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Effect of water activity on production and activity of *Rhizopus oligosporus* polysaccharidases

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Summary. During tempeh fermentation, *Rhizopus oligosporus* produced polysaccharidases to degrade soya bean cell walls; the maximum activity for all polysaccharidases tested occurred 20–30 h after inoculation. *R. oligosporus* was also grown in a soya bean extract model medium to which glycerol was added to control water activity (a_w). The overall activities of the major enzymes produced by the fungus, polygalacturonase, endocellulase and xylanase, appeared to be strongly influenced by a_w . The production of enzymes as well as their specific activities were affected by a_w . The optimum a_w for polygalacturonase and xylanase activity coincided with that for mycelial growth, namely 0.99–1.00. In contrast, the optimum a_w for (endo)cellulase was 0.98, at which mycelial growth was significantly reduced.

Introduction

Water is a fundamental prerequisite for biological activity. Since microorganisms do not recognize the water content of a particular material, but rather the amount of available water, food microbiologists prefer to use the parameter “water activity” instead of the water content.

The influence of water activity (a_w) on microbial growth (Beuchat 1983) and production of metabolites (Mattiasson and Hahn-Hägerdal 1982) is widely recognized. The effect of a_w on production of, for example, aroma compounds has been studied (Gervais and Sarrette 1990); the latter can be considered as resulting from particular metabolic pathways in which enzymes are involved. Considering the protein nature of enzymes, the influence of a_w on protein stability (Laretta-Garde et al. 1987) and activity (Hahn-Hägerdal et al. 1987) as well as the role of water as a vehicle for substrate transport and as a reactant, it may be expected that a_w affects enzymatic transformation during fermentations. Consequently, in fermented foods, enzyme pro-

duction (Kaji and Yoshihara 1969) as well as enzyme activity (Chou and Ho 1988) are of importance in view of the optimization of metabolite production.

Tempeh is obtained by solid substrate fermentation of soya beans with *Rhizopus oligosporus*, a filamentous fungus (Nout and Rombouts 1990). The mycelium is able to penetrate the cell wall of the beans (Jurus and Sundberg 1976) in order to digest the cell constituents and improve the nutritive value of beans (Shurtleff and Aoyagi 1979). The activity of polysaccharide-degrading enzymes (Okolie 1988) possibly contributes to this penetration.

The present report deals with the influence of a_w on the production and activity of polysaccharidases, notably polygalacturonase, cellulase and xylanase by *R. oligosporus* during tempeh fermentation and in model systems.

Materials and methods

Microorganism. *R. oligosporus* NRRL 5905 was grown on malt extract bouillon (MEB, Oxoid CM57) at 30°C for 7 days and maintained on malt extract agar (MEA, Oxoid CM59) at 4°C for a maximum of 4 months.

Preparation of spore suspension for tempeh fermentation. *R. oligosporus* was grown for 2 days on slants of MEA at 30°C. A spore suspension was obtained by shaking slant cultures on a vortex mixer with 10 ml of aqueous 0.05% Tween 80.

Medium and inoculation. Medium 1: in order to determine which polysaccharidases are produced by *R. oligosporus*, it was grown as in the traditional tempeh fermentation process (Nout and Rombouts 1990). Dehulled soya beans were soaked in water overnight at room temperature (25°C), then boiled for 50 min. After discarding the cooking water, the hot beans were spread in a thin layer and cooled to room temperature; subsequently, they were inoculated with a spore suspension of *R. oligosporus* (10^6 cfu/kg of cooked soya beans). The inoculated soya beans were packed firmly in perforated plastic boxes, and incubated at 30°C for 50 h, yielding fresh tempeh.

Medium 2: in order to study the effect of a_w on the production of enzymes and their activities, *R. oligosporus* was grown on a model substrate prepared with soya beans. This method eliminates

problems of heat and mass transfer and measurement of a_w that occur in tempeh. This substrate named soya extract agar (SEA) was obtained by soaking dehulled soya beans overnight (Nout 1989), followed by cooking them in the soak water for 10 min, and by precipitation of soluble proteins at consecutive low(4.5) and high(9.0) pH. The medium contained a minimum of 1% of oligosaccharides, and had a final pH of 4,5; 2% agar (Oxoid L13) was added. After sterilisation for 15 min at 120°C, the medium was poured into petri dishes with perforated covers. SEA plates were inoculated at the centre with a piece of mycelium of 8 mm diameter (Gervais and Sarrette 1990) taken from a previous culture (3 days at 30°C).

