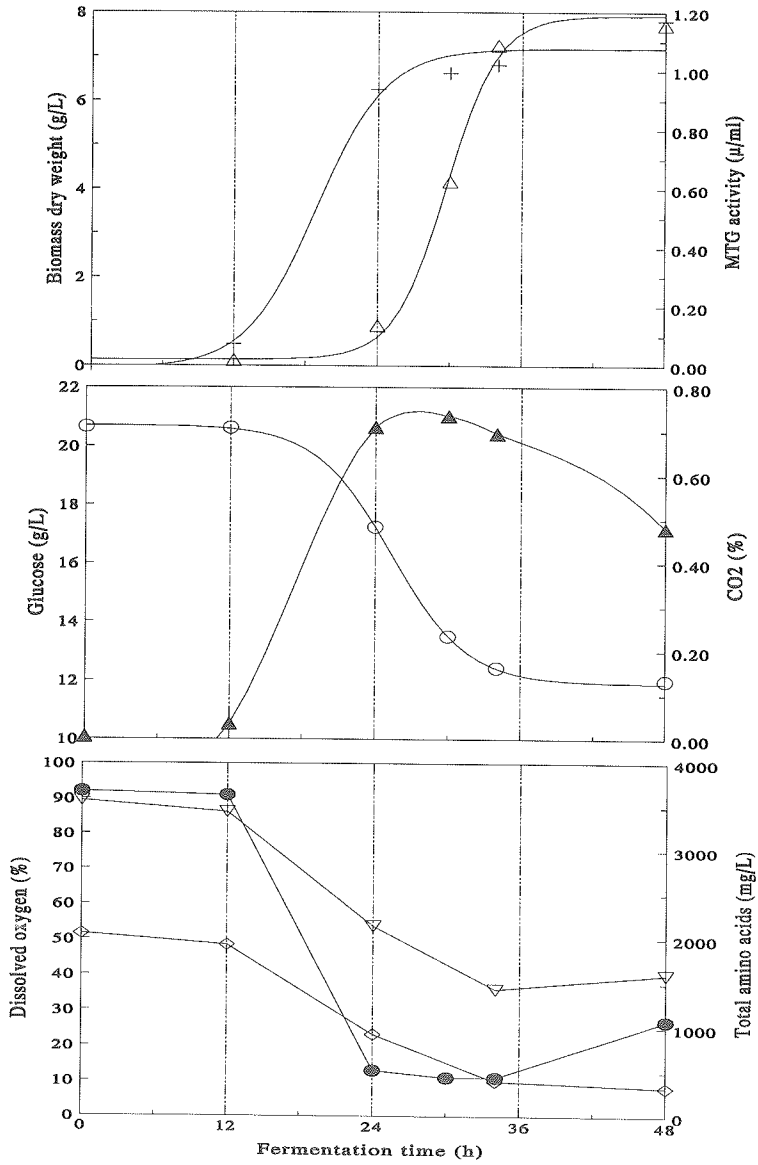

Figure 5. Fermentation profiles. + Cell mass dry weight; ▵ MTG activity; ▲ CO_2 evolution rate; ○ glucose; ◍ dissolved oxygen; ◇ total free amino acid, and ▽ total protein-bound amino acid.

Table 3. Metabolic flow of reactions (1) through (24), determined by direct measurement; a negative sign means that the flow direction is from medium into the cell.

Metabolic flow	Fermentation period		
(mmol · L^{-1} · h^{-1})	0 - 12 h	12 - 24 h	24 - 36 h
r_{glc}	-0.0583	-1.541	-2.34
r_{cell} (g · L^{-1} h^{-1})	0.051	0.464	0.0858
$r_{protein}$ (g · L^{-1} · h^{-1})	0.0267	0.243	0.0449
r_{MTG} (g · L^{-1} · h^{-1})	0	0.00608	0.0861
$r_{Ala}^{c\to m}$	-0.00935	-0.0841	-0.0159
$r_{Arg}^{c\to m}$	-0.00478	-0.15	-0.0205
$r_{Asn}^{c\to m}$	-0.00188	-0.103	-0.0206
$r_{Asp}^{c\to m}$	-0.00302	-0.123	-0.0247
$r_{Cys}^{c\to m}$	-0.00069	-0.00206	0
$r_{Gln}^{c\to m}$	-0.0125	-0.069	-0.0433
$r_{Glu}^{c\to m}$	-0.0158	-0.0861	-0.0549
$r_{Gly}^{c\to m}$	-0.00555	-0.143	-0.0222
$r_{His}^{c\to m}$	-0.0123	-0.0139	-0.0397
$r_{Ile}^{c\to m}$	-0.00318	-0.0794	-0.0603
$r_{Leu}^{c\to m}$	-0.00572	-0.172	-0.1
$r_{Lys}^{c\to m}$	-0.00285	-0.135	-0.0353

$r_{Met}^{c \to m}$	0.00111	-0.00335	-0.0491
$r_{Phe}^{c \to m}$	-0.00908	-0.00908	-0.0877
$r_{Pro}^{c \to m}$	-0.00362	-0.101	0.0209
$r_{Ser}^{c \to m}$	-0.0261	-0.0824	0.00396
$r_{Thr}^{c \to m}$	-0.0049	-0.0405	-0.0405
$r_{Trp}^{c \to m}$	-0.011	-0.00531	-0.00204
$r_{Tyr}^{c \to m}$	-0.0128	-0.0124	-0.0685
$r_{Val}^{c \to m}$	-0.00498	-0.0718	-0.0597

(continued)

Table 4. Metabolic flow of reactions (25) through (41), determined by equation (3); a negative sign means that the flow direction is from medium into the cell.

Metabolic flow	Fermentation period		
(mmol · L^{-1} · h^{-1})	0 - 12 h	12 - 24 h	24 -36 h
$r_{R5P \to His}$	-0.0098	0.00925	-0.0354
$r_{Ser \to Cys}$	0.00447	0.0448	0.00866
$r_{Ser \to Gly}$	0.015	0.0438	0.0123
$r_{PEP \to Trp}$	-0.00846	0.0179	0.00221
$r_{PEP \to Phe}$	-0.00137	0.061	-0.0741
$r_{PEP \to Tyr}$	-0.00772	0.0344	-0.0598
$r_{Pyr \to Ala}$	0.0138	0.126	0.023
$r_{Pyr \to Val}$	0.0104	0.0683	-0.0338
$r_{Pyr \to Leu}$	0.0148	0.0149	-0.0664
$r_{Glu \to Pro}$	0.00925	0.0156	0.0426

$r_{Glu \rightarrow Arg}$	0.00808	-0.0332	0.00106
$r_{Glu \rightarrow Gln}$	-0.00228	0.0242	-0.0261
$r_{Asp \rightarrow Asn}$	0.00328	-0.057	-0.0119
$r_{Asp \rightarrow Met}$	0.0113	0.0899	-0.0319
$r_{Asp \rightarrow Thr}$	0.00797	0.0764	-0.0189
$r_{Asp \rightarrow Lys}$	0.0177	0.0514	-0.00075
$r_{Asp \rightarrow Ile}$	0.00969	0.0375	-0.0387

(Continued)

Table 5. Metabolic flow of reactions (42) through (55), determined by solving metabolite balancing equations, a negative sign before the number means that the flow has a reverse direction

Metabolic flow	Fermentation period		
(mmol. $\cdot L^{-1} \cdot h^{-1}$)	0 - 12 h	12 - 24 h	24 -36 h
$r_{G6P \rightarrow R5P}$	0.0287	0.359	0.0292
$r_{G6P \rightarrow F6P}$	0.0138	1.09	2.29
$r_{F6P \rightarrow GAP}$	0.0205	2.11	4.57
$r_{GAP \rightarrow G3P}$	0.0139	2.05	4.56
$r_{G3P \rightarrow PEP}$	-0.0179	1.6	4.45
$r_{G3P \rightarrow Ser}$	0.000305	0.164	0.0531
$r_{PEP \rightarrow Pyr}$	-0.00305	1.47	4.58
$r_{Pyr \rightarrow AcCoA}$	-0.125	0.957	4.71
$r_{Pyr \rightarrow OAA}$	0.0655	0.291	-0.0405
$r_{AcCoA \rightarrow Cit}$	-0.28	-0.229	4.55
$r_{Cit \rightarrow AKG}$	-0.28	-0.229	4.55
$r_{AKG \rightarrow Glu}$	0.0632	0.208	-0.0793
$r_{AKG \rightarrow OAA}$	-0.287	-0.339	4.48
$r_{OAA \rightarrow Asp}$	0.0573	0.168	-0.109

2. The second phase (12 - 24 h)

During this phase, as shown in Figure 5, cell mass, glucose consumption and CO_2 evolution increased drastically while DO dropped to the lowest point. Amino acid consumption became

more active, which is reflected in the higher intake rate of all amino acids as can be seen in the 12-24 h column of Table 3. Since the cells grow very fast during this period, large quantities of amino acids are needed for cell synthesis. Correspondingly, the production of most amino acids during this period is also active to complement the stoichiometrically required amount, as shown in the pertinent column (12 - 24 h) of Table 4. Consequently, the metabolic flows of the reactions providing amino-acid precursors are also active during this period, such as $r_{G6P \rightarrow R5P}$, $r_{G3P \rightarrow Ser}$, $r_{Pyr \rightarrow OAA}$, $r_{AKG \rightarrow Glu}$ and $r_{OAA \rightarrow Asp}$, as shown in the corresponding column (12 -24 h) of Table 5.

