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Submerged production of pectolytic enzymes by *Aspergillus niger*: effect of different aeration/agitation regimes*

Jožica Friedrich[1], Aleksa Cimerman[1], and Walter Steiner[2]

[1] Boris Kidrič Institute of Chemistry, Hajdrihova 19, Ljubljana, Yugoslavia
[2] Institut für Biotechnologie, Technische Universität Graz, Schlögelgasse 9, Austria

Summary. The production of a pectolytic enzyme complex in a 10-l stirred tank bioreactor was studied using the *Aspergillus niger* mutant A 138. A time course of the fermentation showed that the enzyme synthesis is not associated with growth. Maximal activity was reached after 95 h and from that time on it remained constant. Redox potential and pH values proved to be valuable indicators of the initiation and end of enzyme synthesis. The specific morphology of the fungus, growing in distinct pellets with long peripheral hyphae, resulted in a very dense and viscous broth. It represented a special problem for heat and mass transfer. An attempt was made to overcome this problem by different agitation and aeration regimes. These parameters did not change the morphology but had a marked influence on enzyme synthesis. When, at the time of maximal growth rate, aeration was increased from 0.5 vvm to 1.2 vvm, and agitation from 300 rpm to 500 rpm, the depectinizing activity was doubled in comparison with the results obtained when 0.5 vvm and 300 rpm were used throughout fermentation. When more intensive agitation was employed from the beginning of the process, the depectinizing activity was lowered from 60 to 45 units/ml, together with the viscosity and polygalacturonase activity. However, at the same time, the pectin esterase and pectinlyase yields increased. The required fermentation time was reduced from 95 to 65 h.

Introduction

Enzymes degrading pectic substances are used in fruit and vegetable processing for maceration or juice clarification. They are of microbiological origin and are mainly produced by *Aspergillus niger* in surface or submerged fermentations. The former was thought to be used predominantly (Rombouts and Pilnik 1980), but cultivation under submerged conditions is much easier to study and to control. Based on a review article on the production of pectolytic enzymes by *A. niger* in submerged culture (Hermersdörfer et al. 1984) it can be concluded that East European countries predominate in this research field.

In spite of some existing patents, the submerged production of pectinases is not yet satisfactorily solved. Data on suitable culture conditions for enzyme synthesis and its regulation are in some cases contradictory, which is attributed to the specificity of each fungal strain. In addition, the results are difficult to compare. One reason for this may be the composition of the pectinase complex including various individual components with different catalytic activities. Among these, the most important are polygalacturonases, pectin esterases and pectinlyase. The other reason for difficulties in interpretation is the use of different methods for the determination of enzyme activities.

In our laboratory, research on the production of pectolytic enzymes started with the selection of *A. niger* strains (Friedrich et al. 1986). A productive strain was then mutated and a suitable mutant isolated. Optimization of the medium was performed in shake flask experiments. The present paper deals with experiments in a laboratory stirred tank bioreactor where the time course of the fermentation was studied in detail. Special emphasis was given to the influence of aeration and agitation of the enzyme synthesis. Since the enzyme produced may be used for apple juice clarification, the activity of the whole complex was measured according to the method of Biocon

* This paper is dedicated to Prof. Robert M. Lafferty on the occasion of his 60th birthday

Offprint requests to: J. Friedrich

Ltd, Ireland. This method called the Apple Juice Depectinizing Assay (AJDA) simulates the conditions of the enzyme application and seems to be the most suitable for our purpose.

Materials and methods

Medium. The basic composition of the medium was similar to that used by Tuttobello and Mill (1961). It was optimized to suit the microorganism used. The modified medium composition was as follows: 2% apple pectin, 0.5% sucrose, 1% dry whey, 0.2% NH_4NO_3 and 0.05% $(NH_4)_2SO_4$ in tap water. The pH value of the medium was adjusted to 4.5 with KOH. The substrate was sterilized in the fermentor at 121° C for 30 min with mixing at 200 rpm.

Microorganism. Aspergillus niger pectolytic mutant A 138 from the Collection of the Boris Kidrič Institute of Chemistry, Ljubljana, Yugoslavia, was used as the productive strain. This mutant resulted from UV mutation of the parent strain. It was maintained on beer wort agar slants. Spores from two tubes were suspended in 40 ml sterile tap water (approx. 10^7 spores/ml) and used as an inoculum for 9 l medium.

Fermentation experiments. All fermentation experiments were performed in a 10-l stainless steel stirred bioreactor (Bioengineering, Wald, Switzerland). The temperature was maintained at 32° C. Dissolved oxygen concentration, pH value and redox potential were measured on-line. Foaming was controlled automatically by means of a silicone antifoaming agent. Different aeration and impeller speeds were used as described in the Results and discussion. The fermentations were conducted for 90 to 160 h, i.e. for some time beyond the appearance of maximal enzyme activity. Samples of 100 ml were usually taken twice a day. Growth was examined macro- and microscopically. The broth was filtered and the solid and liquid phases were then analysed.