Control of a_w in the SEA medium. Before sterilisation, an amount of glycerol calculated using the Norrish equation (Norrish 1966) was added to SEA medium in order to produce a_w values ranging from 0.96 to values close to 1.00. After inoculation, petri dishes were incubated at 30°C in chambers with a relative humidity equivalent to the a_w of the respective SEA plates, maintained by glycerol-water mixtures to which sodium azide had been added at 0.01% as a preservative.

Control of a_w in enzyme assay. The influence of a_w on enzyme activities was measured by adding adequate quantities of glycerol to the enzyme-substrate solutions.

Growth measurement in SEA medium. Fungal growth on SEA was measured by either (a) radial growth or by (b) biomass determination. For (a), colony diameters were measured along two perpendicular axes marked on the upper part of the petri dishes. For (b), the culture and its medium were removed from the petri dish, brought to boil in a microwave oven, and subsequently vacuum filtered (Schleicher and Schuell 520B). Three successive washings of the filter were made, each with 50 ml distilled water at 95°C. The filter was dried overnight at 110°C, then placed in a desiccator and weighed.

Enzyme recovery. For tempeh fermentation, a perforated cylindrical mould of 70 mm diameter, closed with perforated covers at both ends was used. The cylinder and covers contained holes with a diameter of 2 mm, spaced 2 cm apart. For sampling, a slice of 30 mm thickness of fermented soya beans was cut from one side of the cylinder and the resulting air space was filled by pushing the cover inwards onto the remaining fermenting beans; the next sample was taken from the other side of the cylinder, so that each sample had undergone similar conditions of aeration.

For the cultures grown on SEA in petri dishes, two samples were taken after 28 and 46 h of incubation.

Samples (70 g each in the case of tempeh fermentation and 60 each in the case of petri dishes) were mixed in a blender (MSE, Beun de Ronde, The Netherlands) at high speed with 200 ml of 50 mM sodium acetate buffer, pH 5.0, then centrifuged at 4°C for 20 min at 10000 rpm. The supernatant was kept and dialysed against 5 l water in an acetate dialysis tube at 4°C for 36 h; the water was changed every 10 h. The dialysis enabled removal of glycerol from SEA media and the obtention of enzyme extracts at a water activity close to 1.00. The dialysed solutions were frozen and subsequently freeze-dried. The dried extract was dissolved in 50 mM sodium acetate buffer, pH 5.0.

Enzyme assays. Activities of enzymes were assayed by determination of reducing sugars released by the hydrolysis of polygalacturonic acid (ICN pharmaceuticals, Cleveland, Ohio, USA) for polygalacturonase activity; Avicell (type SF Serva, Heidelberg, FRG) for exocellulase activity; AF0305 (type Akucell, Akzo, The Netherlands) for endocellulase activity; highly branched xylan (from oat spelts, Sigma, St. Louis, Mo., USA) for xylanase activity; and UFR arabinan obtained by ultrafiltration of clarified apple juice produced from apple pulp using technical pectinase and cellulase enzymes (Voragen et al. 1986) for arabinase activity, respectively.

A mixture of 0.5 ml, containing 100 µl (0.5% w/v) substrate, 50 µl enzyme solution and 350 µl 50 mM sodium acetate buffer, pH 5.0, was incubated for 1 h at 30°C. The release of reducing sugars was determined with the Nelson Somogyi method (Spiro 1966) using a Beckman DU-64 spectrophotometer at 520 nm.

Activities of β -D-glucosidase, α -D-galactosidase, β -D-xylosidase, α -L-arabinofuranosidase and α -D-glucosidase were measured using their corresponding p-nitrophenol (PNP) derivatives as substrates. An amount of 0.4 ml enzyme solution was added to 0.1 ml PNP-substrate solution (0.1% in 50 mM acetate buffer, pH 5.0) and incubated for 1 h at 30°C. The reaction was stopped by addition of 0.5 M glycine buffer, pH 9.0 and 0.002 M EDTA. The released PNP was measured at 400 nm, and quantified using a molar absorption coefficient of 13700.

