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Production of a Novel Pyranose 2-Oxidase by Basidiomycete *Trametes multicolor*

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ABSTRACT

During a screening for the enzyme pyranose 2-oxidase (P2O) which has a great potential as a biocatalyst for carbohydrate transformations, *Trametes multicolor* was identified as a promising, not-yet-described producer of this particular enzyme activity. Furthermore, it was found in this screening that the enzyme frequently occurs in basidiomycetes. Intracellular P2O was produced in a growth-associated manner by *T. multicolor* during growth on various substrates, including mono-, oligo-, and polysaccharides. Highest levels of this enzyme activity were formed when lactose or whey were used as substrates. Peptones from casein and other casein hydrolysates were found to be the most favorable nitrogen sources for the formation of P2O. By applying an appropriate feeding strategy for the substrate lactose, which ensured an elevated concentration of the carbon source during the entire cultivation, levels of P2O activity obtained in laboratory fermentations, as well as the productivity of these bioprocess experiments, could be enhanced more than 2.5-fold.

Index Entries: *Trametes multicolor*; pyranose 2-oxidase; screening; culture medium development

INTRODUCTION

Pyranose 2-oxidase (P2O, glucose 2-oxidase, pyranose:oxygen 2-oxidoreductase, EC 1.1.3.10) catalyzes the C-2 oxidation of several aldopyranoses to form the corresponding 2-keto derivatives, with the preferred

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substrate being D-glucose, which is oxidized to D-*arabino*-2-hexosulose (2-keto-D-glucose, D-glucosone). During this oxidation, electrons are transferred to molecular oxygen to yield hydrogen peroxide. P2O has been purified and characterized from several sources *(1–6)*. Typically, it is a rather large glycoprotein that contains covalently bound flavin adenine dinucleotide.

There is very good indication of a likely involvement of P2O in lignocellulose degradation. A possible role of this enzyme could be as a major source of hydrogen peroxide *(7)*. This view is supported by the fact that P2Os typically are rather unspecific, accepting all major sugars found in lignocellulose as substrates, and that their main localization is in the periplasmic space, or even extracellular, which has been demonstrated *in situ* during wood decay for several organisms *(7,8)*.

A completely different physiological function of P2O has been suggested by Baute et al. *(9,10)*, who showed that glucose can be converted to the antibiotic cortalcerone, via glucosone, by the fungus *Corticium caeruleum*. The formation of this antibiotic substance could also be proven in a number of other fungi (13.65% of the species tested in a screening). Moreover, the existence of the enzyme pyranosone dehydratase, which transforms glucosone into cortalcerone, has been shown in *Phanerochaete chrysosporium (11,12)*. However, a number of organisms that exert P2O activity do not possess this ability to convert glucosone into cortalcerone, apparently lacking the second enzyme of this pathway. These findings indicate that glucosone may play a more significant role as an intermediate in glucose metabolism in fungi, cortalcerone biosynthesis being probably one of the possible alternative pathways *(11)*.

P2O offers an attractive potential as a biocatalyst for the specific oxidation of unprotected sugars. The dicarbonyl compounds formed by these enzymatic transformations can be used as fine chemicals, or as building blocks in synthetic carbohydrate chemistry *(13,14)*. Because chemical syntheses of these compounds are laborious and result in relatively low yields, together with a number of byproducts, the enzymatic conversion represents an interesting alternative to the chemical route. Because of the high regioselectivity of the enzyme employed, a close-to-complete formation of three different dicarbonyl sugars, with yields of 85–99% by using P2O from *Peniophora gigantea*, has been recently reported *(15)*. Furthermore, some of the dicarbonyl sugars have proposed applications in food technology. 2-Ketoglucose can be used as the key intermediate in the production of fructose, mannitol, or sorbitol, and has attracted considerable attention in this respect *(16–18)*.

It was the objective of our work to identify a suitable producing strain of the enzyme P2O, which has a great potential for carbohydrate transformations. Additionally, growth conditions favoring the enhanced formation of this enzyme in an appropriate organism should be investigated in detail.

MATERIAL AND METHODS

Chemicals

All chemicals were of the highest purity available, and were obtained from Merck, Darmstadt, Germany, unless otherwise stated. 2,2'-Azino-*bis*(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), syringaldazine, malt extract, corn-steep liquor, avicel (microcrystalline cellulose), cellobiose, and xylitol were obtained from Sigma (St. Louis, MO); horseradish peroxidase, grade I, was from Boehringer Mannheim (Mannheim, Germany); tryptone was from Oxoid (Basingstoke, UK); soybean meal (Provasoy™) was from Vamo Mills (Izegem, Belgium); beechwood xylan was from Lenzing AG (Lenzing, Austria); casamino acids were from Marcor (Hackensack, NJ); casein, casein hydrolysate, and NH_4Cl were from Fluka (Buchs, Switzerland). Sunflower oil and skim milk were purchased at a local supermarket. Whey powder was a gift from Bundeslehranstalt für Alpenländische Milchwirtschaft (Rotholz, Austria).