Analytical methods. The amount of biomass was determined by drying the filter cake at 105° C overnight and then weighing. For soluble dry matter determination, an aliquot of the filtrate was evaporated, dried and weighed. Ammonia nitrogen was determined by the Berthelot reaction according to Keller et al. (1967). The method of Lowry (1951) was used for soluble protein determinations. The activity of the enzyme complex was measured by AJDA following the instructions of Biocon, Cork, Ireland. Clear apple juice supplemented with 0.2% pectin (Fluka, Neu-Ulm, FRG) was used as the enzyme sustrate. The enzyme/substrate mixture was incubated at 45° C. The time necessary for depectinization of the substrate was measured and compared with an enzyme of known activity. The depectinization of the substrate was followed by precipitation of pectin using isopropanol until no precipitate could be observed. In addition to AJDA, viscosity reduction was also used for the determination of the enzyme complex activity by a standard method (Schormüller 1967). Polygalacturonase (PG) activity was determined according to Wang and Keen (1970). Pectin esterase (PE) activity was measured according to the method described in the product information sheet no. AM-062-80 of Biocon. By this method, the methanol released during the enzymatic reaction, was determined. Pectinlyase (PL) was measured according to Ayers et al. (1966).

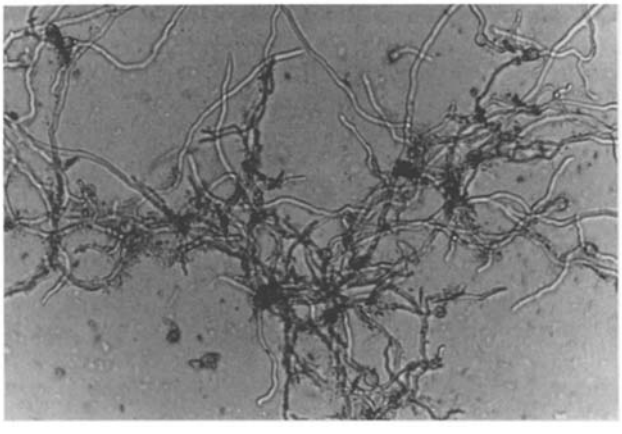

Fig. 1. *Aspergillus niger* mycelium after 17 h of growth, ×90

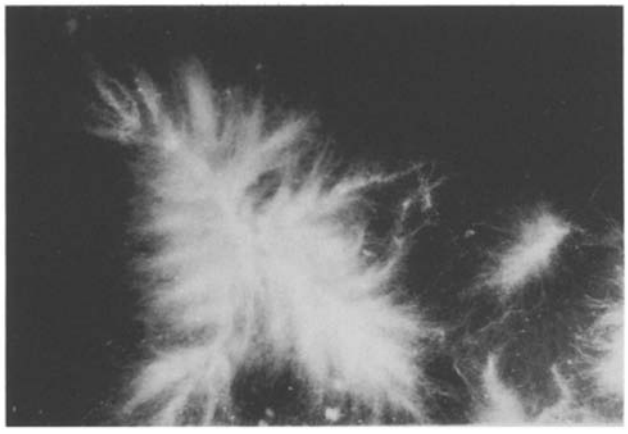

Fig. 2. Fungal pellet after 41 h, ×6

Results and discussion

Experiments with constant aeration and agitation

Fermentation experiments were performed with 9 l broth aerated with 5 l/min (approx. 0.5 vvm) and agitated at 300 rpm. During cultivation, *A. niger* A 138 showed different morphological stages. At about 17 h of incubation, the first hyphal interlacements were observed by microscopic examination. Substrate particles were attached to them. Sporadically rounded hyphal parts appeared as shown in Fig. 1.

At 25 h a few pellets could be seen macroscopically. From that time on, the biomass began to grow intensively. At 40 h the broth was filled with loose pellets of irregular size and shape as shown in Fig. 2. These branched pellets with their long peripheral hyphae caused a high broth viscosity which still increased after new smaller tuft-like aggregates had formed (Fig. 3). It appeared as if

Fig. 3. Part of a disintegrated pellet after 65 h, × 12

the pellets had disintegrated. However, the amount of biomass continued to increase and it was assumed that parts of the growing pellets split off forming more small mycelial masses. Hardly any mixing of the broth was visible; only after about 80 h did the viscosity of the broth begin to decrease. Apparently, this coincided with a slow but continuous decrease in biomass that was detected at the same time.

