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Studies on immobilization of the thermophilic bacterium *Thermus aquaticus* YT-1 by entrapment in various matrices

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Summary. Immobilization of the thermophilic bacterium *Thermus aquaticus* YT-1 has been studied using various entrapment techniques. Alginate, κ-carrageenan, agar, agarose and polyacrylamide were tested as supports during operation at 65° C at which the cells are known to produce protease when grown free in solution. Alginate showed toxic effects and no viability was observed after entrapment in Ca alginate or even after exposure of free living cells to sodium alginate. Polyacrylamide was observed to be the best support. Protease activity was closely related to the appearance of free cells in the medium.

Introduction

Many microorganisms have been immobilized successfully by entrapment methods. The potential of using immobilized cells in industrial processes is regarded as very large (Cheetham 1980). Cells at different stages (viable, resting, dead, etc.) have been successfully entrapped in various matrices (Mattiasson 1983; Brodelius and Vandamme 1987). Immobilized viable cells have stayed alive for a long time in the immobilized stage when the conditions were optimal.

In the literature there is very limited treatment of immobilized thermophilic microorganisms (e.g. Sonnleitner and Fiechter 1986). This is strange since this group of organisms offers many attractive characteristics for process design. An elevated process temperature may correspond to a faster reaction rate, a better solubility for non-gaseous compounds and a reduced incidence of microbial contamination. Furthermore, thermostable enzymes have been reported to be more resistant towards protein denaturants as compared to enzymes from mesophilic organisms (Stellwagen 1984). There is, therefore, a need to investigate immobilization techniques for thermophilic organisms and to study such immobilized preparations under operation at elevated temperatures.

Thermus aquaticus YT-1 was selected as a model organism, since it has been reported to be the best producer of extracellular proteases among many different strains investigated (Matsuzawa et al. 1983). The reaction studied was the production of protease excreted into the medium, because such processes involve the whole metabolic machinery and will thus give information on cell viability. Another reason is that proteases per se are a most important group of industrial enzymes and it would therefore be attractive to design continuous processes where relatively little carbon source is used for maintenance of the producing cells and more is converted into the desired product.

Materials and methods

Chemicals

Sodium alginate (Munacol SS/LD/2), agar, agarose and paraffin oil were supplied by BDH, Poole, UK. Acrylamide, BIS (*N,N*-methylene-bis-acrylamide), ammonium persulphate and TEMED (*N,N,N',N'*-tetramethylethylenediamine) were purchased from Bio-Rad, Richmond, Calif, USA. κ-Carrageenan, soybean oil, yeast extract, tryptone, peptone and casein were obtained from Sigma, St. Louis, Mo, USA.

Cultivation of T. aquaticus YT-1

Cells of *T. aquaticus* YT-1 (DSM 625) were grown aerobically at 65° C in medium containing basal salts (Degryse et al. 1978)

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Table 1. Various cultivation media for *Thermus aquaticus* YT-1

Medium (pH 7.2)	Components (w/v in 0.20 *M* phosphate buffer, pH 7.2, and basal salts)
A	0.25% Yeast extract, 0.25% tryptone
B	0.40% Yeast extract, 0.80% tryptone
C	0.40% Yeast extract, 0.80% peptone
D	2.0% Casein
E	0.40% Yeast extract, 0.80% tryptone, 2.0% casein
F	0.50% Glutamic acid
G	0.25% Yeast extract, 0.25% tryptone[a]

[a] %w/v in 0.05 *M* phosphate buffer, pH 7.2, and basal salts

and different nutrients; yeast extract, tryptone, casein, and peptone in 0.20 *M* potassium phosphate buffer, pH 7.2, as indicated in Table 1. The inocula were obtained from 2-day-old cultures at 65° C in a medium containing 0.25% tryptone, 0.25% yeast extract in phosphate buffer and basal salts. The dry weights of cells were determined at intervals, and the supernatants of the cultures were separated by centrifugation (6000 *g*, 15 min) and filtration through a 0.2 μm Millipore membrane.

