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# Changes in Protein Secretion of *Aspergillus niger* Caused by the Reduction of the Water Activity by Potassium Chloride

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## Summary

The effect of the rapid reduction of the water activity ($a_w$) on the extracellular protein and amylolytic activity of *Aspergillus niger* was studied. An $a_w$ value gradient from 0.90 to 0.99 in KCl solutions was applied for the mycelium treatment. It was found that the $a_w$ reduction considerably influenced the protein secretion. This phenomenon was dependent on the age of the treated mycelium and the range of the $a_w$ gradient. The highest protein and enzyme secretion yields were obtained at $a_w = 0.98$ using a 72-h old mycelium. In comparison with the non-treated mycelium, the increase in the secretion amounted to about 60% for the amylolytic activity and 37% for the soluble protein, respectively. It was shown that the mycelium incubated in KCl solutions of an $a_w$ value from 0.90 to 0.99 had the ability for regeneration in fresh CZAPEK-DOX medium. The effect of the osmotic shock on the protein secretion was limited only for the treated cell population and declined in the mycelium which was regenerated after the transfer into the culture medium.

## Introduction

The term "water activity" ($a_w$) was introduced into microbiology by SCOTT [17] and was rapidly accepted by microbiologists as a very convenient concept to define the water relation in culture media and to estimate the amount of water available for biochemical reactions. Water activity and related parameters, namely membrane potential and osmotic pressure, can be externally regulated by the control of the concentration of low molecular solutes occurring in the culture medium, in particular by salts, for example, NaCl, KCl, $CaCl_2$ and others.

Water activity is recognized as a fundamental factor affecting the growth and metabolism of fungi [2, 3, 6]. The influence of the water activity on the enzyme stability and activity is also widely recognized. Considerably few reports refer to the effect of the water activity on the production and secretion of extracellular enzymes [1, 5, 13, 16].

Water plays an important part in the transmembrane transport of metabolites. Its role in this process is complex and not entirely elucidated. It can be assumed that the changes in the water activity of a culture medium affect the membrane protein structure and alter

the hydrophilic surface layer of the plasmalemma. The solutes appearing in the medium can also influence the ionic channels in the membrane and can change the active mass transport [16]. Both factors can play a role in being specific stimuli for protein secretion. Far-reaching changes in the membrane structure caused by rapid $a_w$ reduction can damage the physiological functions in the mass transfer control of the membrane.
The aim of this study is to define the influence of rapid changes in water activity caused by the potassium chloride concentration on the ability of *Aspergillus niger* to perform the mycelium regeneration and the secretion of amylolytic enzymes during and after osmotic treatment.

## Materials and Methods

### *Microorganism*

*Aspergillus niger* 193 from our own collection was used in this investigation. The culture was grown on malt extract medium (DIFCO, USA) at 30 °C for one week and was then maintained at 4 °C for a maximum of 3 months.

### *Culture Conditions*

The cultures were incubated in 500 $cm^3$ ERLENMEYER flasks containing 70 $cm^3$ of modified CZAPEK-DOX liquid medium (g/l): $NaNO_3$, 3.0; KCl, 0.5; $MgSO_4 \times 7\ H_2O$, 0.5; $CaCl_2$, 0.15; $KH_2PO_4$, 1.0; $FeSO_4 \times 7\ H_2O$, 0.01 and soluble potato starch 10.0. The water activity of the medium was 0.999. Each flask was inoculated with spore suspension to obtain $1 \times 10^7$ spores per 1ml. The cultivation was performed at 30 °C for 3 days on a rotary shaker at 200 rpm.
The regeneration of the mycelium after osmotic treatment was performed under the same conditions by using modified CZAPEK-DOX medium seeded with wet mycelium conforming to 0.1 g of cell dry matter.
The biomass production by the regenerated mycelium was expressed as a percentage of cell biomass introduced with the inoculum. The results presented an average of three repetitions of each experimental variant.