3. The third phase (24 - 36 h)

After 24 h of fermentation, the growth rate decreases, while in contrast, the amount of MTG increases to the highest level until 36 h, as shown in Figure 5. In addition, the consumption of glucose and amino acids continued during this phase. Within this phase, most amino acids, except Cys, Pro and Ser, are still taken up by the cell from the medium, as shown in the pertinent column of Table 3. The synthesis of more than half of the amino acids, however, is not active during this period, as shown in the corresponding columns of Tables 4 and 5. As stated, production of MTG particularly occurred during this phase, even though growth had stopped. The supply of amino acids during this period is not sufficient for further cell growth but apparently can still meet the requirement of MTG synthesis. The reason is that the stoichiometric requirements of amino acids for the synthesis of MTG is less than 10% of that for cell mass (Zhu *et al.* 1996). In contrast to the previous phase (12 - 24 h), the reactions providing amino acid precursors were not active during this phase, such as $r_{G6P \rightarrow R5P}$, $r_{G3P \rightarrow Ser}$, $r_{Pyr \rightarrow OAA}$, $r_{AKG \rightarrow Glu}$ and $r_{OAA \rightarrow Asp}$, as shown in the 24 - 36 h column of Table 5. The main flow of carbon goes in the direction of the TCA cycle which plays the main role in providing energy during this phase. This also explains why there is still a relatively high CO_2 evolution rate during this phase. Also DO was at the low range. In addition, compared to the phase of 12 - 24 h, the metabolic flow $r_{G6P \rightarrow R5P}$ is much less active during this phase. One of the reasons is that the reaction of G6P → R5P that supplies building blocks for cells is not as active as in the previous period. Another reason is likely that at the early stage of fermentation the pentose-phosphate pathway plays the main role in providing energy while at the later stage TCA takes over this function, which corresponds to similar conclusions obtained from studies on the

metabolism of animal cells (Bonarius *et al.* 1996).

4. The fourth phase (36 - 48 h)

During the last phase (36 - 48 h), MTG production also stopped, as well as glucose and amino acids consumption. CO_2 evolution rate dropped drastically and DO increased. Because of the inaccuracy of cell mass measurement in this phase, the metabolic flows within this period cannot be determined. Protein bound amino acids increased during this period while free amino acids decreased slightly. This also indicates that cross-linking might have occurred during this period causing the increasing of protein-bound amino acids. The profile of CO_2 evolution and DO indicates that the strain became inactive at this phase.

From Figure 5 it can be seen that MTG production occurs in particular when growth has stopped. The production has a retardation compared to growth. This indicates that the accumulation of a certain amount of cell mass is the essential prerequisite for production.

After 36 h fermentation, the total amount of amino acids in the medium is 1.834 g/L (see Figure 5 and Tables 6, 7). At that time the amount of MTG is about 1200 units/L. If we take the specific activity of purified MTG to be 20000 units per gram protein (Ando *et al.* 1989), the total amount of MTG in the medium is about 0.06 g/L and thus only contributes a minor part to the total amino acid measured in the medium. Theoretically, the rest amino acids will be still sufficient for a further cell mass increase. If we assume that these proteins can be completely converted to cell protein and the cell contains 52.4% protein, the increase in amount of cell mass could be 3.5 g/L.

However, the concentration of most free amino acids measured after 36 h was in the range of 10^{-3} to 10^{-2} mmol/L only, as shown in Table 6, except Gln, Glu, Asn, Asp, Met and Trp. Theoretically, according to the schematic pathway shown in Figure 2, certain amino acids such as Met, Thr, Lys, Asn, Ile, Gln, Arg and Pro should not be in shortage so long as Glu and Asp are in surplus. However, only a few of them, namely Gln, Glu, Asn, Asp, Met and Trp, are still in relatively high concentrations after 36 h. Most free-form amino acids are readily taken up by the cells during the logarithmic growth phase. The lower availability of free

amino acids is probably the limiting factor for further cell growth. Most residual amino acids measured at the later stage of the fermentation were in the protein-bound form, as shown in Figure 5 and Table 7. The reason of preventing them from being accessible for the microorganism can probably be that they were cross-linked by MTG. Another reason might be that the enzymes needed for hydrolysing peptones were cross-linked by MTG. After a certain

Table 6. Free amino acids at different fermentation phase (mg/L)

	0 h	12 h	24 h	36 h	48 h
Ala	55	55	11	5	4
Arg	243	245	30	1	1
Asn	166	171	89	85	77
Asp	205	210	109	104	95
Cys	8	8	3	4	4
Gln	60	58	67	41	35
Glu	76	73	86	52	44
Gly	79	83	10	1	4
his	63	64	9	5	5
Ile	107	110	58	10	10
Leu	224	231	9	1	3
Lys	156	159	17	3	1
Met	88	91	74	26	14
Phe	136	83	80	16	14
Pro	17	18	0	0	0
Ser	71	58	5	8	10
Thr	44	44	36	1	1
Trp	55	38	25	29	5
Tyr	135	67	75	1	1
Val	69	70	42	4	2

Table 7. Protein bound amino acids at different fermentation phase (mg/L)

	0 h	12 h	24 h	36 h	48 h
Ala	114	104	58	47	57
Arg	176	164	65	51	45
Asn	235	227	143	114	143
Asp	289	279	176	140	176
Cys	25	24	26	25	30
Gln	334	314	184	134	162
Glu	424	399	234	171	205
Gly	123	114	58	47	57
his	120	96	125	55	60
Ile	159	151	78	31	35
Leu	269	253	122	53	62
Lys	214	206	100	62	71
Met	54	53	64	24	24
Phe	165	200	185	75	40
Pro	236	230	108	137	150
Ser	153	133	82	84	108
Thr	130	123	73	50	62
Trp	24	14	14	5	3
Tyr	155	195	160	85	55
Val	174	166	93	47	54

period of fermentation, especially after the synthesis of MTG, the concentration of free amino acid remained almost constant. Although there was still a certain amount of protein-bound amino acids in the medium, they were apparently not accessible to the microorganism. This indicates that these amino acids cannot be simply provided through the pathways as described in Figure 2. However, the strain, *S. mobaraense*, does have the ability to synthesize all amino acids according to the results shown in Table 4. The limitation for the synthesis of these

amino acids seems then the shortage of nitrogen, in this case free ammonium. This suggests the importance of using other nitrogen sources such as ammonium and/or other fermentation techniques to stimulate further growth and product formation. The strategy and technique dealing with this phenomenon will be described in our subsequent paper.

ABBREVIATIONS AND SYMBOLS

Metabolites

Glc	glucose
G6P	glucose-6-phosphate
R5P	ribose-5-phosphate
F6P	fructose-6-phosphate
GAP	glyceraldehyde-3-phosphate
G3P	3-phosphoglycerate
PEP	phosphoenol-pyruvate
Pyr	pyruvate
AcCoA	acetyl coenzyme A
Cit	citrate
AKG	α-ketoglutarate
OAA	oxaloacetate
MTG	microbial transglutaminase

Greeks

$\theta_{a,\,i}^{cell}$	stoichiometric coefficient of the *i* th amino acid for the synthesis of cell
$\theta_{a,\,i}^{MTG}$	stoichiometric coefficient of the *i* th amino acid for the synthesis of MTG
$\Delta c_{a,i}$	concentration difference in the medium of the *i* th amino acid at two sampling times
Δc_{cell}	concentration difference of cell mass at two sampling times
Δc_{MTG}	concentration difference of MTG at two sampling times

Rates

$r_{a,\,i}^{m\neg c}$ intake rate of the i th amino acid from medium to the cell

$r_{a,\,i}^{\Delta t}$ production rate of the i th amino acid during the fermentation period t_2 and t_1, except for Asp, Glu and Ser

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APPENDIX

Equations for the determination of metabolic flows of reaction (42) through (55)

$$r_{G6P\rightarrow R5P} - r_{R5P\rightarrow cell} - r_{R5P\rightarrow His} = 0 \quad (42)$$

$$r_{G6P\rightarrow F6P} + r_{G6P\rightarrow cell} + r_{G6P\rightarrow R5P} - r_{Glc} = 0 \quad (43)$$

$$r_{F6P\rightarrow GAP} - 2 \times (r_{G6P\rightarrow F6P} - r_{F6P\rightarrow cell}) = 0 \quad (44)$$

$$r_{GAP\rightarrow G3P} - r_{F6P\rightarrow GAP} + r_{GAP\rightarrow cell} = 0 \quad (45)$$

$$r_{G3P\rightarrow PEP} - r_{GAP\rightarrow G3P} + r_{G3P\rightarrow cell} + r_{G3P\rightarrow Ser} = 0 \quad (46)$$

$$r_{G3P\rightarrow Ser} - r_{Cys} - r_{Gly} - r_{Trp} - r_{Ser}^{c\rightarrow m} - r_{Ser\rightarrow protein} = 0 \quad (47)$$

$$r_{PEP\rightarrow Pyr} - r_{G3P\rightarrow PEP} + r_{PEP\rightarrow cell} + r_{Trp} + r_{Phe} + r_{Tyr} = 0 \quad (48)$$

$$r_{Pyr\rightarrow AcCoA} - r_{PEP\rightarrow Pyr} + r_{Pyr\rightarrow Cell} + r_{Pyr\rightarrow OAA} + r_{laA} + r_{Val} + r_{Leu} - r_{Met} + r_{Ile} + r_{Lys} = 0 \quad (49)$$

$$r_{Pyr\rightarrow OAA} + r_{AKG\rightarrow OAA} - r_{OAA\rightarrow Cell} - r_{OAA\rightarrow Asp} - r_{OAA\rightarrow Cit} = 0 \quad (50)$$

$$r_{AcCoA\rightarrow Cit} - r_{Pyr\rightarrow AcCoA} + r_{AcCoA\rightarrow Cell} + r_{Cys} + r_{Leu} + r_{Arg} = 0 \quad (51)$$

$$r_{Cit \rightarrow AKG} = r_{AcCoA \rightarrow Cit} = r_{OAA \rightarrow Cit} \tag{52}$$

$$r_{AKG \rightarrow Glu} - r_{Glu}^{c \rightarrow m} - r_{Gln} - r_{Arg} - r_{Pro} - r_{Glu \rightarrow protein} - r_{Lys} - r_{Ile} - r_{Val} - r_{Ala} = 0 \tag{53}$$

$$r_{AKG \rightarrow OAA} - r_{Cit \rightarrow AKG} + r_{AKG \rightarrow Cell} + r_{AKG \rightarrow Glu} - r_{Ile} - r_{Val} - r_{Leu} - r_{Arg} - r_{Lys} = 0 \tag{54}$$

$$r_{OAA \rightarrow Asp} - r_{Asp}^{c \rightarrow m} - r_{Asn} - r_{Met} - r_{Thr} - r_{Lys} - r_{Ile} - r_{Asp \rightarrow Protein} = 0 \tag{55}$$