For growing the cells on plates, 2.8% agar was added to a medium containing 0.25% yeast extract and 0.25% tryptone in phosphate buffer and basal salts. The cells were grown on agar plates at 65° C.

Assay of proteolytic activity

The assays were performed by the azocasein digestion method (Millet 1970), using 0.5% azocasein in 0.20 *M* TRIS-HCl, pH 7.2, (0.50 ml) and 1 ml enzyme sample. After incubation for 1 h at 70° C, 10% trichloroacetic acid (1 ml) was added to terminate the enzyme-catalysed reaction. The precipitate was separated by centrifugation (13 000 *g*, 5 min). The absorbance at 440 nm of the supernatants was measured. Proteolytic activity was expressed as AU (Azocasein Units). One unit of proteolytic activity is defined as the amount of enzyme required to produce an increase in absorbance at 440 nm of 1.0/h under standard assay.

Immobilization of T. aquaticus YT-1

All processes were performed under sterile conditions.

Cultivation of T. aquaticus YT-1 for immobilization. The microbial cells were grown in a fermentor and in shake flasks. Fermentor cultivations were carried out in a Chemoferm FLC-B-3 fermentor (Chemoferm AB, Hägersten, Sweden) with a working volume of 2 l and the pH was maintained at 7.2 by automatic titration with 2 *M* HCl. The aeration rate was 0.5 vvm and the stirrer speed was 300 rpm. The cells were harvested at maximum growth in medium A and separated from the culture supernatant by centrifugation (10 000 *g*, 20 min). The cells were washed with 0.2 *M* potassium phosphate buffer, pH 7.2, and immobilized by entrapment in various matrices.

Calcium alginate. Cells (2 g wet wt) were suspended in sodium alginate (8 ml; 6.5% in water) and pumped through a syringe into 0.5 *M* $CaCl_2$. The beads containing the entrapped cells were allowed to stabilize for 2 h. After washing with sterile water, the beads were transferred into medium A supplemented with 1 m*M* $CaCl_2$ and then incubated in a shake flask at 65° C. As a control a culture of free cells (1% inoculum) was incubated under the same conditions. Proteolytic activity was frequently estimated during 2 weeks. In order to determine viability of the cells after entrapment in calcium alginate, some beads were transferred into 0.1 *M* citrate-phosphate buffer, pH 7.2, and stirred for 15 min, thereby complexing the calcium ions and solubilizing the alginate (Aldercreutz et al. 1985). The suspension of cells obtained was cultivated by plating on nutrient agar and by inoculating in liquid medium. The assays for protease activity were carried out on the supernatant.

κ-Carrageenan. An aqueous suspension of κ-carrageenan (2% w/v in water) was warmed at 50° C. The gel obtained was mixed with the cells (2 g wet wt) and dropped into 0.1 *M* KCl. The beads were allowed to harden overnight in the KCl solution and were then washed with sterile water and medium A, respectively. The beads containing the immobilized cells were incubated at 65° C in medium A supplemented with KCl and NH_4Cl as indicated in Table 2.

Agar and agarose. After formation of the polymer solution (8 ml of 2%) at 50°–60° C, the cells (2 g wet wt) were immobilized as described by Nilsson et al. (1983).

Polyacrylamide. For block polymerization, 8 ml of a stock solution of monomers (acrylamide 35.20 g and BIS 2.40 g in sterile water 100 ml) were mixed with microbial cells (2 g wet wt), ammonium persulphate (40 μl; 0.40 g/ml) and TEMED (100 μl). The mixture was allowed to polymerize for 15 min in an 800-ml glass beaker, and the heat evolved was eliminated by using an ice bath. The polymer sheet was cut and washed respectively with 70% EtOH, sterile water and growth medium.

For bead polymerization, the microbial cells were entrapped according to the method of Nilsson et al. (1983); but the concentration of the cells and the chemicals were as described above.

Thermostability of matrices. Immobilized cells (0.5 g) were treated with microbial cultivation medium A (10 ml) at 65° C with shaking. The stability of supports was determined by studying the dissolution and fragmentation of the beads.