### *Mycelium Separation*

At an appropriate time the mycelium was filtered through a SCHOTT F4 filter, and the supernatant was used for protein and enzyme assays. The mycelium was washed three times with 100 ml of fresh medium at a pH of 5.0 and used in experiments with osmotic treatment and in control experiments.

### *Osmotic Treatment*

In order to cause an osmotic shock, the mycelium was transferred into a range of solutions with reduced water activity. The concentration of wet mycelium treated with osmotic shock was 150 g/l. The control of the water activity of the incubation solutions was carried out by adding adequate quantities of KCl (Tab. 1). To calculate the water activity values, the osmotic coefficients were adopted from ROBINSON and STOKES [12]. The $a_w$ values were estimated by a SINA-SCOPE instrument (SINA, Zurich, Switzerland). The mycelia were incubated at 30 °C for 30 min, with gentle shaking (50 rpm). The control samples were incubated in fresh CZAPEK-DOX mineral medium ($a_w = 0.999$) at a pH of 5.0. The results of these experiments are mean values of nine repetitions.

Tab. 1. Molalities of potassium chloride for various $a_w$ values

| $a_w$ | Molality | | Adequate water potential* [–MPa] |
|---|---|---|---|
| | Ideal solute | Potassium chloride | |
| 0.99 | 0.57 | 0.30 | 1.41 |
| 0.98 | 1.13 | 0.62 | 2.82 |
| 0.96 | 2.31 | 1.26 | 5.71 |
| 0.94 | 3.54 | 1.88 | 8.65 |
| 0.92 | 4.83 | 2.50 | 11.66 |
| 0.90 | 6.17 | 3.11 | 14.74 |

*Calculated for T = 30 °C

*Cell Disintegration*

After incubation, the mycelium was disrupted by sonication carried out at 20 °C for 30 min, with the vibration amplitude of 48 μm in buffer solution using an ultrasonic disintegrator (TECHPAN, Warsaw, Poland). The influence of the sonication on the crude enzyme activity was examined to correct the results obtained in the investigations. For this purpose, a sample of a 72-h old culture medium was centrifuged at 10,000 rpm for 10 min, and the supernatant was used for the enzyme activity assay according to the method described below. A sample was treated with sonication using the same sonication conditions described and a non-treated sample was used as the control. The difference in the amylolytic activity was determined to be 5.8%. All the results obtained, using enzyme solutions extracted from mycelium by sonication, were decreased by this coefficient.

*Enzyme Activity Assay*

The total amylolytic activity was determined by the hydrolysis of 1% soluble starch solution (MERCK, Germany). The starch depolymerization was carried out at 50 °C for 60 min, and the results were expressed as units equivalent to the micromoles of the glucose released per minute of the reaction and calculated per one ml of the culture filtrate used. The production of reducing sugars was measured in the reaction with dinitrosalicylic acid according to MILLER'S method [10].
It was found that the incubation of the amylolytic enzymes in KCl solutions has an unfavourable effect on its catalytic activity. The loss of activity was a linear function of the increasing KCl concentration. At a maximum potassium chloride concentration of 230 g/ml, adequate to $a_w = 0.90$, the loss of amylolytic activity was determined as 21% of the control sample. In experiments performed with solutions containing potassium chloride, the correction coefficients for each solute concentration were applied to reduce the influence of the solutes on the enzymatic reaction course.

*Determination of Soluble Protein*

The soluble protein content was determined according to the LOWRY *et al.* method [8]. Bovine serum albumin was used as the standard. The results were corrected to eliminate the errors caused by changes in the assay conditions due to the presence of $a_w$ depressors used in the reaction solutions.

*Determination of the Dry Weight*

The dry weight of the mycelium was determined gravimetrically after two-step drying (for reaching a constant weight), first at 60 °C and next at 105 °C.