All activities are expressed in international units (U). Measurements of enzyme activities were repeated three times: differences between repetitions differed by less than 7%. All experiments were carried out in duplicate: the variation coefficient between duplicates was less than 20%.

Results

Enzymes produced in tempeh

Since soybean cell walls contain 30% pectic substances, 50% hemicelluloses and 20% celluloses on a dry weight basis (Kikuchi et al. 1971), we investigated polygalacturonase, cellulase, xylanase and arabinase activities during tempeh fermentation. No pectate lyase and no exocellulase activities were measured. From Fig. 1 it can be seen that polygalacturonase activity was detected after 10 h of fermentation. A few hours later, endocellulase, xylanase and arabinase activities could be detected. The maximum activity for all enzymes was found between 20 and 30 h of fermentation.

Figure 2 shows activities of glycosidases produced in tempeh. Stachyose is one of the antinutritional compounds found in soya beans. During tempeh fermentation, the production of α -D-galactosidase contributes to its degradation. The α -galactosidase activity we measured after 24 h of tempeh fermentation corresponds with that found by Van de Riet et al. (1987).

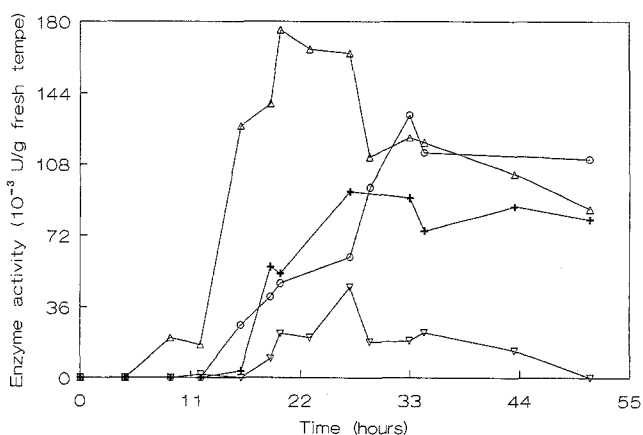


Fig. 1. Time course of polygalacturonase (Δ), endocellulase (\circ), xylanase (+) and arabinase (∇) activity of *Rhizopus oligosporus* during tempeh fermentation: U, International units

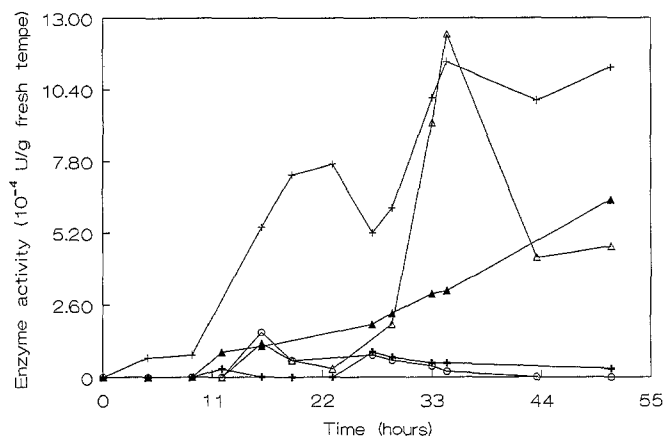


Fig. 2. Time course of β -D-glucosidase (+), α -D-galactosidase (Δ), β -D-xylosidase (O), α -L-arabinosidase (+) and α -D-glucosidase (\blacktriangle) of *R. oligosporus* during tempeh fermentation: U, International units

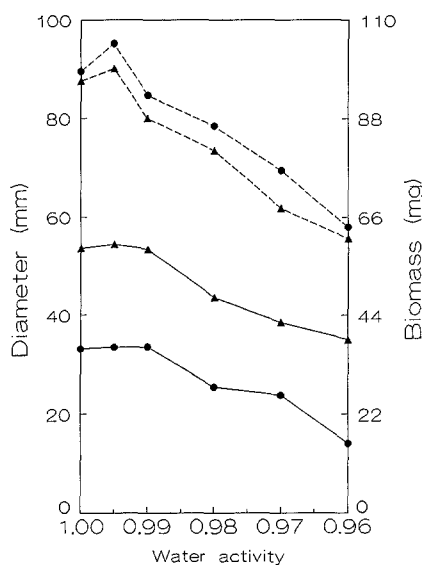


Fig. 3. Effect of water activity on the growth of *R. oligosporus* on soya extract agar medium: \blacktriangle , diameter of the colony; \bullet , biomass (dry weight); —, after 28 h of fermentation; ----, after 46 h of fermentation

Influence of a_w on mycelial growth and enzymes produced on SEA medium

Mycelial growth. Figure 3 shows that the diameter and dry weight of *R. oligosporus* colonies decreased with lower a_w values.