Protease production by immobilized T. aquaticus YT-1

In this experiment, bacterial cells were immobilized by entrapment in polyacrylamide by block and bead polymerization. The immobilized cells were treated with 70% EtOH for 1 min, and washed with sterile water and cultivation medium before use in the following processes.

Batch cultivation. The immobilized cells (2 g wet wt cells) were incubated at 65° C in a Sakaguchi flask containing medium A (100 ml). At certain times of incubation the proteolytic activity was determined after removal of free cells by centrifugation and filtration.

Continuous culture. The experiment was carried out under sterile conditions and the system composed of a vertical column (2.2 × 6.5 cm) containing immobilized cells (2 g wet wt cells) which was kept in an incubator at 65° C. Cultivation medium A was passed through the column at a flow rate of 3.50 ml/h. The proteolytic activity of the eluted fractions from the top of the column was determined every 12 h.

Table 2. Properties of Immobilized *T. aquaticus* YT-1 in various supports

Support	Immobilization technique	Gel strength[a]	Supplement to medium A	Stability at 65° C	Cell growth
Calcium alginate	Ionotropic gelation	++++	1 m*M* $CaCl_2$ 0.05 *M* Phosphate buffer, pH 7.2	>1 month	—
κ-Carrageenan	Ionotropic gelation	+	—	15 min	+
		+	0.5% NH_4Cl	1 h	+
		++	2% NH_4Cl	1 day	−
		+	0.5% KCl	1 h	+
		++	0.75% KCl	1 day	+
		+++	1%–2% KCl	>1 week	−
Agar	Solidified in oil phase	++++	−	>1 week	+
Agarose	Solidified in oil phase	+++++	−	>1 week	+
Polyacrylamide	Block polymerization	++++[b]	−	>1 month	+
		+++++[c]	−	>2 months	+
	Bead polymerization	+++++	−	>2 months	+

[a] Manually crushed
[b] 10%–20% Acrylamide
[c] 30%–40% Acrylamide

Results and discussion

Cell growth and protease secretion from free cells

The ability of *T. aquaticus* YT-1 to utilize various substrates as carbon sources for growth was first studied by Brock and Freeze (1969). In this experiment we attempted to optimize the medium for higher protease production by immobilized *T. aquaticus* YT-1. Since the maximum activity of protease was constant at 65° C and decreased at 70° C (Matsuzawa et al. 1983), we decided to cultivate the cells at 65° C. The result of growth yield and protease activity measurements are shown in Fig. 1. This figure shows that *T. aquaticus* YT-1 grew well in media containing yeast extract and protein hydrolysate (media A, B, C and D) whereas a high concentration of protein hydrolysate (medium E) decreased the final cell density. The biomass yield did not increase proportionally to the concentration of nutrient (media A and B). Tryptone (medium B) was a better protein hydrolysate for biomass yield and also better for protease production than peptone (medium C). Glutamic acid 0.5% (medium F) was used as a source of carbon and nitrogen but microbial growth yield was lower than with yeast extract and tryptone (medium A). Medium G supplemented with 1 m*M* $CaCl_2$ was used for stabilization of the calcium alginate beads.

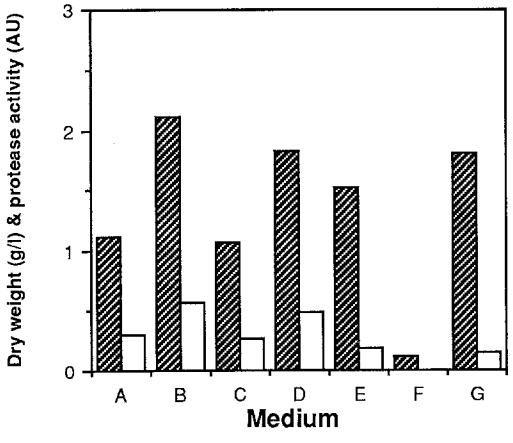

Fig. 1. Growth yield (▨) and protease activity (□) of *Thermus aquaticus* YT-1 in various media (Table 1): AU = Azocasein Units

The growth yield of *T. aquaticus* YT-1 in a batch fermentor using medium B (65° C, pH 7.5, air 0.5 vvm) was higher than in shake flasks (2.69 g dry wt/l vs 2.15 g dry wt/l). This result might be explained by the fact that *T. aquaticus* YT-1 grew better at higher oxygen supply.