*Examination under the Transmission Electron Microscope*

Fragments of *A. niger* mycelium after cultivation were rinsed with buffer and fixed in 2% glutaraldehyde buffered in 0.1 M sodium cacodylate at a pH of 7.4 and at a temperature of 4 °C for 24 h. The samples were further fixed with 2% osmium tetroxide in the same buffer for 2 hours. After rinsing, the objects were temporarily contrasted with 1% uranyl acetate for 2 hours. Dehydration was performed by treating the samples with ethyl alcohol, acetone and propylene oxide followed by fixation in the epoxy resin – Epon 812 [9]. The polymerized objects were cut with the LKB ultramicrotome, type Ultratom III. Ultrathin layers were contrasted with uranyl acetate and lead citrate [11, 19]. The ultrathin layers were observed and pictures were taken under the transmission electron microscope JEOL, type 7A at a voltage of 60 kV.

## Results

*The Regeneration of the Mycelium after Osmotic Treatment*

A rapid $a_w$ reduction caused by the transfer of mycelium into potassium chloride solutions produces a dramatic deterioration in the growth conditions for the fungus, in particular in the range of low $a_w$ values. It was interesting to estimate the ability of the mycelia to return to a normal healthy state after osmotic shock. For this purpose, the mycelia intended for osmotic treatment were separated from the media of different culture ages, i.e. from two-, three-, and four-day old cultures and transferred into KCl solution with reduced water activity (osmotic shock). According to the kinetic growth curve it can be noticed that the first two mycelial samples were withdrawn from the exponential phase of growth and the third sample was taken from the advanced stationary phase (Fig. 1). After osmotic treatment, the mycelia were transferred into fresh CZAPEK-DOX medium, and newly formed biomass was determined.
The data presented in Tab. 2 indicate that mycelium regeneration is strongly affected by previous osmotic treatment. It should be stressed that the whole range of osmotic shock

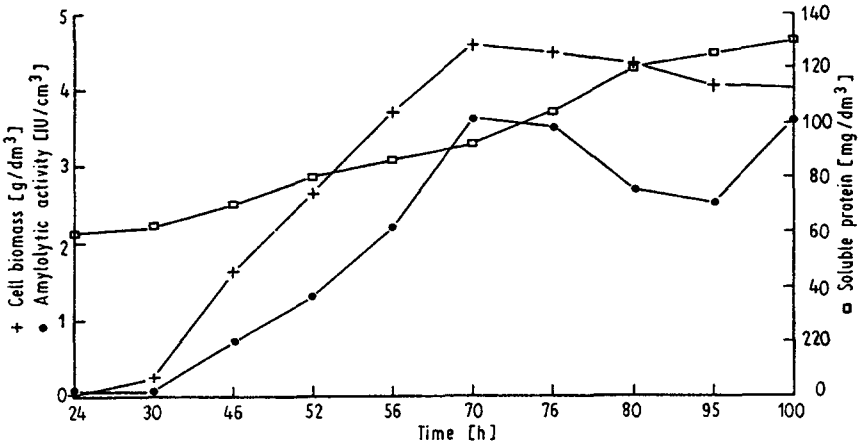


Fig. 1. Kinetics of *Aspergillus niger* growth, and the enzyme and extracellular protein production in submerged culture

applied to the mycelium treatment did not kill the fungus, and it retained an ability for regeneration. However, the higher the osmotic shock previously applied was, the slower the detected mycelium regeneration was. Independent of the age of the mycelium used for the osmotic pretreatment, a fast return to a normal healthy state was only observed in the cases when the mycelium was previously incubated at an $a_w$ above 0.96, whereas the $a_w$ reduction below 0.92 generated strong metabolic stress. A small cell mass decrease was revealed, especially in the first day of cultivation, when the 96-h old mycelium was pretreated and afterwards used for fresh medium inoculation. It was found that all the mycelia previously osmotically treated grew slower than the untreated mycelia, especially in the first twenty-four hours. Independent of the $a_w$ reduction range, a similar biomass concentration in all samples was detected after 48 h of cultivation. This confirms that the fungus has a good ability of regenerating mycelium after osmotic stress.