Microbial Strains and Culture Conditions

All fungal organisms were from the culture collection of the Institute of Applied Microbiology, Universität für Bodenkultur Wien, where they are deposited under the indicated strain numbers. The wild-type strain of *T. multicolor* (= *T. zonata*) MB 49, which was used for most parts of this study, was isolated from hardwood in southern Germany. Stock cultures were maintained on glucose-maltose Sabouraud agar, and were transferred every 8 wk. Inoculated plates were incubated at 25°C for 4–6 d, and then stored at 4°C.

For the initial screening tests, two different culture media were used. Medium A contained (in g/L): maltose, 20; D-glucose, 10; $MgSO_4 \cdot 7H_2O$, 0.5; inositol, 0.05; peptone from casein, 0.2; yeast extract 2.0; KH_2PO_4, 0.4; $ZnSO_4 \cdot 7H_2O$, 0.001; $FeCl_3 \cdot 6H_2O$, 0.01; $MnSO_4 \cdot H_2O$, 0.005. Medium B contained (in g/L): D-glucose, 5.0; D-galactose, 5.0; D-xylose, 5.0, L-sorbose, 5.0, yeast extract, 5.0, malt extract, 10.0 *(19)*. For the initial screening performed on agar plates, medium B was supplemented with agar (20 g/L) and ABTS (0.25 g/L), before autoclaving. Peroxidase (1,000 IU/L) was added by sterile filtration, when the autoclaved medium had cooled to 50°C *(19)*. All optimization experiments were done in 300-mL baffled conical flasks containing 100 mL of medium. These were inoculated with a piece (1 cm^2) from an actively growing, 4–6-day-old colony of *T. multicolor* on Sabouraud agar. The inoculated flasks were continuously shaken on an orbital shaker at 110 rpm (stroke 25 mm) and 25°C for 12 d. Mycelia were harvested by centrifugation, washed twice using saline, and then disrupted for the determination of P2O activity (*see* Enzyme Assay).

Bioprocess Experiments

Fermentation studies were carried out in a 20-L laboratory fermenter (MBR Bio Reactor, Wetzikon, Switzerland) with a working volume of 15 L, and equipped with four disk turbine impellers, each with six flat blades. The basal culture medium for these cultivations contained whey powder, which was added so that the final lactose concentration was 25 g/L, peptone from casein (10 g/L), and KH_2PO_4 (1.0 g/L). The temperature was controlled at 25°C, and the pH was allowed to float. Aeration was automatically varied from 0.1 to 1.0 vol of air/fluid vol/min to maintain a pO_2 of 40% of air saturation. Foaming was controlled by using 10% v/v aqueous polypropylene glycol P2000 (Fluka).

Enzyme Assay

Mycelia (2 g wet wt) were resuspended in 10 mL of potassium phosphate buffer (50 m*M*, pH 6.5, containing 10 m*M* EDTA), homogenized, and then disrupted using a French press operating at 1,310 bar (19,000 lb/in^2) cell pressure. Following three passages through the French press cell (4°C), debris were removed by centrifugation (30,000*g*, 4°C, 20 min). The clear supernatant thus obtained was used for enzyme activity determination. P2O activity was spectrophotometrically determined at 30°C, using ABTS as described by Danneel et al. *(6)*. One IU of P2O activity is defined as the amount of enzyme necessary for the oxidation of 2 μmol of ABTS/min under the given conditions. Laccase activity was determined by oxidation of syringaldazine *(20)*. β-Galactosidase activity was assayed using *p*-nitrophenyl-β-D-galactopyranoside (8 m*M* final concentration) as the substrate.

Other Analyses

Protein determinations were done according to the dye-binding method of Bradford *(21)*, using bovine serum albumin (fraction V; United States Biochemical Corp., [USB] Cleveland, OH) as standard. Lactose was assayed by using a commercial kit (Boehringer Mannheim).