Proteolytic activity was higher in the medium containing both yeast extract and protein hydrolysate (medium B) than in the case of casein hydrolysate only (medium D). The microbes grew in 0.50% glutamic acid (medium F) but no protease

activity was detected after 4 days of cultivation. In this latter medium there would be no need for protease activity since the cells already have a carbon and nitrogen source in excess.

Since medium A is composed of relatively low concentrations of nutrients and gives relatively high protease activity, it has been used as the cultivation medium for immobilized *T. aquaticus* YT-1.

Effects of immobilization on microbial growth

No attempt has previously been made to produce protease using immobilized thermophilic extracellular protease-producing bacteria: *T. aquaticus* YT-1 was immobilized in the present study in order to accomplish this.

Entrapment is the most gentle technique for preserving the viability of cells during an immobilization step. Calcium alginate is the most frequently used support because of the non-toxicity to living cells (Vorlop and Klein 1983), and the simplicity of the method. The spherical particles obtained are suitable for column packing (Klein et al. 1983), have high mechanical strength (Klein and Washausen 1979; Cheetham 1979), and have good abrasion resistance (Klein and Eng 1979). Recently, the extracellular protease-producing bacteria *Serratia marcescens* and *Myxococcus xanthus* have been successfully entrapped in calcium alginate (Vuillemard et al. 1988). There are so far no literature reports on the toxicity of alginate to microorganisms. After entrapment of *T. aquaticus* YT-1 in 6.5% sodium alginate, no proteolytic activity was detected from immobilized cells during 2 weeks.

The determination of viability of immobilized cells after solubilization with citrate-phosphate buffer showed no growth when plating on nutrient agar or by cultivation in normal liquid medium. In a control experiment when free cells were cultivated with 0.5%–6.5% sodium alginate in the nutrient medium, it was also observed in this case that no growth occurred. The explanation for this observation is still not clear, but one possibility is that the alginate complexes some di- or multivalent ions needed by the cells. Other alternatives such as restriction in respiration and growth due to the high polymer concentration (Hahn-Hägerdal et al. 1982) or a negative effect of a locally high concentration of ions (Brock and Freeze 1969; Alfredsson et al. 1988) cannot be fully eliminated yet. However, in control experiments when the cells were grown in the presence of κ-carragenan, no such harmful effects were observed. It is thus unlikely that the observed effect is due to a high polymer concentration per se, but rather to specific properties of the alginate.

κ-Carrageenan has been used for immobilization of the extracellular protease-producing bacterium, *Myxococcus xanthus;* the cells remained viable and enzymatic activity was enhanced (Younes et al. 1984). In the case of *T. aquaticus* YT-1, the cells grew in nutrient medium containing 0.2%–3.0% κ-carrageenan but the κ-carrageenan beads dissolved in water or nutrient medium after incubation at 65° C for 15 min. In order to improve the thermostability of the beads, NH_4Cl and KCl were added to the medium. The result (Table 2) shows that KCl was the best hardening agent. Concentrations of KCl greater than 1% kept the beads stable at 65° C for more than 1 week. However, these concentrations of salt inhibited microbial growth, therefore entrapment of *T. aquaticus* YT-1 in κ-carrageenan is not recommended if protease production is required.

Agar and agarose beads were stable at 65° C for more than 1 week. Entrapment of *T. aquaticus* YT-1 in agar and agarose preserved the viability of the cells. However, on the second day of incubation at 65° C free cells started to appear. Proteolytic activity was observed after 3 days of incubation.

Although polyacrylamide is toxic to some microorganisms, it has been used frequently for cell immobilization (Mattiasson 1983; Brodelius and Vandamme 1987). Kokubu et al. (1981) used this matrix to immobilize the extracellular protease-producing bacterium, *Streptomyces fradiae,* which has a temperature optimum of 30° C. The result in Table 2 shows that the preparation of cells entrapped in polyacrylamide was stable at 65° C for more than 1 month.