Tab. 2. Regeneration of mycelium after osmotic shock expressed as the percentage of mycelium mass introduced with inoculum

| Pretreatment ($a_w$) | Biomass production [% of inoculum biomass] | | | | | |
|---|---|---|---|---|---|---|
| | 48-h old mycelium | | 72-h old mycelium | | 96-h old mycelium | |
| | Cultivation time | | | | | |
| | 24 h | 48 h | 24 h | 48 h | 24 h | 48 h |
| 0.99 | 252 | 264 | 225 | 258 | 130 | 280 |
| 0.98 | 231 | 252 | 185 | 261 | 128 | 287 |
| 0.96 | 210 | 248 | 159 | 233 | 120 | 280 |
| 0.94 | 203 | 236 | 138 | 240 | 111 | 271 |
| 0.92 | 194 | 241 | 124 | 225 | 93 | 252 |
| 0.90 | 185 | 235 | 111 | 216 | 79 | 240 |
| Without treatment | 270 | 294 | 274 | 290 | 390 | 438 |

The inoculum contained 0.1 g of dry biomass; the value was calculated as 100%.

The best resistance to osmotic shock was shown in the 48-h and the 72-h old mycelia. During the first day of cultivation, only a slow growth was detected followed by an acceleration of the growth rate on the second day. It appeared in particular in cultures where the mycelia grew which were previously treated with strong osmotic shock. The 96-h old mycelium showed the worst regeneration ability in fresh medium in the first cultivation after twenty-four hours.

*The Production of Amylases and Total Extracellular Protein during Osmotic Shock*

The results presented in Tab. 3 showed a significant influence of the $a_w$ value controlled with potassium chloride on the amylolytic activities and soluble protein secretion. The secretion yields were dependent both on the $a_w$ reduction scale and on the mycelium

age. In the $a_w$ range from 0.99 to 0.90, the best amylolytic activity secretion was detected at $a_w = 0.98$. It was found that the highest secretion yields of amylase were produced both by the 72-h and the 96-h old mycelia.
In comparison with control samples, the maximum activities produced by two-day, three-day and four-day old mycelia treated with osmotic shock were 1.6, 1.6 and 1.2 times higher, respectively. Thus, the 96-h old mycelium has a greater secretion yield than the younger one.
The secretion of total soluble protein (LOWRY) was also dependent on the $a_w$ gradient and mycelium age. The highest amount of secretion of protein was found at $a_w = 0.98$ for two-day and three-day old mycelia, and at $a_w = 0.96$–$0.94$ for the four-day old one. It was also noticed that the highest protein amount was secreted by the four-day old mycelium. The increase in the secretion yield between the maximum values and control samples reached 17–37% dependent on the mycelium age.

Tab. 3. Enzyme and total protein secretion by *Aspergillus niger* at reduced water activity caused by potassium chloride

| $a_w$ | Total amylolytic activity [IU/cm³] | | | Soluble protein (LOWRY) [mg/cm³] | | |
|---|---|---|---|---|---|---|
| | Mycelium age | | | Mycelium age | | |
| | 48 h | 72 h | 96 h | 48 h | 72 h | 96 h |
| 0.99 | 1.58 | 1.96 | 2.00 | 0.097 | 0.126 | 0.222 |
| 0.98 | 2.06 | 2.52 | 2.26 | 0.109 | 0.129 | 0.233 |
| 0.96 | 1.72 | 2.14 | 2.14 | 0.102 | 0.119 | 0.241 |
| 0.94 | 1.58 | 2.14 | 1.98 | 0.087 | 0.114 | 0.241 |
| 0.92 | 1.50 | 2.10 | 2.00 | 0.088 | 0.100 | 0.186 |
| 0.90 | 1.50 | 1.96 | 1.98 | 0.082 | 0.090 | 0.174 |
| Control | | | | | | |
| 0.999 | 1.30 | 1.58 | 1.86 | 0.093 | 0.094 | 0.205 |

The data obtained showed some dissimilarities in the secretion of amylolytic enzymes and total soluble protein (Tab. 3). The secretion of soluble protein gave the best yields when the 96-h old mycelia were treated and was distinctly higher than those obtained with the younger mycelia. However, the differences in the secretion yields of amylolytic activities acquired with the 72-h old and the 96-h old mycelia were not significant.