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CHAPTER 5

FED-BATCH FERMENTATION DEALING WITH NITROGEN LIMITATION IN MICROBIAL TRANSGLUTAMINASE PRODUCTION BY *Streptoverticillium mobaraense*

ABSTRACT

In the later stages of a batch fermentation for microbial transglutaminase production by *Streptoverticillium mobaraense* the availability of nitrogen source accessible to the microorganism becomes critical. Fed-batch fermentation is investigated with the aim at avoiding this substrate limitation. When peptone is used as a nitrogen source in the feed, no significant improvement of growth and transglutaminase production is observed. This is probably due to crosslinking of the nitrogen source by the transglutaminase produced. Using an inorganic nitrogen source alone does not give satisfactory growth and production either. A fed-batch fermentation method is thus developed to deal with this problem. In the batch phase of the fermentation an initial medium containing peptone, designed on the basis of the stoichiometric requirements of the microorganism, is used to ensure optimal growth. In the feeding phase ammonium sulphate is used instead to avoid the crosslinking effect. The feed composition, mainly the amount of nitrogen and carbon source, is also based on the stoichiometric requirements of the organism, taking into account the replacement of peptone by ammonium sulphate. Using this fed-batch fermentation technique, cell-mass dry weight and transglutaminase production could be increased by 33% and 80%, respectively, compared

to those in a batch fermentation.

Key words: Fed-batch fermentation, transglutaminase, *Streptoverticillium mobaraense*, stoichiometry, medium design

INTRODUCTION

Through analysis of a batch fermentation for the production of microbial transglutaminase (MTG; protein-glutamine γ-glutamyltransferase, EC 2.3.2.13) by *Streptoverticillium mobaraense* (Zhu *et al.* 1995), we found that amino acids play an important role in the synthesis of cell mass and MTG (Zhu *et al.* 1996 a). On the basis of mass balances, we also studied the metabolism of these amino acids for the synthesis of cell mass and MTG in a batch fermentation (Zhu *et al.* 1997). The results show that the concentrations of free amino acids were very low in the later phase of the batch fermentation, which were probably caused by crosslinking catalysed by MTG. Free amino acids were probably limiting for further growth and MTG synthesis.

One of the techniques to deal with substrate limitations is fed-batch fermentation (Whitaker 1980; Moser 1985; van 't Riet and Tramper 1991). Fed-batch techniques have been intensively studied and applied during the last decades. More recently, Xie and Wang (1994) developed an integrated strategy for a fed-batch fermentation dealing with an animal-cell culture system. In their case, glucose and glutamine are the major carbon and nitrogen source. However, these two materials are also responsible for the production of toxic by-products, namely lactate and ammonium, especially when their concentration in the medium is high. Therefore, the strategy should be to develop a medium that not only provides these main materials based on the stoichiometry of the cell line for growth and production, but also to control the culture environment to eliminate the production of lactate and ammonium. In their study, the authors used first a medium based on the stoichiometry of the cell line to obtain an optimal cell growth. Then the fed-batch phase was realized by controlled feeding of glucose

and glutamine to eliminate the toxic effect of ammonia and lactate. This strategy provides the stoichiometric requirement of the cell while controlling the key components in the medium to a minimal level to avoid producing toxic by-products. The principle of this integrated fed-batch technique can be also applied to other fermentation processes where an optimal culture environment is required. For MTG production, peptone is a favourable nitrogen source used in the initial medium for optimal growth of the microorganism (Zhu *et al.* 1996 a). In the later phases of a batch fermentation, free amino acids are not available anymore in the medium, which probably causes the growth to stop. As MTG produced may crosslink peptides and/or amino acids, these materials may be unsuitable as nitrogen source in a fed-batch process. Therefore, we used nitrogen sources other than peptone/peptide, such as inorganic nitrogen sources, in a fed-batch fermentation process. This paper presents our work done on the development of initial and feeding media for fed-batch production of MTG.

MATERIALS AND METHODS

Microorganism

Streptoverticillium mobaraense (CBS 20778) was obtained from the Nederlands Centraalbureau voor Schimmelcultures (CBS, Baarn, Netherlands). The microorganism was cultured on oat-meal agar at 30 °C for 7 days. Spore suspension was prepared in sterile water and used for inoculation.

Assay

Biomass dry weight

Biomass dry weight was measured by filtering samples through a pre-weighed filter paper (Whatman, GF/C). The residue on the filter was washed with distilled water and dried at 105 °C until constant weight.

Residual sugar

Starch was hydrolysed by gluco-amylase (Sigma) before measurement. Glucose released was measured according to the DNS method described by Miller (1960). Glucose was used for

preparing the calibration curve.

MTG activity

The colorimetric procedure described by Grossowicz *et al.* (1950) was used for MTG activity measurement. A calibration curve was made using L-glutamic acid γ-monohydroxamate. One unit of MTG is defined as the formation of 1 μmol L-glutamic acid γ-monohydroxamate per minute at 37 °C.

Amino acids

All amino acids in the sample were measured by an amino-acid analyser, Biotronik LC 6001 (Maintal, Germany). Since Asn and Gln are expressed as Asp and Glu, respectively, due to hydrolysis, their concentrations were calculated following the reported ratio (Bonarius *et al.* 1996).

Crosslinking of amino acids by MTG

Purified MTG was added to solutions containing all 20 amino acids and incubated at 37 °C for 20 min. The same solution was treated at the same condition without adding MTG as reference. The amount of amino acids in the experimental group and the reference group was analysed by the method described above.

Selection of an inorganic nitrogen source

A series of experiments was done in Erlenmeyer flasks to select a suitable inorganic nitrogen source for fed-batch fermentation. All media contained soluble starch 2% (w/w), $MgSO_4$ 0.2%, K_2HPO_4 0.2% and KH_2PO_4 0.2%. Addition of other components such as peptone, ammonium sulphate, vitamins and spore elements was done in a way to check their effect on growth and MTG production (see Results and Discussion). One hundred ml medium was sterilized in a 500 ml Erlenmeyer flask at 121 °C for 20 min. After inoculation, the cultivation was done on a shaker at 28 °C and 220 rpm for 48 h. The culture broth was filtered through a pre-weighed filter paper for cell-mass dry-weight measurement and the supernatant was used for MTG activity measurement.

Fermentation

Batch culture was carried out as described by Ando *et al.* (1989) with modifications (Zhu *et al.* 1996 a). The medium was composed of (g/L) soluble starch (20), peptone (20), $MgSO_4 \cdot 7H_2O$ (2), KH_2PO_4 (2), K_2HPO_4 (2), yeast extract (2) and was supplemented with certain amino acids (Zhu *et al.* 1996 a). Fermentation was done in a 5.0 L (working volume) Bioflo II fermenter (New Brunswick, NJ). Aeration was maintained at 1.0 L/min. Agitation was set at 400 rpm for the first 18 h and turned to 500 rpm afterwards. Sterile polypropylene glycerol (PPG) in a concentration of 0.5 g/L was used for automatic foam control. CO_2 evolution rate was measured and recorded automatically by a gas analyser. pH was set at 6.5 and controlled automatically using 1.0 M HCl and 1.0 M NaOH. After 20 h, the fermentation was switched to fed-batch.

Feed design

For fed-batch fermentation using peptone as a nitrogen source in the feed, the amount of peptone used in the feed was calculated based on the stoichiometric requirements (Zhu *et al.* 1996 a) for a desired cell-mass concentration of 15 g/L with necessary amino-acid supplementation.

For fed-batch fermentation using ammonium sulphate as a nitrogen source in the feed, the feed contained all other components except peptone which was substituted by $(NH_4)_2SO_4$. The amount of $(NH_4)_2SO_4$ and soluble starch used in the feed was calculated based on the demand of nitrogen and glucose for a target cell-mass concentration of 15 g/L (see Appendix). The feed had the following composition (g/L): ammonium sulphate 10, $MgSO_4$ 2, KH_2PO_4 2, K_2HPO_4 2 and yeast extract 2. Soluble starch was not added to the feed because the residual sugar in the medium was still high enough to reach the desired cell-mass concentration.