After entrapment of *T. aquaticus* YT-1 in block or bead polymerization, free cells leaked out after 32 and 36 h of incubation, respectively. Proteolytic activity was detected after the appearance of free cells. The microbial cells leaked out even though the preparation of immobilized cells was treated with 70% EtOH, and the concentrations of monomer and cross-linking agents were varied from 10%–40% and 10%–20% respectively.

Proteolytic activity from entrapped T. aquaticus YT-1 in polyacrylamide

Figures 2 and 3 illustrate the cell growth and proteolytic activity in systems with free cells and im-

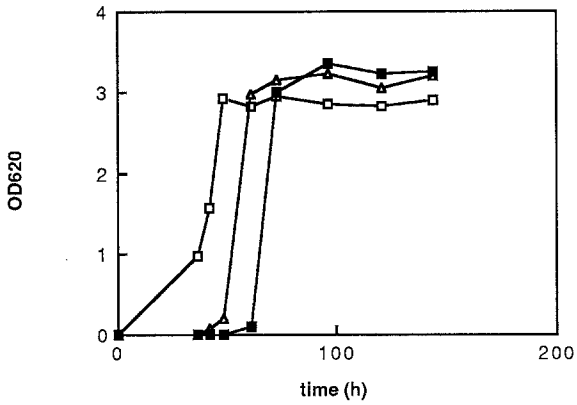

Fig. 2. Comparison of cell growth in medium A from free (□) and immobilized *T. aquaticus* YT-1 with polyacrylamide by block polymerization (△) and by bead polymerization (■)

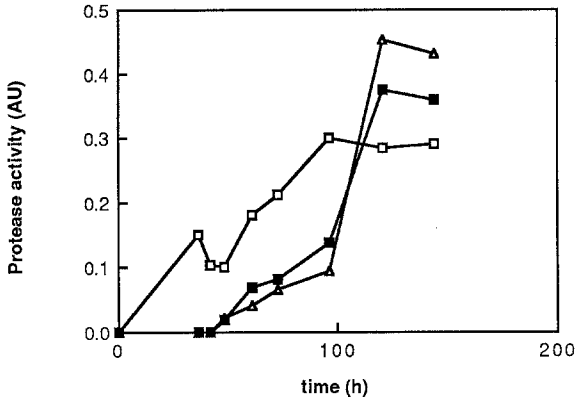

Fig. 3. Comparison of protease activity of free (□) and immobilized *T. aquaticus* YT-1 with polyacrylamide by block polymerization (△) and by bead polymerization (■): AU = Azocasein Units

mobilized cells in medium A, respectively. The cells in the free cell system grew and secreted proteases before the cells in the immobilized cell system, but the maximum growth was almost the same. The maximum protease activity in the immobilized cell system was slightly higher than in the free cell system. This result indicates that entrapment with polyacrylamide increases the protease activity of *T. aquaticus* YT-1.

After 2 days of continuous processing in the system with immobilized cells, cells leaked out from the beads and were allowed to flow out in the eluted fractions. The proteolytic activity of the eluted fractions indicated that no proteases were secreted from these cells during several days of processing. The true immobilized cells obviously act as a source for continuous inoculation and the cells thus produced can be found in the eluate. As the production of protease is delayed, i.e. only partially coupled to cell growth (cf. Figs. 2, 3) it is a matter of choosing the proper residence time in order to maintain continuous protease production with immobilized *T. aquaticus*. Further studies on this matter are needed.

In conclusion, from the experiments reported above it is clear that the selection of a polymer material for entrapment of thermophilic microorganisms is quite different from that of mesophilic organisms. It is furthermore interesting to note that alginate was harmful to the bacterium tested, in spite of the fact that alginate of the same quality has successfully been used for immobilization of, for example, mammalian cells. The immobilized bacteria at the present stage of development are not suitable for large scale production of proteases.

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