Influence of a_w on hydrolytic reactions of enzymes. As previously mentioned, the effect of a_w on the production of enzymes should be distinguished from the effect on their activity. Since polygalacturonase, endocellulase and xylanase were the predominant polysaccharidases produced by *R. oligosporus* in tempeh, we investigated the effect of a_w on their hydrolytic capacity by the a_w control of reaction mixtures through glycerol addition. Two kinds of enzymes were used in this experiment: en-

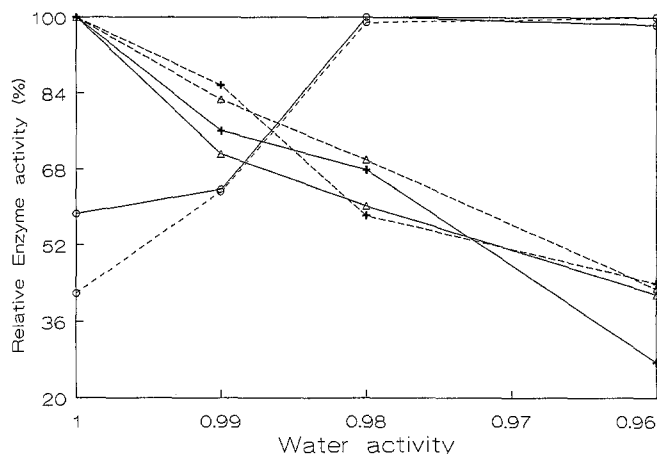


Fig. 4. Effect of water activity (a_w) on the percentage of the product released in hydrolytic reactions of polygalacturonase (Δ), endocellulase (O) and xylanase (+): —, enzyme produced in medium at $a_w = 1.00$; ----, enzyme produced in medium at $a_w = 0.98$

zymes produced by the fungus grown on a medium at a_w close to 1.00 and corresponding enzymes formed by the fungus grown at a_w 0.98. Figure 4 shows that the activity of polygalacturonase, xylanase and cellulase were influenced by the a_w of the reaction mixture in which they were incubated. Maximum polygalacturonase activity was observed at an a_w close to 1.00, whereas it decreased significantly at lower a_w , to 42% at $a_w = 0.96$. A similar effect of a_w was detected with the xylanases. There was a decrease in relative enzyme activity at reduced a_w , of the same order of magnitude as observed with the polygalacturonases. The behaviour of cellulases was very different. Whereas the maximum relative enzyme activity was observed at a a_w between 0.98 and 0.96, it decreased with higher a_w to only approximately 50% at a_w close to 1.00.

There were no significant differences between the behaviour of enzymes produced at $a_w = 0.98$ and enzymes produced at a_w close to 1.00, except for cellulase activity measured at a_w close to 1.00 and xylanase activity measured at $a_w = 0.96$. The measurement of kinetic parameters of polygalacturonase activity incubated at a_w close to 1.00 and $a_w = 0.96$ revealed that the maximum reaction velocity (V_{max}) was affected by a_w . For instance, at $a_w = 0.96$, $V_{max} = 3.05$ nmol of reducing sugars/min, but at $a_w = 1.00$, V_{max} was 4.25 nmol of reducing sugars/min. However, the K_m values (0.41 mg/ml) were the same under both reaction conditions.

Influence of a_w on enzyme production. The objective of this experiment was to assess the influence of a_w on the production of some extracellular enzymes by *R. oligosporus*. The fungus was grown in SEA medium at different a_w ranging from 1.00 to 0.96. Subsequently, enzymes were extracted and their activity was measured at a_w close to 1.00 without addition of any a_w depressor. All results concerning cellulase activity were corrected for the influence of a_w on enzyme assays presented above. Figure 5 presents the production of the enzymes