RESULTS AND DISCUSSION

Fed-batch fermentation using peptone as nitrogen source in the feed

From a batch fermentation (Zhu *et al.* 1996 a; 1997) it was found that after 24 h growth slowed down and the biomass dry weight reached a final concentration of 7.5 g/L. MTG production reached its peak of 1 unit/ml at 34 h. Since glucose was in surplus in the medium, the nitrogen source was considered as a possible limiting factor. As shown by the results of previously conducted batch fermentations (Zhu *et al.* 1997), in the later stage most free amino acids were at a very low concentration. This probably caused growth and MTG production to stop. Therefore, fed-batch fermentation was applied in order to avoid nitrogen limitation. The concentration of peptone in the feed was designed for a target cell-mass concentration of 15 g/L with necessary amino-acid supplementation based on the stoichiometric model (Zhu *et al.* 1996 a). The total input of amino acids in this fed-batch system was thus much higher than that in a batch fermentation. Three fed-batch experiments were done and identical results were obtained. The growth and MTG production profiles from one of the three experiments are shown in Figure 1. Growth slowed down at about 28 h and reached a final cell concentration of 7.5 g/L. This is the same as that in a batch fermentation. MTG production, however, stopped already at 30 h and reached a peak of 0.6 unit/ml, which is even lower than that in a batch fermentation. The technique of fed batch fermentation using peptone as a nitrogen source in the feed did thus not improve the performance. These unsatisfactory results imply that limitation still occurs in the system with peptone in the feed. Therefore, the amino acid concentration in the fermentor was measured (Table 1).

After 24 h fermentation, concentrations of most free amino acids were very low in the fed-batch fermentation using peptone as a nitrogen source in the feed, which explains why no improvement was accomplished. The reason for these low concentrations, even though the amount of peptone added was higher than that in a batch fermentation, is very likely crosslinking of amino acids into non-accessible amino acids and peptides under the influence of the transglutaminase produced.

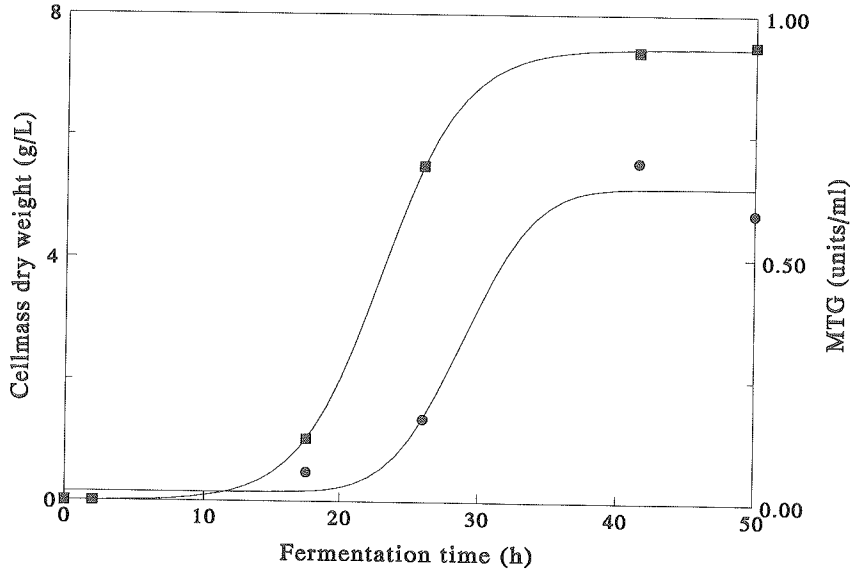

Figure 1. Growth and MTG production profiles of a fed-batch fermentation using peptone in the feed. ■ Cell mass dry weight (g/L); ● MTG (unit/ml)

To support this hypothesis, an incubation experiment of amino acids with transglutaminase was done. The amino-acid solution had the composition given in Table 2. Purified MTG was added to a concentration of 1 unit/ml. A reference solution was incubated under the same condition without adding MTG. The results given in Table 2 show that amino-acid concentration decreased after 20 min incubation with MTG. In the fermentations, free amino acids in the medium disappear by MTG-catalysed crosslinking. This can be demonstrated by an approximate calculation.

Table 1. Free-amino-acid measurement (mg/L) of fed-batch fermentation using peptone in feed

	0 h	16 h	24 h	40 h	48 h
Ala	50	45	6	6	6
Arg	210	180	2	1	1
Asp	330	290	90	90	110
Glu	110	100	120	80	120
Gly	70	60	2	1	2
His	50	40	2	1	1
Ile	90	120	50	5	2
Leu	220	215	40	1	4
Lys	150	130	2	2	2
Met	90	55	10	4	4
Phe	155	95	50	9	7
Ser	60	45	3	2	2
Thr	35	35	15	2	3
Tyr	155	75	40	1	1
Val	75	65	2	1	1

Based on the results in Table 2, the cross-linking rare of amino acids can be expressed as:

$$\left(\sum_{1}^{20} C_{a,i}^{t=0} - \sum_{1}^{20} C_{a,i}^{t=20}\right) \times 3 = 1083 \ (mg \cdot L^{-1} \cdot h^{-1})$$

The addition rate of amino acids by feeding can be calculated as following:

$$\frac{f \times \sum_{1}^{20} C_{a,i}^{feed}}{V} \approx 37.0 \ (mg \cdot L^{-1} \cdot h^{-1})$$

The calculation is done using a feeding rate of 0.04 L/h, two times peptone as much as in a batch fermentation and neglect the change of total volume in the fermenter.

Table 2. Cross-linking effect of MTG on amino acids

Amino acids	Reference group (mg/L)	Experimental (mg/L)
Ala	111	90
Arg	80	65
Asp	221	180
Glu	322	261
Gly	143	116
His	91	76
Ile	46	36
Leu	166	133
Lys	110	91
Met	183	146
Phe	92	75
Ser	127	104
Thr	47	38
Tyr	60	47
Val	107	87

It is clearly to see from the above estimation that the addition rate of amino acids is much lower than the rate of amino acids to be cross-linked. Consequently, the availability of free amino acids can be expected to be low in a fed batch using peptone in the feed.

Fed-batch fermentation using inorganic nitrogen source in the feed

In order to carry out a fed-batch fermentation using a nitrogen source that cannot be crosslinked by MTG, experiments were done to select a suitable nitrogen source in the feed.

The experiment was carried out in 500 ml shake flasks containing 100 ml medium. The composition of the media and the results of growth and MTG production are given in Table 3. Experiment No. 1 shown in Table 3 was done using medium designed based on the stoichiometric model (Zhu *et al* 1996 a) and is used here as the reference.

Table 3. Selection of a nitrogen source

	Medium composition (%w/w) and results						
No.	Peptone	$(NH_4)_2SO_4$	Yeast extract	Vitamins*	Spore elements**	Growth	MTG
1	2	-	0.2	-	-	+	+
2	-	1	0.2	-	-	±	±
3	-	1	-	added	-	-	-
4	-	1	-		added	-	-
5	-	1	-	added	added	-	-
6	-	1	-	-	-	-	-
7	-	-	1.0	-	-	±	-
8	2	-	-	added	added	-	±

* Vitamin solution added to the medium (Zhu *et al.* 1996 b)
** Spore element solution added to the medium (Cook & Hütter 1981)
\+ Good growth and MTG production
± Reasonable growth and MTG production
\- Poor growth and MTG production
In all experiment soluble starch (2%) was used as main carbon source.

The results in Table 3 show that ammonium sulphate can be used as a nitrogen source when yeast extract is also added to the medium. In the absence of yeast extract, neither peptone nor ammonium sulphate as nitrogen source gave good results with respect to growth and MTG production. This indicates that yeast extract is essential for growth and MTG production. It must be noted, however, that yeast extract alone used as a nitrogen source did not give satisfactory growth and MTG production, as shown in Table 3. The function of yeast extract is probably to provide growth factors which are important in the culture of *S. mobaraense* for

MTG production. Replacement of yeast extract with vitamins and/or spore elements, however, did not give satisfactory results. Therefore, yeast extract is used throughout the experiments as a necessary component in the feed.

Based on the results shown in Table 3, it can be concluded that ammonium sulphate can be used as a nitrogen source in the culture of *S. mobaraense* for MTG production. The growth and MTG production level is, however, lower than that using peptone as a nitrogen source. Therefore, it is better to use initially in the batch phase a medium with peptone as nitrogen source to ensure optimal growth. Then the fermentation is switched to the feeding phase in which ammonium sulphate is used to avoid the crosslinking effect.

The profiles of a fed-batch fermentation using ammonium sulphate as nitrogen source in the feed are shown in Figure 2. The batch phase of the fermentation, phase I shown in the figure, started with 4 L stoichiometrically designed medium (Zhu *et al.* 1996 a) and was run until shortly before the growth stops in a batch phase (at 20 h). Then the fermentation was switched to the feeding phase, phase II shown in the figure. The feeding rate was controlled at 0.040 L $\cdot$ h^{-1} so that the total volume of the medium in the fermenter would reach the maximum working volume of the vessel at 44 h; at that time the feeding was stopped. The culture was harvested after 50 h.