During fermentation, foaming of the broth caused a substantial problem. Automatic foam control was not effective during the critical period when foaming was most intensive. This coincided with the period of maximal growth rate of the fungus and lasted from about 25 to 40 h. During this period, foaming had to be carefully controlled by adding an antifoaming agent when necessary. If only a small quantity of the broth was lost with the foam, the fermentation was continued. However, it was observed from the results in such cases that the conditions in the broth changed so much that prolongation of the experiment did not yield useful results.

Measurements of dry biomass showed that after a lag period of about 20 h, growth increased until 75 h of incubation. Later on, a continuous decrease in total biomass was observed.

Enzymatic activity measured by AJDA, appeared with a delay of some 30 h with regard to biomass. Maximal activity was reached at 95 h of fermentation, about a day after the biomass maximum. An activity of 30 AJDA units/ml was attained. Enzyme activity remained constant for the next 65 h when the experiment was stopped. However, if aeration and agitation rates were decreased at the time when the broth became less viscous, enzyme activity was substantially reduced.

The loose filamentous pellets causing the high broth viscosity resulted in substantial reduction in heat and mass transfer. Oscillations in temperature and pH measurements appeared. Constant aeration and agitation in the tested range proved to be unsuitable, and therefore experiments with different aeration/agitation regimes were performed.

Experiments with variations in aeration and agitation rates

The initial conditions were 5 l/min (approx. 0.5 vvm) air flow and 300 rpm, as in previous fermentations. At 40 h the concentration of disolved oxygen began markedly to decrease and then aeration was increased to 10 l/min (approx. 1.2 vvm) and the impeller speed to 500 rpm. No appreciable changes in morphology were observed in comparison to those in experiments with constant lower aeration/agitation rates. At about 50 h of fermentation the broth became very thick. However, no oscillations of on-line measured parameters were observed. It could be concluded that there were no serious heat and mass transfer limitations under these conditions.

As shown in Figs. 4 and 5 the course of fermentation was improved after increased aeration and agitation. The slopes of all curves became more pronounced. The redox potential reached higher values, as in previous experiments, the shape of the curve being similar. The increase at the beginning was only due to aeration and not to fungal activity, since it was observed that the redox potential increased even if no inoculation of the medium was performed. The curve normally showed two peaks. Following the first peak, enzyme activity appeared.

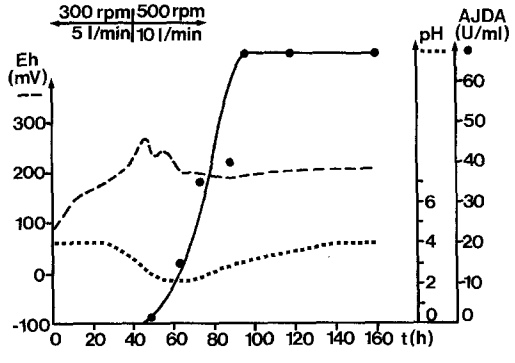

Fig. 4. Time course of redox potential (Eh), pH and enzyme activity (AJDA) during fermentation with variations in aeration and agitation rates

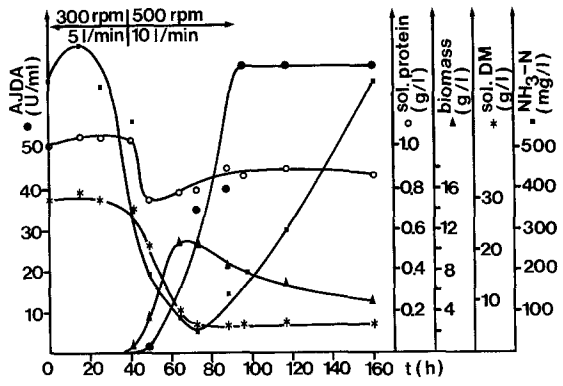

Fig. 5. Time course of biomass, soluble dry matter (DM), ammonia nitrogen (NH_3-N), soluble protein and enzyme activity (AJDA)

The pH curve showed a substantial decrease during the period of intensive growth. After remaining at a minimal value of pH 2.2 for a period of 15–20 h, it then began to rise. In spite of the very low pH value, which was already in the range of rapid inactivation of polygalacturonase (Gould 1975), enzyme activity continued to increase. Maximal activity was reached about a day after the pH value began to rise. Since the pH values and redox potentials were measured on-line, they served as valuable indicators of the initiation and end of enzyme synthesis.

Biomass, soluble dry matter, ammonia nitrogen, soluble protein and enzyme activity during fermentation are presented in Fig. 5. Ammonia nitrogen showed a peak at the beginning, presumably due to the reduction of nitrate to ammonia by assimilation (Berry 1975). After 25 h it was intensively consumed and reached a minimal value coinciding with the biomass maximum. As soon as the biomass concentration began to decrease, the ammonia nitrogen started to rise. This was supposed to be the result of autolysis, but this assumption could not be confirmed since the proteolytic activity was very low. Some other effects were thus involved.