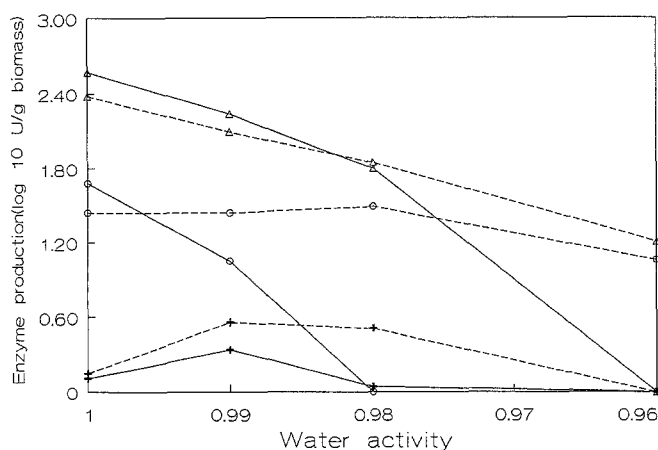


Fig. 5. Effect of water activity on enzyme production per unit of mycelial dry weight: Δ , polygalacturonase; \circ , endocellulase; +, xylanase; —, after 28 h of fermentation; ----, after 46 h of fermentation: U, International units

expressed per unit of biomass dry weight. After 28 h of fermentation at $a_w = 0.96$, the mycelial growth was very poor and it was not possible to measure any enzyme activity. In the culture medium used, an a_w close to 1.00 favoured the production of polygalacturonase. In the a_w range 1.00–0.98, there were no great differences in enzyme activity after 28 h or 46 h. With xylanase, we observed an increased enzyme production at 46 h compared to 28 h for all a_w values, and this increase was even more pronounced at low a_w . At 28 h, the optimum a_w for xylanase production was close to 0.99, and at 46 h there was no significant difference between $a_w = 0.99$ and $a_w = 0.98$. After 46 h, there was more enzyme produced at a_w close to 1.00 than at $a_w = 0.96$. With cellulase, results after 28 h did not show the same pattern as those after 46 h. Whereas the endocellulase production was strongly reduced at $a_w = 0.98$ after 28 h of fermentation, the a_w had no significant effect after 46 h.

Discussion

Polygalacturonase was first detected in tempeh, followed by endocellulase, xylanase and arabinase. Similar patterns were observed by Cooper and Wood (1975) who found that with *Verticillium albo-atrum*, production of polysaccharidases occurred in a sequential manner and that the first enzyme to be detected was polygalacturonase. This sequential appearance probably reflects the physicochemical accessibility of bean cell wall polymers, resulting in induction as successive substrates become available during progressive degradation of the cell wall. Polygalacturonase is also a major enzyme in *R. stolonifer* (Manachini et al. 1987) and in *R. arrhizus* (Sachde et al. 1987). Whitaker (1978) mentioned earlier that *R. oligosporus* produces pectinase activity.

Although the enzyme activities that we detected are not very high compared with those of fungi used commercially for the production of polysaccharidases, and

R. oligosporus is known mainly for its protease and lipase production, our findings show undoubtedly that the *R. oligosporus* enzymes can degrade soya bean cell-wall components.

The fact that certain enzymes such as β -D-glucosidase do not show a gradual increase during the fermentation but rather have two maximum values, could be due to the production of two or more isoenzymes that degrade the same substrate but are not synthesized at the same time. Their synthesis might depend on the nature of the compounds to be hydrolysed in the medium.

It appears that the moment at which the highest enzyme activities were found (20–30 h), did not correspond with that chosen to stop tempeh fermentation, usually 2 days. We hypothesize that the enzymatic disruption of bean cell walls occurs during the early stages, and that the conversion of proteins and lipids takes place subsequently. This hypothesis is supported by the data of Chou and Ho (1988), who found that the highest yield of α -galactosidase during sufu preparation by *Actinomyces taiwanensis* was observed after 24 h of fermentation, whereas the highest yield of lipase and protease occurred after 48 h. Another explanation is offered by Sudarmadji (1977) who prepared tempeh with a pure culture of *R. oligosporus* incubated at 32°C, and observed the appearance of the mycelium on beans at the same time as in our experiment, i.e. after 20 h of incubation. He measured the temperature at the centre of the cakes (for 41 h) and concluded that the fermentation process may be differentiated into three phases. During the first phase (0–30 h), there was a rapid increase in total free fatty acids (maximum lipase activity), mould growth (estimated visually) and temperature at the centre of the cake. The end of this phase corresponded to the moment at which we found maximum activities of polysaccharidases. At the start of the second phase, tempeh was ready to be harvested (30–50 h), attaining its typical flavour, colour and texture during this period. The third phase (> 50 h) corresponded to the spoilage of tempeh by bacteria and to the development of an unpleasant taste. According to Sudarmadji (1977), mould growth slows down at the end of the first phase; this stage corresponds to the onset of decreasing enzyme activities found by us. Thus, it appears that there is a good relationship between polysaccharidase activities and growth of mycelium. This consideration is of importance since there is no possibility of measuring mycelial biomass directly during tempeh fermentation.