In phase I, the profiles are the same as in a batch fermentation described previously (Zhu *et al.* 1996 a, 1997). During phase II, the supply of nitrogen was provided by the remaining amino acids, and by ammonium sulphate and yeast extract in the feed. Growth continued until 30 h while in batch fermentation it stopped at 24 h (Zhu *et al.* 1997), as shown in Figure 3. Cell-mass dry weight reached about 10 g/L, which is an increase of about 33% as compared to that in a batch fermentation. MTG production occurred until 40 h while in a batch fermentation it stops at 34 h. An MTG concentration of 1.8 unit/ml was reached, which is an increase of 80% compared to 1.0 unit/ml in a batch fermentation. The continuation of growth and MTG production in the feeding phase can probably be attributed to the supply of inorganic nitrogen which cannot be crosslinked by MTG. Experiments were also done using the same amount of peptone and ammonium sulphate, respectively in batch and fed-batch phases with a 4 times

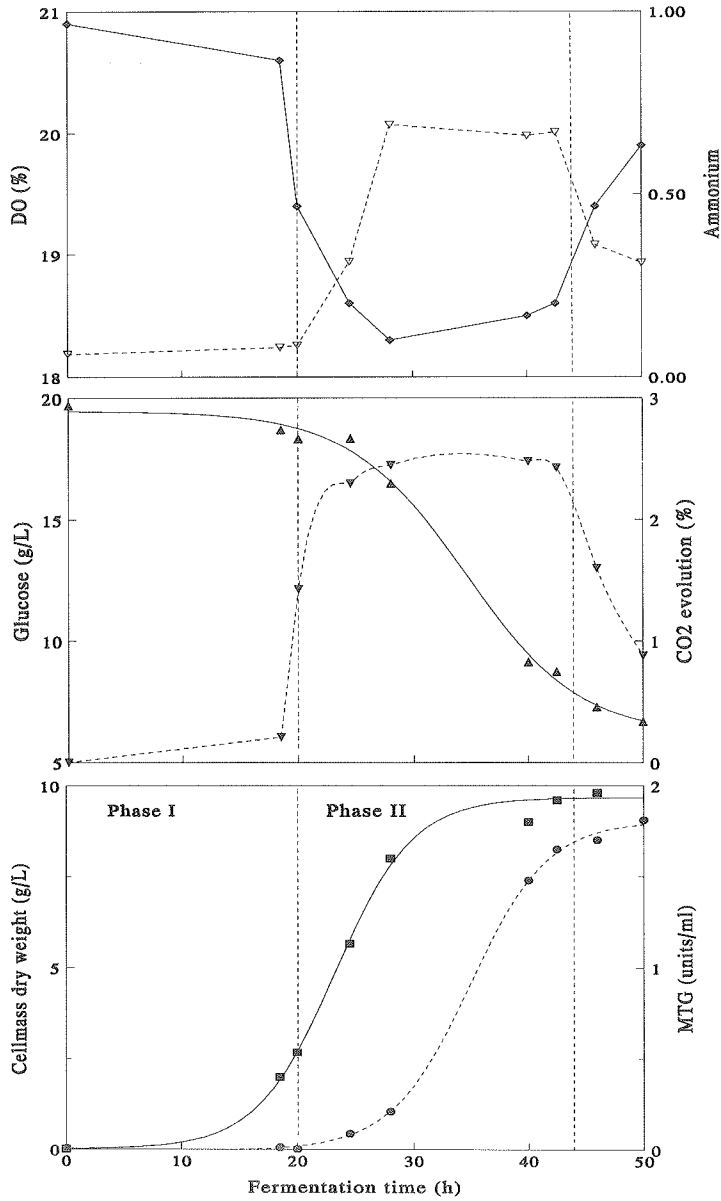

Figure 2. Profiles of a fed-batch fermentation using ammonium sulphate in the feed. ■Cell-mass dry weight (g/L); ● MTG (unit/ml); ▲ Glucose (g/L); ▼ CO_2 in outgas (%), ◆ DO (%); ▽ Ammonium (g/L).

higher total amount of yeast extract (results not shown here). Neither cell mass nor MTG increased. This also supports the need of an inorganic nitrogen source for the continuation of growth and MTG production.

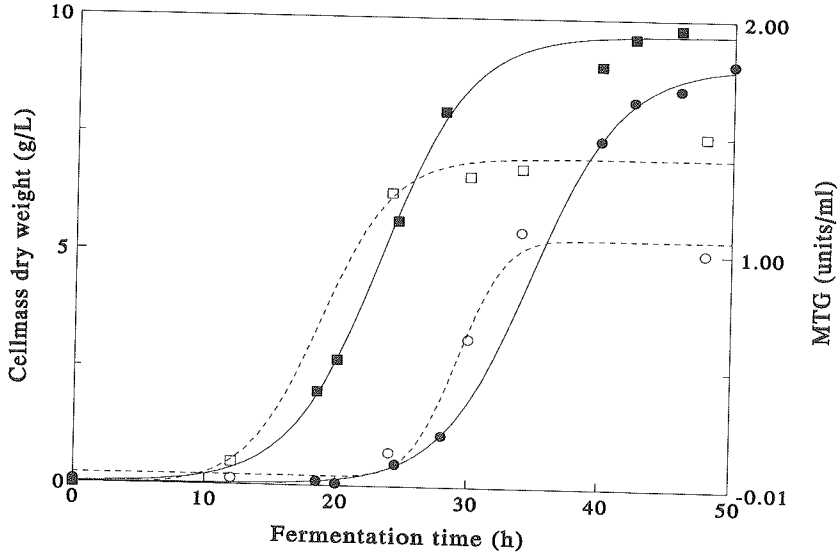

Figure 3. Comparison of growth and MTG production between fed-batch fermentations using ammonium sulphate and batch fermentation. ■ Cell-mass dry weight in fed-batch fermentation using ammonium sulphate in feed (g/L); □ Cell-mass dry weight in batch fermentation (g/L); ● MTG in fed-batch fermentation using ammonium sulphate in feed (unit/ml); ○ MTG in batch fermentation (unit/ml).

In a fed-batch fermentation using ammonium sulphate as a nitrogen source in the feed, there is a certain amount of amino acids freely available throughout the fermentation. The reason of less crosslinking by MTG is probably the surplus of ammonium in the medium. Because ammonium is a by-product of the crosslinking reaction, the existence of a large amount of

ammonium possibly inhibits further crosslinking.

Although the final cell-mass concentration of 10 g/L is lower than the espected 15 g/L on the basis of the stoichiometric model, MTG production is significantly improved. Fed-batch fermentation using $(NH_4)_2SO_4$ thus proves to be effective in MTG production. This method is successfully used now up to 20 L scale in our laboratory for routine production of MTG for research application purposes. The possibility to switch nitrogen source from peptone to ammonium sulphate offers also other advantages such as a reduced viscosity of the fermentation broth with consequent reduction of the power input needed for maintaining a sufficiently high dissolved oxygen level and it further reduces the cost of the medium.

APPENDIX: STOICHIOMETRIC DESIGN OF THE FEED

In order to avoid a reduced availability of the nitrogen source, ammonium sulphate is used as a main nitrogen source during the feeding phase following a batch phase that uses an initial medium. In principle, the contribution of nitrogen to the synthesis of all amino acids can be expected to occur from ammonium. Therefore, more carbon source must be consumed during this phase than in the batch phase where peptone is used as nitrogen source. The amount of glucose and ammonium needed in the feed can be calculated based on the stoichiometric requirements for the synthesis of cell mass and MTG.

At a certain stage of a fed-batch fermentation, neglecting the volume change caused by sampling and pH regulation, the total cell-mass concentration in the fermenter can be expressed as:

$$C_x^t = \frac{C_x^b V_b + \Delta M_x^f}{V_b + V_f} = \frac{C_x^b V_b + \Delta M_x^f}{V_t} \qquad (g \cdot L^{-1}) \qquad (1)$$

where C_x^t is the cell-mass concentration at time t (h), C_x^b (g/L) is the cell-mass

concentration at the end of the batch phase, V_b is the broth volume of a batch fermentation (L), ΔM_x^f is the increase of cell mass during the feeding period (g), and V_f is the total volume of feed (L), with $V_b + V_f = V_t$, the total volume (L).

If a target final cell-mass concentration of C_x^t (g/L) is desired at the end of the fed-batch fermentation, the increase of cell mass realized during the feeding period can be calculated by re-arranging the above equation:

$$\Delta M_x^f = C_x^t V_t - C_x^b V_b \quad (g) \tag{2}$$

From this value, the amounts of all medium components needed in the feed can be calculated on the basis of stoichiometric requirements (Zhu *et al.* 1996 a). However, due to the replacement of nitrogen source with ammonium sulphate the stoichiometric requirements for nitrogen and glucose should be corrected.

The estimation of the amount of inorganic nitrogen source required, in our case the amount of $(NH_4)_2SO_4$, can be calculated based on the stoichiometric requirements of amino acids for the synthesis of cell mass and MTG. Table A-I gives the requirements of all 20 amino acids for the synthesis of 1 g cell mass (dry weight), the requirement of NH_3 for the synthesis of these amino acids, the requirement of precursors for the synthesis of these amino acids and the requirement of glucose for the synthesis of corresponding precursors (Ingraham *et al.* 1983). Note that all these precursors are intermediate metabolites in the metabolic pathways from glucose. Therefore, the amount of glucose needed for the synthesis of these intermediate metabolites can also be estimated, as shown in Table A-I. This will be explained in more detail in the following text.

For the determination of the amount of ammonium required for the synthesis of 1 g cell mass (dry weight), data given in Table A-I can be used. The following equation is used:

$$A_t = \sum_{i=1}^{20} a_i \times m_{a,i} \times 10^{-6} \quad (mol \bullet g^{-1}) \tag{3}$$

Here A_t is the total amount (mol • g^{-1}) of ammonium for the synthesis of 1 g cell mass (dry weight), a_i is the amount of the i th amino acid needed for the synthesis of 1 g cell mass (µmol/g) and $m_{a,i}$ is the amount of ammonium needed for the synthesis of the i th amino acid (µmol/µmol).

The total amount of ammonium sulphate needed in the feed can be calculated based on Equations (2) and (3):

$$N_a = \frac{\Delta M_x^f \times A_t \times M_a}{2} \quad (g) \tag{4}$$

where N_a is the amount of ammonium sulphate in the feed, M_a is the molecular weight of ammonium sulphate and the factor of 2 is the fact that one molecule ammonium sulphate provides 2 molecule ammonium.

$$N_{glc}^{p,f} = \Delta M_x^f \times \sum_{i=1}^{20} a_i \times g_{a,i} \times M_{glc} \times 10^{-6} \quad (g) \tag{5}$$

In a similar way the amount of glucose needed for the synthesis of protein can be calculated: where $N_{glc}^{p,f}$ is the amount of glucose in the feed for the synthesis of cellular protein during the feeding period (g), $g_{a,i}$ is the amount of glucose needed for the synthesis of the i th amino acid and M_{glc} is the molecular weight of glucose.