The consumption of soluble substances began after 25 to 30 h of incubation and the biomass slowly started to rise. At 40 h when aeration and agitation were increased, the fungus began to grow rapidly. The respiration rate was highest at about 50 h, at which time the pellets began to disintegrate and enzyme activity appeared. It began to rise after the time of the maximal growth and respiration rates.

A continuous increase in enzyme activity was observed. After the time of the biomass maximum the enzymes were still produced for another 20 h. The process could be stopped after 90–100 h fermentation when an activity of 60–68 AJDA units/ml was achieved. Soluble protein consumption lasted until 50 h of fermentation. Later the amount increased, a fact which was in accordance with enzyme release in the filtrate as seen from activity measurements.

When evaluating the effect of aeration and agitation, it could be concluded that the increasing rates in the phase of accelerated growth led to a doubling of the enzyme activity reached within the same fermentation time. However, the amount of biomass did not increase much and it was concluded that pectolytic activity was not proportional to the amount of biomass. The same result was reported by Tuttobello and Mill (1961).

The substantial improvement in enzyme activity was the reason for the subsequent experiments where the aeration/agitation conditions were increased from the beginning of fermentation. During the first phase only the mixing rate was increased from 300 rpm to 500 rpm. The initial aeration was 5 l/min (approx. 0.5 vvm) as in previous experiments. During the phase of accelerated growth it was increased up to 10 l/min (approx. 1.2 vvm). Foaming was very intensive and represented a serious problem. Careful control was needed during the critical period from 25 to 40 h when the antifoaming agent had to be added at short intervals of only a few minutes. Morphology was not affected by the changed agitation. Enzyme activity at the end of fermentation (45 AJDA units/ml) was lower than in previous experiments.

The only positive effect of enhanced initial agitation was a shorter fermentation time. The maximal activity was reached only 65 h after inoculation. The comparison of different aeration/agitation regimes and their effect on enzymatic activity

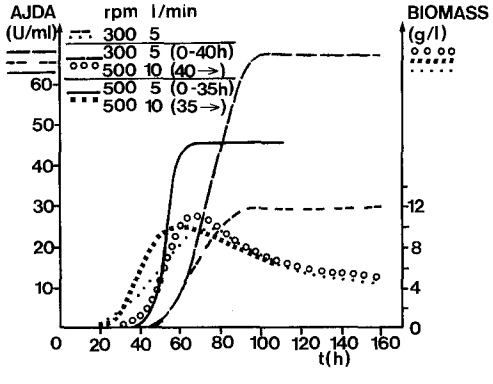

Fig. 6. Time courses of enzyme activity (AJDA) and biomass at different aeration/agitation regimes

Table 1. Pectolytic activity measured by viscosity reduction, polygalacturonase (PG), pectin esterase (PE), pectinlyase (PL) and AJDA in relation to the aeration/agitation regime

Aeration/ agitation regime	Viscosity reduction (%)	PG (mg/ml min)	PE (μg/ml min)	PL (E_{230})	AJDA (units/ml)	Fermentation time (h)
I	66–75	0.50	12–17	0.06–0.09	11–30	96
II	77–83	0.65–0.81	26–32	0.03–0.05	60–68	88–96
III	60–73	0.40–0.53	28–46	0.11–0.14	30–45	65

I = 5 l/min, 300 rpm; II = 5 l/min, 300 rpm in the first 40 h, 10 l/min up to the end; III = 5 l/min, 500 rpm in the first 35 h, 10 l/min, 500 rpm up to the end

and biomass growth is shown in Fig. 6. It can be seen that the effect was most marked on the enzyme activity whereas the maximal amount of biomass was not much affected. Zetelaki-Horváth and Békássy-Molnár (1975), however, found that higher agitation speed resulted in higher mycelial and enzyme yields when growing *A. awamori* for polygalacturonase production.

In addition to AJDA, the viscosity reduction method was used to determine the activity of the pectolytic enzyme complex. The activities of single enzymes such as polygalacturonase, pectin esterase and pectinlyase were also measured. The results of aeration and agitation effects on different pectolytic enzyme activities are shown in Table 1. It can be concluded from Fig. 6 and Table 1 that optimal results were obtained when the conditions of aeration and agitation were moderate at the beginning of fermentation and increased during the phase of maximal growth rate. Pectolytic activity measured by AJDA, viscosity reduction, or polygalacturonase activity achieved the highest values using such a fermentation regime. However, pectin esterase and pectinlyase were more active during conditions of more intensive agitation from the beginning of the fermentation. The same conditions were also optimal from the standpoint of the fermentation time.

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