*The Production of Amylases and Total Extracellular Protein by Mycelium Regenerated after Osmotic Treatment*

It was interesting to find out whether the increase in enzyme secretion observed during osmotic shock would continue in the mycelium transferred into the regeneration medium. The results of this experiment are presented in Tabs. 4 and 5.
It was found that the production of amylolytic activity by the regenerated mycelia was significantly influenced by the previous osmotic treatment. We have identified two important factors that determine the enzyme secretion by the mycelium regenerated in the

Tab. 4. Amylolytic activity produced in submerged culture by the regenerated mycelium of *Aspergillus niger* after osmotic treatment

| Pretreatment ($a_w$) | Amylolytic activity [U per g of dry mycelium] | | | | | |
|---|---|---|---|---|---|---|
| | 48-h old mycelium | | 72-h old mycelium | | 96-h old mycelium | |
| | Cultivation time 24 h | 48 h | 24 h | 48 h | 24 h | 48 h |
| 0.99 | 268 | 214 | 319 | 515 | 483 | 166 |
| 0.98 | 410 | 220 | 413 | 547 | 327 | 176 |
| 0.96 | 429 | 224 | 557 | 561 | 484 | 224 |
| 0.94 | 457 | 238 | 714 | 606 | 523 | 263 |
| 0.92 | 465 | 264 | 829 | 643 | 762 | 291 |
| 0.90 | 493 | 285 | 1234 | 668 | 1209 | 356 |
| Without treatment | 277 | 214 | 321 | 536 | 243 | 118 |

Tab. 5. Extracellular protein produced in submerged culture by the regenerated mycelium of *Aspergillus niger* after osmotic treatment

| Pretreatment ($a_w$) | Extracellular protein [mg per g of dry mycelium] | | | | | |
|---|---|---|---|---|---|---|
| | 48-h old mycelium | | 72-h old mycelium | | 96-h old mycelium | |
| | Cultivation time 24 h | 48 h | 24 h | 48 h | 24 h | 48 h |
| 0.99 | 14 | 20 | 83 | 86 | 68 | 25 |
| 0.98 | 31 | 21 | 101 | 94 | 54 | 28 |
| 0.96 | 36 | 25 | 171 | 126 | 91 | 32 |
| 0.94 | 40 | 27 | 207 | 127 | 121 | 48 |
| 0.92 | 46 | 32 | 231 | 128 | 186 | 91 |
| 0.90 | 60 | 42 | 250 | 137 | 220 | 103 |
| Without treatment | 17 | 25 | 58 | 98 | 43 | 17 |

fresh medium: (i) the age of the mycelium previously treated and (ii) the range of $a_w$ reduction used in pretreatment.

Comparing the mycelia of different culture ages, it can be ascertained that the highest amylolytic activities were obtained using the 72-h old mycelium. The mycelium previously treated at $a_w = 0.90$ produced about 1,234 U of enzyme activity per gram of cell dry matter after 24 h of cultivation. A similar result was obtained with the 96-h old mycelium.