However, the amount of glucose calculated from Equation (5) is only for the synthesis of cellular protein, which was in the stoichiometric model contributed from amino acids (Zhu *et al.* 1996 a). Therefore, the amount of glucose used in the feed must include the requirement for other cellular components and energy. A mass balance covering all these considerations

can be expressed as:

$$N_{glc}^{t} = \theta_{glc} \times C_{x}^{t} \circ V_{t} + N_{glc}^{p,f} = \theta_{glc} \times M_{x}^{t} + N_{glc}^{p,f} \quad (g) \tag{6}$$

where N_{glc}^{t} is the theoretical requirement of glucose when a target final cell-mass concentration C_{x}^{t} is expected and θ_{glc} is the stoichiometric coefficient of glucose for the synthesis of cell mass under the stoichiometric model (Zhu *et al.* 1996 a). Since residual glucose can still be detected at the end of a batch phase, the amount of glucose in the feed can be corrected by:

$$N_{glc}^{f} = N_{glc}^{t} - C_{glc}^{b,e} \times V_{b} \quad (g) \tag{7}$$

where N_{glc}^{f} is the amount of glucose in the feed corrected by considering the residual glucose $(C_{glc}^{b,e})$ when the fermentation is switched from batch to fed-batch.

Table A-I. Contribution of glucose and NH_3 to the synthesis of amino acids

Protein amino acids	Amount in 1 g cell (μmol) a_i	Precursors (μmol/μmol)*	NH_3 (μmol/μmol)** $m_{a,i}$	Glucose (μmol/μmol)*** $g_{a,i}$
Ala	488	1 Pyr	1	5
Arg	281	1 AKG	4	1
Asn	229	1 OAA	2	0.5
Asp	229	1 OAA	1	5
Cys	87	1 G3P	1	5
Gln	250	1 AKG	1	1

Glu	250	1 AKG	2	1
Gly	582	1 G3P	1	5
His	90	1 R5P	3	1
Ile	276	1 OAA, 1 Pyr	1	1
Leu	428	2 Pyr, 1 AcCoA	1	15
Lys	326	1 OAA, 1 Pyr	2	1
Met	126	1 OAA	1	5
Phe	176	1 E4P, 2 Pep	1	2
Pro	210	1 AKG	1	1
Ser	205	1 G3P	1	5
Thr	241	1 OAA	1	5
Trp	54	1 R5P, 1 E4P, 1 Pep	2	25
Tyr	131	1 E4P, 2 Pep	1	2
Val	402	1 Pyr	1	5

(Continued)

* μmols of precursors needed for the synthesis of 1 μmol corresponding amino acid;

** μmols of ammonium needed for the synthesis of 1 μmol corresponding amino acid;

*** μmols of glucose needed for the synthesis of 1 μmol corresponding precursors.

NOMENCLATURE

a_i the amount of the *i* th amino acid needed for the synthesis of 1 g cell mass (μmol)

A_t total amount of ammonium for the synthesis of 1 g cell mass dry weight (mol)

C_x^b cell-mass concentration of a batch fermentation, (g/L)

C_x^t cell-mass concentration at time t (g/L)

f feed rate (L/h)

$g_{a,i}$ the amount of glucose needed for the synthesis of the *i* th amino acid (mol/mol)

$m_{a,i}$ the amount of ammonium needed for the synthesis of the *i* th amino acid (μmol/μmol)

M_a molecular weight of ammonium sulphate

$M_{a,i}$ molecular weight of the *i* th amino acid

M_{glc} molecular weight of glucose

ΔM_x cell-mass change during two sampling intervals (g)

ΔM_x^f the increase of cell mass during the feeding period (g)

N_a the amount of ammonium sulphate in the feed (g)

$N_{glc}^{p,f}$ the amount of glucose in the feed for the synthesis of cellular protein during the feeding period (g)

N_{glc}^t the theoretical requirement of glucose when a target final cell mass concentration C_x^t is expected (g)

Δt sampling intervals (h)

V_b broth volume of a batch fermentation (L)

V_f total volume of feed (L)

V_t the total volume (L)

ΔV volume change during two sampling times (L)

Greeks letters

θ_{glc} the stoichiometric coefficient of glucose for the synthesis of cell mass under the stoichiometric model (mmol/g)

$\theta_{a,\,i}^{cell}$ stoichiometric coefficient of the *i* th amino acid for the synthesis of cell mass (mmol/g)

$\theta_{a,\,i}^{MTG}$ stoichiometric coefficient of the *i* th amino acid for the synthesis of MTG (mmol/g)

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CHAPTER 6

TRANSGLUTAMINASE AS A POTENTIAL TOOL IN DEVELOPING NOVEL PROTEIN FOODS

SUMMARY

There is a worldwide urgent need of novel protein foods in both developed and developing countries. In the development of novel protein ingredients and novel protein foods, crosslinking enzymes, in particular transglutaminase can play an important role in the improvement of texture, flavour, nutritional value and shelf-life and in the elimination of allergenicity. Modern biotechnology techniques enable the mass production of the enzyme, making it available as a potential tool for the development and production of novel protein foods. Here we present an overview on the demand for novel protein foods, their desired characteristics and the potential of using transglutaminase in the development of novel protein foods. Special attention is given to the realization of producing transglutaminase of microbial origin at an economic price.

Keywords: transglutaminase, crosslinking enzymes, novel protein foods, protein modification, sustainable development.

In developed countries both the government and consumers show increasing concerns about the supply of food proteins of animal origin. Consumers are aware of the environmental burden caused by animal manure which is one of the serious negative consequences of consuming animal proteins. Second is the common awareness of health. The cause of disorders such as high blood pressure, diabetes, obesity and heart attack can be attributed to a diet rich in foods of animal origin. Foods of animal origin have also the disadvantage of being expensive to produce because of the biological inefficiency of converting plant proteins to animal proteins. Obviously, the protein consumption pattern in developed countries has to be changed, not only for the sake of a worldwide sustainable development, but also in the pursuit of an improved environment and health. As an example, a programme has been launched in the Netherlands aimed at a sustainable development in order to reduce the environmental burden by a factor of 20 by the year 2035 (Janssen 1997). In the food sector, the consumption of food protein of animal origin is expected in 2035 to be 40% lower (Janssen 1997). Considering the fact that the consumers in 2035 will need the same amount of protein as today's consumers, the reduction of consumption of food protein of animal origin by 40% must be compensated by using other protein sources which give rise to much less environmental burden (Janssen 1997). Nowadays, average *per capita* annual consumption of meat in EU countries is about 90 kg (Hughes 1995). This indicates that a relatively large amount of meat and meat products will be replaced by proteins of other origin. In contrast to the situation in developed countries, protein supply in developing countries is usually insufficient and causes malnutrition (Steinkraus 1994). Strategies to deal with these protein deficiency problems have been to increase the production of food protein of animal origin and to develop meat substitutes. Average *per capita* annual consumption of meat in developing countries such as China is currently about 20 kg (Zhu & Paardekooper 1996). However, to avoid making the same mistakes as developed countries have made, it is advisable for developing countries to consider the consequence for sustainability of increasing the production of protein foods of animal origin. Yet another important factor for the development of non-animal food proteins is the ever increasing world population. The expansion of urban areas has resulted in a dramatic decrease of the tillable land. As a consequence, a more efficient and economic way of protein production and resource utilization is required. Both developed and developing countries thus need to explore new

resources of food proteins and to develop novel protein foods.

Criteria for developing novel protein foods

Nowadays, the development of a new product is always market-oriented. This requires the consideration of trends in consumer acceptance and preferences. Mankind's attitude towards food acceptance and preference leads to an instinctive behaviour as long as people have a certain level of welfare and food is not the only measure for survival. As long as about 2500 years ago, the great Chinese philosopher Confucius had several principles in his diet (Confucius 500 BC). Confucius' principles concerning food acceptance were (1) do not eat tainted fish and meat; (2) do not eat what looks bad; (3) do not eat what smells bad; (4) do not eat what is poorly cooked; (5) do not eat at inappropriate times; (6) do not eat what is not correctly cut, and (7) do not eat without the appropriate sauces (spices). To summarize, food acceptance in Confucius' time was already directed by appearance, flavour, freshness and processing methods. Although at the time of Confucius there was no rational awareness of the physiological energy requirement and of the nutritional importance of proteins, vitamins and minerals, these principles reflect the importance of acceptance and preference in food selection even in ancient times. In modern society, consumers pay additional attention to aspects such as nutritional value, health, animal welfare and the environment. In designing strategies for developing sustainable food production systems and novel protein foods, one of the crucial factors is the trend in consumer preferences and acceptance. The current trend includes the following aspects: (1) higher demands for more individually directed products; (2) demands for balanced foods such as low-energy, low-fat and low-carbohydrate foods; (3) convenience foods; (4) health foods; (5) natural production processes; (6) the so-called "grazing habits"; (7) the increasing proportion of the elderly people, and (8) demands for luxury foods as well as foods for low-income families (Hughes 1995, ten Hoor 1994, Labuza 1994, Salmon 1992, McMahon 1996).