Concerning the influence of a_w on the growth of the fungus on SEA medium, Wang et al. (1974) reported already that the protease production and mycelial growth of *R. oligosporus* grown on a solid substrate, were more influenced by the moisture content of the medium than by incubation time. Wang et al. (1974) also indicated that many members of the order Mucorales, to which *R. oligosporus* belongs, require a relative humidity near 100% to grow best. We found an optimum a_w for growth of 0.995. This corresponds with the findings of Gervais et al. (1988) for *Trichoderma viride*.

Concerning the effect of a_w on the hydrolytic reactions of enzymes, it appears that a_w affects the V_{max} of

the hydrolysis but not the affinity of the enzyme for the substrate. As only the velocity was affected, the influence of a_w probably resulted from a first-order non-competitive inhibition. Hydrolytic enzymes split off a part of the substrate molecule while forming an acyl-enzyme intermediate. In this experiment, reduced a_w did not affect the rate of enzyme-substrate complex and acyl-enzyme formation. The K_m value found in our experiments is comparable with the values found by Manachini et al. (1987) and Liu and Luh (1978) who worked with an endo-polygalacturonase produced by *R. stolonifer* and *R. arrhizus*, respectively.

The fact that the production of polygalacturonase is not very different after 28 h or 46 h means that already after 28 h, the fungus had produced the major part of the polygalacturonase enzyme; this corresponds with Fig. 1 where polygalacturonase was the first enzyme to be synthesized in tempeh. Only at $a_w = 0.96$ was the polygalacturonase production significantly more important after 46 h than after 28 h. However, this was due to the long lag phase for mycelial growth at this a_w . The different behaviour of cellulase production between 28 h and 46 h of fermentation could be related to the fact that cellulase synthesis by fungi occurs very often after polygalacturonase and xylanase synthesis. In tempeh the maximum production of cellulase also occurred after that of polygalacturonase, but its maximum activity was close to that of xylanase. On the whole, it appears that 28 h of fermentation is too short a period to allow comparisons, and that samples extracted after 46 h are more representative of enzyme production capability.

With regard to the production of enzymes per unit of biomass dry weight, $a_w = 0.98$ stimulated cellulase production by the mycelium, $a_w = 0.99$ was optimum for xylanase, and $a_w = 1.00$ was optimum for polygalacturonase production. These optima are more pronounced if this production is measured at the same a_w of the growth medium and concerns the total biomass of each batch. Nevertheless, a_w seems to affect more the enzymatic reaction than the production of enzymes by a fungus. The osmoregulation of mycelial cells in order to preserve metabolic pathways, synthesis of proteins for example, might attenuate these differences.

Grajek and Gervais (1987) studied the influence of a_w on enzyme production by *Trichoderma viride* TS and obtained similar results to ours. The maximum production of *T. viride* polygalacturonase and xylanase occurred at $a_w = 1.00-0.99$. We conclude that the optimum a_w for the production and hydrolytic activity of these enzymes appears to be very close to the optimum a_w for mycelial growth. Cellulase behaves differently, considering that the optimum a_w for enzyme production and activity is clearly below the optimum a_w for mycelial growth. This was suggested earlier by Hulme and Stranks (1971) with *Myrothecium verrucaria*.

Our results lead to several questions referring to the mechanism of the effect of a_w on enzyme synthesis and enzyme excretion. With regard to the latter, questions arise concerning the formation and the functioning of the fungal plasma membrane. It has been shown (Charlang and Horowitz 1974) that a_w affects the fluidity of

the membrane. Whereas these findings would offer a basis for a hypothesis on the role of a_w in mechanisms of protein export localized in the plasma membrane, there is still a complete lack of understanding with regard to its effect on enzyme synthesis.

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