It was also observed that the differences in the amylolytic activity produced on the first day of cultivation by osmotically pretreated mycelia were higher than those detected on the second day of cultivation. Thus, using the 72-h old mycelium, the activity range detected after 24 h of cultivation varied from 319 U at $a_w = 0.99$ to 1.234 U at $a_w = 0.90$, whereas after 48 h of cultivation, the activities varied from 515 U to 668, respectively. The similar patterns were obtained using the 48-h and the 96-h old mycelium.
The results listed in Tab. 4 show that the greater the $a_w$ reduction applied during osmotic treatment was, the greater the enzyme secretion detected was. This physiological behaviour was also observed after both 24 h and 48 h of cultivation It was also noticed that the enzyme production negatively correlated with the production of cell biomass (Tab. 2). The highest enzyme activity appeared in the culture medium seeded with mycelia previously treated at $a_w = 0.90$, whereas the fungus growth in these cultures was very low, and even a small reduction in the biomass concentration was determined.
The pattern of extracellular protein secretion by mycelia regenerated in fresh medium as a function of the mycelium age and the $a_w$ reduction during pretreatment is given in Tab. 5. The data obtained shows many similarities with the pattern of enzyme secretion. Thus, the greatest protein production was detected in the medium which was seeded with the 72-h old pretreated mycelium. It was also found that the lower the $a_w$ value applied in the osmotic treatment was, the higher the protein secretion determined was in the culture medium. Another similarity was a greater protein secretion in the first 24 hours than in the second.

## Discussion

The data obtained in this study showed that the reduction of the water activity in the external environment affected the protein secretion by the fungal cells. In other literature there are some reports on the influence of the osmolarity on the active transport of *Eucaryota* [14, 15, 19]. Unfortunately, no data were found concerning the ion transport in the cells of filamentous fungi in response to osmotic stimulation.
The data presented in this paper show the characteristic behaviour of mycelium exposed to rapid changes in osmotic pressure; in a relatively narrow range of osmotic pressure values, equal to $a_w$ values between 0.96 and 0.98, a significant increase in protein secretion was observed, whereas at the higher osmotic pressure, equal to $a_w$ values below 0.94, the secretion yield was reduced. We hypothesize that with the rapid increase of external osmotic pressure, the first reaction of the cell is to thicken the cytoplasm by quickly removing the water from the cell, and finally, the concentration of solutes within the cell increases. This suggestion was confirmed by the disappearance of transport vehicles from the cytoplasm caused by the treatment of the mycelium by osmotic shock (Fig. 2). It was also found that the reduction of $a_w$ values below 0.94 caused considerable damage to the organization of subcellular structures.
The reduction of the enzyme secretion yield at low water activity values can be explained as disturbances in the control of the mass transport by the cell. Our experiment with recycling the mycelium previously treated by osmotic shock to the fresh culture medium showed a lack of correlation between the protein secretion yield and a return to a normal healthy state for mycelium treated at an $a_w$ below 0.92 (Tabs. 3–5). An inverse