Strategies for developing novel protein foods meeting consumers' desires

To make a novel protein food that can be accepted by consumers according to the above criteria, the following aspects must be considered: (1) nutritional value; (2) palatability; (3) safety; (4) good texture and flavour characteristics and (5) other beneficial effects such as

health promotion and disease prevention. To achieve these qualities, in particular to make novel protein foods that are comparable or even better and more attractive than meat and meat products, the processing of protein raw materials and subsequently the processing of the commercial products are the key steps. In developing novel protein foods, new protein sources, such as proteins of plant and microbial origin that might be completely different from conventional ones, will be explored and processed to food products. Candidate protein sources for novel protein foods must have the following characteristics: (1) wide availability; (2) low cost; (3) tradition of usage; (4) good nutritional value; (5) safety for human use; (6) good functional properties; and (7) the suitability to be incorporated in conventional foods while maintaining the quality consumers expect (Wolf 1992). In addition to conventional physical and chemical treatment in the modification of proteins, enzymatic processing becomes more attractive and favourable for both the industry and the consumers. The reason is that enzymatic modification generally requires milder conditions and is unlikely to lead to the formation of undesired by-products. Among enzymatic methods, the use of crosslinking enzymes is gaining more and more attention in the modification of food proteins (Ikura 1988, Motoki & Seguro 1994, Matheis & Whitaker 1987, Feeney & Whitaker 1989, Zhu *et al.* 1995, Singh 1991).

Protein-crosslinking enzymes with special emphasis on transglutaminase

Crosslinking of proteins means usually the covalent bonding of a protein to itself or to another proteins (Feeney & Whitaker 1989). In addition to changes in molecular size, protein crosslinking often results in important changes in chemical, functional and nutritional properties of proteins (Singh 1991).

Crosslinking enzymes include transglutaminase (EC 2.3.2.13), lipoxygenase (EC 1.13.11.12), polyphenol oxidase (EC 1.14.18.1), peroxidase (EC 1.11.1.7), lysyl oxidase (EC 1.4.3.13), protein- disulfide reductase (EC 1.8.4.2), protein-disulfide isomerase (EC 5.3.4.1) and sulfhydryl oxidase (EC number not assigned).

Among these crosslinking enzymes, only transglutaminase, lipoxygenase, polyphenol oxidase and peroxidase are commercially available. Lipoxygenase, polyphenol oxidase and peroxidase

are already applied for the quality improvement of dough for bread. However, the use of these three enzymes can also lead to nutritional impairment of proteins (Matheis & Whitaker 1987). No reports exist on the use of lysyl oxidase in the processing of food proteins. Sulfhydryl oxidase acts in synergy with glucose oxidase on dough rheology. Among the very few references concerning protein-disulfide reductase and protein-disulfide isomerase hardly any studies can be found on the modification of food proteins.

In contrast, transglutaminase has been intensively studied with respect to its purification, characterization and application in processing of food proteins. Transglutaminase (protein-glutamine γ-glutamyltransferase, EC 2.3.2.13) is an enzyme capable of catalysing acyl transfer reactions introducing covalent cross-links between proteins (Ikura 1988, Nonaka *et al.* 1989) as well as peptides and various primary amines. When the ε-amino groups of lysine residues in proteins act as an acyl acceptor, ε-(γ-Glu)-Lys bonds are formed both intra- and intermolecularly. Without primary amines in the reaction system, water becomes the acyl acceptor and the γ-carboxyamide groups of glutamine residues are deamidated, becoming glutamic acid residues (Ikura 1988). Transglutaminase-catalysed reactions are schematically shown in Figure 1 (adapted from Ikura 1988). Transglutaminase catalyses crosslinking reactions between different food proteins including whey proteins, soya proteins, gluten, myosin and actomyosin (Zhu *et al.* 1995). Crosslinking of proteins catalysed by transglutaminase can lead to textured products, help protect lysines in food proteins through various chemical reactions, modify solubility and functional properties and produce food proteins with better nutritive values through crosslinking of different proteins containing complementary limiting essential amino acids (Matheis & Whitaker 1987, Singh 1991).

The potential of applying transglutaminase can be found in several reviews (Ikura 1988, Motoki & Seguro 1994, Zhu *et al.* 1995, Nielsen 1995, Kuraishi *et al.* 1996). Some representative examples are briefly listed in Table 1. For more details in the application of transglutaminase in modifying conventional food proteins, readers can refer to an intensive review by Nielsen (1995).

(a)

$$R\text{-}Glu\text{-}CO\text{-}NH_2 + H_2N\text{-}R' \rightarrow R\text{-}\ Glu\text{-}\ CO\text{-}\ NHR' + NH_3$$

(b)

$$R\text{-}Glu\text{-}CO\text{-}NH_2 + H_2N\text{-}Lys\text{-}R' \rightarrow R\text{-}\ Glu\text{-}\ CO\text{-}\ NH\text{-}Lys\text{-}R' + NH_3$$

(c)

$$R\text{-}Glu\text{-}CO\text{-}NH_2 + H_2O \rightarrow R\text{-}\ Glu\text{-}CO\text{-}\ OH + NH_3$$

Figure 1. Transglutaminase catalysed reactions

Table 1. Application potentials of transglutaminase

Applications	References
Texture improvement	(Kuraishi *et al.* 1996, Sakamoto & Soeda 1991, Seguro & Motoki 1991, Takagaki & Narukawa 1990, Matuui *et al.* 1990, Ichihara *et al.* 1990, Wakameda *et al.* 1990 a, b)
Flavour improvement	(Sakamoto & Soeda 1991, Takagaki *et al.* 1990, Kobata *et al.* 1990, Soeda 1992)
Shelf life improvement	(Kato *et al.* 1991)
Nutrition improvement	(Noguchi *et al.* 1992)
Elimination of protein allergenicity	(Yamauchi *et al.* 1991)
Simulation of luxury foods	(Tani *et al.* 1990)
Supplementation of essential amino acids	(Singh 1991)
Improvement of yoghurt quality	(Kuraishi *et al.* 1996)
Low calorie foods	(Takagaki *et al.* 1990, Yamanak & Sakai 1992)

Searching and developing novel crosslinking enzymes

Sources of transglutaminase

Until the late 1980s, animal tissue was the only source of transglutaminase. Up to now, commercial transglutaminase is solely isolated and purified from Guinea pig liver and is very expensive even for laboratory research activities. Another form of transglutaminase is the so-called factor XIII isolated from plasma, placenta and platelets (Traoré &Meunier 1992). Transglutaminase has also been found in plant tissues such as *Helianthus tuberosus* (Falcone *et al.* 1993) and white lupine seeds (Siepaio & Meunier 1995). Tissue-type transglutaminase has also been found in fish (Yasueda *et al.* 1995). In 1989, the production of transglutaminase through microbial fermentation with *Streptoverticillium* species was reported (Anto *et al.* 1989). The primary structure of transglutaminase from *Streptoverticillium* sp. has been studied and its amino acid composition and sequence determined (Kanaji *et al.* 1993). More recently, production of transglutaminase by *Oomycetes* species has been claimed (Bech *et al.* 1996).

Efforts in improving transglutaminase production

Since neither transglutaminase of animal origin nor transglutaminase of plant origin is easily amenable to mass production and consequently to industrial application, the production of transglutaminase by microbial fermentation seems to be the most promising alternative. Ando *et al.* (1989) screened about 5000 strains and only found *Streptoverticillium* species capable of producing the enzyme. Expression of transglutaminase has been studied in several other organisms including *Escherichia. coli*, *Aspergillus*, *Bacillus*, *Streptomyces*, *Saccharomyces* and lactic-acid bacteria (Bech *et al.* 1996, Bjornvad & Prento 1996, Takehana 1994). Only a few studies can be found in the literature on the biochemical engineering aspects of the production of the enzyme by microorganisms. A stoichiometric model was recently reported for the medium design in the production of transglutaminase by *Streptoverticillium mobaraense* (Zhu *et al.* 1996). This model simplifies the microbial reaction network by lumping all reactions into one equation, analogous to the study done by Xie and Wang (1994). A schematic description of the method is shown in Figure 2. By quantitatively calculating the relationship between substrate and cell mass, product and energy needed for the process, the medium can be designed rationally to meet the stoichiometric requirements for cell growth

and enzyme production. Using this rationally designed medium, transglutaminase concentration at the harvest of a batch fermentation was significantly improved. Furthermore, an integrated process of fed-batch fermentation was used for further improvement of transglutaminase production (Zhu *et al.* in preparation, 1997), which resulted in a further increase of transglutaminase concentration at the harvest of fermentation and a decrease of the substrate cost.

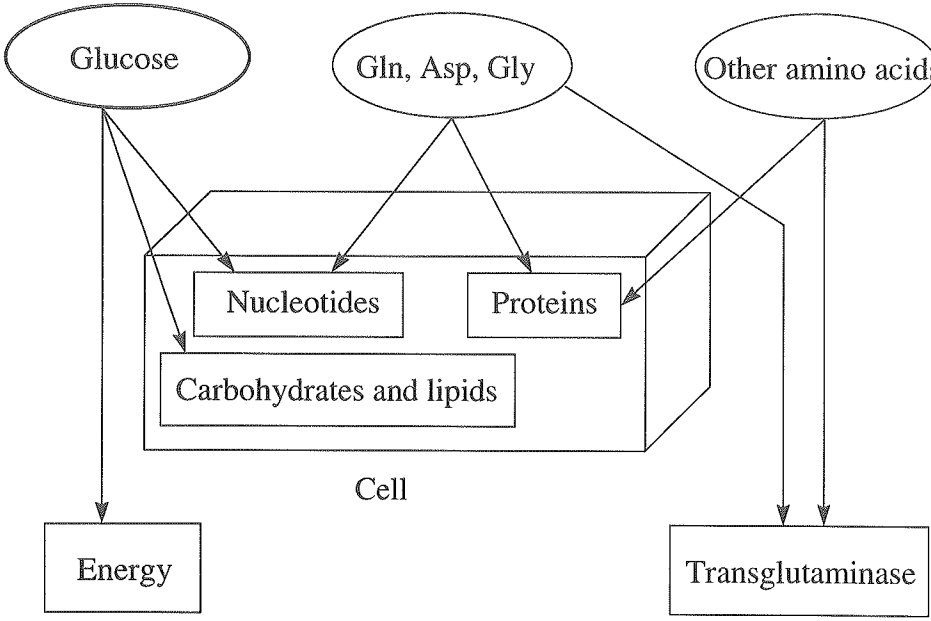

Figure 2. Stoichiometric model

Trends in the production of crosslinking enzymes

More efforts will be made in the search for new highly-productive strains for transglutaminase production. Considering governmental regulations and consumer concerns about food safety, emphasis should be first placed on searching for strains within the scope of so-called food-grade microorganisms. More effective screening methods should be developed such as more sensitive detection methods to reduce the cost of labour and time.