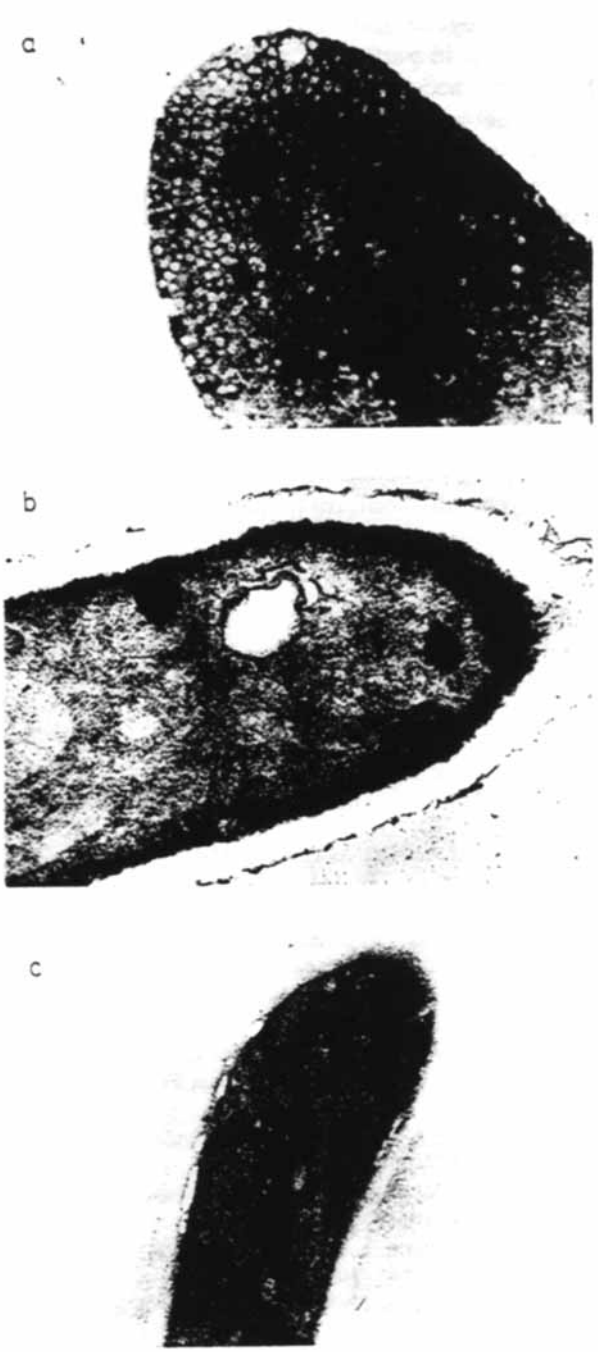


Fig. 2. Longitudinal section of the apical region of a hypha of *Aspergillus niger*
(a) before osmotic shock, (b) after osmotic treatment at $a_w = 0.98$, and (c) after osmotic treatment at $a_w = 0.90$
Magnitudes: (a) 25,000 x, (b) 24,000 x, (c) 22,400 x.

dependence between mycelium regeneration and protein secretion was observed. It appeared after 24 h of cultivation, in particular, when the greatest protein and amylolytic activity secretion was obtained in spite of the growth absence. These findings demonstrate that osmotic treatment caused an increase in plasma membrane permeability, resulting in soluble protein extraction. After the physiological adaptation of the cells to the new culture conditions, a significant decrease in protein secretion was noticed. It can be explained by the repairing of the damage of the membrane structure caused by osmotic shock and the restoration of the control function of membrane. This hypothesis is confirmed by some observations. At first, the changes in enzyme activity produced by untreated mycelium after 24 h and 48 h of cultivation were relatively small, whereas the same changes produced by treated mycelium were very significant. Secondly, the reduction in the amount of enzyme and soluble protein released was accompanied by considerable mycelium regeneration. It seems that the effect of osmotic shock on the increase in protein is only limited for the treated cell population. There are some reports on the effect of the $a_w$ reduction in enzyme secretion [4, 7, 13]. The data presented by these authors are generally conform to the results obtained in this study.

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## Advances in Biosensors

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In the last few years, the research and application of biosensors has rapidly increased. From this point of view it was a logical consequence to start a new series of "Advances in Biosensors". For this reason A. P. F. TURNER from the Biotechnology Centre of the Cranfield Institute of Technology was a predestined editor. By reading the first three volumes, it can be concluded that the start of this series was a success. In the first two "official" volumes all the possible areas of biosensor development, research on biosensors and application of such measuring systems were presented, e.g. the topics discussed in volume 1 are Whole-Organism Based Biosensors and Microbiosensors (KUBO *et al.*); Electrochemical Biosensors: Application to Some Real Problems (MASCINI and MOSCONE); Enzymatic Amplification and Elimination in Biosensors (SCHUBERT *et al.*); Biosensors Based on Modified Electrodes (KULYS); Mediated Electrochemistry: A Practical Approach to Biosensing (CARDOSI and TURNER); Eclectic Immunoassay: an Electrochemical Approach (JENKINS *et al.*); Optical Immunosensors: An Overview (ROBINSON); Electrochemical, Piezoelectric and Fibre-Optic Biosensors (GUILBAULT and SCHMID).
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