Also rDNA techniques are applied to find alternatives for producing transglutaminase by

food-grade microorganisms. Expressing of transglutaminase has been studied in food-grade strains such as *Aspergillus* and *Saccharomyces cerevisiae* strains, as well as in non-food-grade strains such as *E. coli,* and *Streptomyces* sp. (Yasueda *et al.* 1995, Bech *et al.* 1996, Takehana *et al.* 1994, Washizu *et al.* 1994, Jagadeeswaran & Haas 1990, Ikura *et al.* 1990).

Food fermentation is widely applied in oriental countries, especially for foodstuffs of plant origin. Fermentation is also a very promising method for producing novel protein foods. Finding transglutaminase in strains used in food fermentation will be an ideal way of introducing protein crosslinks in food proteins. As an alternative, if it is impossible to find crosslinking activities in food-grade organisms, rDNA techniques can probably offer possibilities for expression of crosslinking activity in food-grade organisms normally used in food fermentation. It must be noted, however, that the consumer attitude towards products generated through genetic engineering varies widely across the world.

Crosslinking reactions catalysed by transglutaminase can be easily controlled by reaction conditions such as temperature, incubation time, enzyme concentration and source of substrate with different amino acid residuals. As a consequence, desired final products can thus be designed to meet consumers' demands with respect to nutritional value, texture, flavour, appearance and shelf-life.

Conclusions

The human race is facing the challenge to create a sustainable world. In the sector of food supply, both developed and developing countries have to deal with the sustainable supply of food proteins. Although they have completely different problems, they have the same aim, namely to explore and utilize new protein sources for novel protein foods. The development of such novel protein foods has to meet consumer demands. Protein modification by crosslinking enzymes is a very powerful and promising method to make novel protein foods of good texture, flavour, appearance, nutritional value and shelf-life. Among the many crosslinking enzymes, only transglutaminase has shown so far its potential for developing novel protein foods. Modern biotechnology offers promising possibilities to find new crosslinking enzymes which are safe and can be produced from cheap substrates and on a

large scale.

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SUMMARY

Transglutaminase (EC 2.3.2.13) catalyses *in vitro* cross-linking reactions in various proteins. This enzyme has been used in attempts to improve the functional properties of protein foods. Up to now, commercial transglutaminase has been obtained from animal tissues. The complicated separation and purification procedure results in an extremely high price of the enzyme, which hampers a wide application in food processing. Recently, studies on the production of microbial transglutaminase (MTG) have been launched. The enzyme obtained from microbial fermentation has been applied in the treatment of various food proteins. Food treated with MTG appeared to have an improved flavour, appearance and texture. In addition, MTG can increase shelf-life and reduce allergenicity of certain foods. However, much work has to be done before this interesting enzyme can be produced on an industrial scale in an economic manner. This thesis focuses on the development of an economic and practical process for MTG production.

The study is directed mainly to process development and optimization with respect to MTG production in order to improve the productivity and to minimize the cost.

First, a stoichiometric model was developed for the application of medium design in MTG production by *Streptoverticillium mobaraense*. The model avoids dealing with all the metabolic reactions involved by simply lumping them into a single reaction. With the help of the analysis of the nutrients' roles and biochemical knowledge of the micro-organism, all stoichiometric coefficients in the model were calculated. Together with the experimental measurements, these coefficients were used for medium design. With this designed medium, MTG activity and specific productivity were increased significantly.

Second, analysis of amino-acid metabolism was done using mass balances. Depletion of free amino acids in a batch fermentation is probably the limiting factor for further cell growth and MTG production. The cause of this limitation is probably the cross-linking of amino acids by the MTG produced.

Third, an integrated fermentation technique was applied for further optimization of MTG production. A strategy was developed on the basis of the stoichiometric requirements for cell synthesis and MTG production and of the consideration that the product itself causes cross-linking of the substrates. By applying fed-batch techniques using an inorganic nitrogen source in the feed that cannot be cross-linked, MTG production was further increased significantly.

Finally, on the basis of our studies and others' work, trends with respect to the production and application of transglutaminase are signalled in the last chapter.

SAMENVATTING

Transglutaminase (EC 2.3.2.13) katalyseert *in vitro* crosslinking reacties van verschillende eiwitten. In het verleden is men erin geslaagd dit enzym te gebruiken voor het verbeteren van de functionele eigenschappen van eiwitrijke voedingen. Tot nu toe is daarbij gebruik gemaakt van transglutaminase van dierlijke afkomst. Een brede toepassing van het enzym in de voedingsindustrie wordt echter belemmerd door de hoge kostprijs, die veroorzaakt wordt door de ingewikkelde winnings- en zuiveringsprocedure. Kort geleden is onderzoek op gang gekomen om het enzym langs microbiële weg te produceren. Dit microbieel transglutaminase (MTG) is toegepast bij het bewerken van verschillende voedingseiwitten. Met MTG behandeld voedsel ziet er aantrekkelijker uit, heeft een steviger textuur en heeft verbeterde smaakeigenschappen. Bovendien wordt door toevoeging van MTG bij bepaalde producten de houdbaarheid verlengd en de allergene werking verlaagd. Voordat dit bijzondere enzym echter op een economisch verantwoorde manier op industriële schaal kan worden geproduceerd moet nog veel onderzoek worden verricht. In dit proefschrift is de ontwikkeling van een praktisch en economisch proces voor de productie van MTG het centrale thema.

Het onderzoek is vooral gericht op het ontwikkelen van een productieproces voor MTG en het optimaliseren daarvan door verhoging van de productiviteit en zoveel mogelijk verlagen van de kosten.

Allereerst werd een stoichiometrisch model ontwikkeld om een geschikt medium voor de productie van MTG met *Streptoverticillium mobaense* te kunnen ontwerpen. In het model zijn alle betrokken stofwisselingsreacties teruggebracht tot één enkelvoudige reactievergelijking. Met behulp van de biochemische kennis over het micro-organisme en bestudering van de rol van de nutriënten konden alle stoichiometrische coëfficiënten worden berekend. De verkregen coëfficiënten vormden, samen met experimentele waarnemingen, de basis voor een medium-ontwerp. Met dit medium waren zowel de specifieke productiviteit als de specifieke activiteit van MTG aanzienlijk hoger dan met het oorspronkelijke medium.

Vervolgens werd het aminozuur-metabolisme bestudeerd met behulp van massabalansen. Een gebrek aan vrije aminozuren is bij een batch-fermentatie waarschijnlijk de beperkende factor waardoor na een bepaald moment verdere groei en MTG-productie niet meer plaatsvindt. Vermoedelijk worden de aminozuren door het gevormde MTG verknoopt.

Als derde onderdeel werd de MTG productie verder geoptimaliseerd, waarbij gebruik gemaakt werd van een geïntegreerde fermentatietechniek. Er werd een strategie ontwikkeld die enerzijds berust op de stoichiometrische eisen voor celsynthese en MTG-productie en anderzijds op de overweging dat de substraten door het product verknoopt kunnen worden. Door gebruik te maken van fed-batch-fermentatie met in de toevoer een anorganische stikstofbron (die niet kan worden verknoopt) kon de MTG-productie verder worden verhooogd.

Ten slotte werden nieuwe ontwikkelingen met betrekking tot de productie en toepassing van transglutaminase gesignaleerd. Deze worden in het laatste hoofdstuk besproken.

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CURRICULUM VITAE

The author of this thesis was born on 1 December 1955 in Shanghai, China. His primary and secondary education took place in the same city. After finishing high school in 1973, it was not possible for him to start an academic education, because the 10-year Cultural Revolution (1966-1976) deprived all high-school graduates of the rights of entering universities. After the re-establishment of the national entrance examination system for university studies in China in 1978, he passed the examination and started to study at East China University of Science and Technology in Shanghai (then called East China University of Chemical Technology), majoring in biochemical engineering. After finishing the study in 1982, he worked as an assistant in the Chemistry Department until 1983 and then in the Biochemical Engineering Department until 1986, at the same university. In December 1986, he was selected as a visiting scholar to the Institute of Microbiology at the University of Münster, Germany, where he studied under the guidance of Professor H.-J. Rehm. After his return to China in December 1987, he worked as a lecturer in the Group of Food Science and Technology, Biochemical Engineering Department, East China University of Science and Technology in Shanghai. In April 1993, he began to work as a guest researcher at the Industrial Microbiology Division of TNO Nutrition and Food Research Institute in Zeist, Netherlands. In September 1994, his research was conducted as a PhD study under the guidance of Professor J. Tramper of Wageningen Agricultural University, Netherlands. Since December 1996, he has worked in the same institute as a (post-doctoral) scientific researcher in the Industrial Microbiology Division.

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