RESULTS

Screening

In an initial screening procedure, in which a number of fungi were tested for the ability to produce carbohydrate-oxidizing enzyme activities, agar plates containing the chromogen ABTS (2,2′ azino-*bis*[3-ethylbenzthiazoline-6-sulfonic acid]) and peroxidase were employed. By using this method, positive strains could be easily identified by a characteristic purple-blue dye, which is formed by the peroxidase-catalyzed reaction of the chromogen and H_2O_2. It has been previously shown that this screening

method is suitable for both extra- and intracellular carbohydrate oxidases *(19)*. This preliminary screening procedure included a total of 38 species of basidiomycetes from 24 genera. Approximately two-thirds of those basidiomycetous fungi tested were found to form carbohydrate oxidases under the conditions selected for the screening. However, the incubation time, which was necessary for the first color response to be detectable, varied greatly from 1 to 7 d for the different strains. Carbohydrate oxidizing enzyme activities were detected among several *Armillaria* sp, *Phanerochaete* sp, *Phellinus* sp, *Pholiota* sp, *Trametes* sp, as well as in *Asterophora lycoperdoides, Daedaleopsis tricolor, Flammulina velutipes, Fomes fomentarius, Ganoderma applanatum, Lenzites betulina, Marasmius alliaceus, Oudemansiella mucida, Phlebiopsis gigantea,* and *Schizophyllum commune.* Based on these results, selected organisms were grown in shaken-flask cultures, using different growth media, and P2O activity was assayed from the mycelial extracts. In this experiment, 19 species were investigated, 11 of which formed intracellular P2O activity (Table 1). Only organisms with unequivocally positive P2O activity are listed. Gluconic acid formed by the activity of glucose 1-oxidase, which also yields hydrogen peroxide and thus interferes with the P2O assay employed, could only be detected in *P. chrysosporium* MB 59; hence, this organism is not shown in Table 1.

Culture Medium Development

T. multicolor, which formed both the highest specific, as well as total, activity of P2O in the screening experiment, was selected for further studies. To investigate the effect of the carbon (C) source on P2O formation in this organism, various carbohydrate substrates, including mono-, oligo-, and polysaccharides, were added to a basal medium containing yeast extract (2.0 g/L), malt extract (1.0 g/L), and peptone from casein (3.0 g/L). The concentration of the latter complex nitrogen (N) source was increased, compared to the initial screening experiment, since it was found to favorably affect P2O formation. The results of these experiments are given in Table 2. Growth of *T. multicolor* was good on most of the substrates tested. Intracellular P2O activity was formed by the organism during growth on all of the various C sources employed, albeit to a greatly varying extent. Best results pertaining to the total activity of P2O were obtained for whey powder, which contains approx 80% lactose, as well as for D-xylose, lactose, and cellobiose. Since whey powder not only showed excellent results, but also is a cheap, technical substrate, it was selected for further experiments.

Since one of the objectives of this work was to identify medium components that positively influence P2O formation in *T. multicolor,* the effect of various, mainly complex N sources, which were shown to significantly affect the formation of this enzyme in another fungus *(22)*, was studied as well. The indicated N sources were added to a medium containing whey powder (30 g/L) and KH_2PO_4 (1.0 g/L). Their concentrations were based on their total N concentration, as estimated by the Kjeldahl method, thus

Table 1
Formation of Pyranose 2-Oxidase Activity in Different Fungi

Organism	Medium	Growth[a] (g/L)	Time (h)	Enzyme activity (IU/mL)[b]	(IU/mg)[c]	(IU/L)[d]
F. fomentarius MB 79	B	26.8	474	0.008	0.083	1.3
L. betulina MB 78	B	31.2	526	0.209	0.230	39.1
O. mucida MB 121	B	23.2	386	0.615	0.346	84.9
O. mucida MB 122	B	20.4	553	0.416	1.30	50.9
P. chrysosporium MB 56	A	20.8	233	0.037	0.023	4.6
P. chrysosporium MB 56	B	49.3	334	ND	ND	ND
P. gigantea MB 70	A	25.6	526	0.005	0.052	0.7
P. gigantea MB 70	B	35.2	261	0.187	0.245	39.5
P. mixta MB 116	B	10.4	549	0.006	0.006	0.4
S. commune MB 145	B	113.6	214	0.023	0.015	15.7
T. hirsuta MB 50	B	82.4	236	0.018	0.165	8.9
T. multicolor MB 49	A	104.0	286	0.267	2.67	167.0
T. multicolor MB 49	B	48.7	168	0.574	0.892	167.8
T. pubescens MB 88k	B	188.2	387	0.025	0.096	28.4
T. pubescens MB 90	B	217.6	214	0.032	0.178	40.8
Trametes versicolor MB 53	A	43.6	187	0.208	0.186	54.7
T. versicolor MB 53	B	119.2	236	0.053	0.363	37.9
T. versicolor MB 54	A	41.9	186	0.197	0.277	49.6
T. versicolor MB 54	B	249.2	261	0.010	0.048	15.0

All organisms were incubated at 25°C for the time indicated.
[a] Mycelial wet wt.
[b] Volumetric activity determined in the crude enzyme preparation (mycelial extract).
[c] Specific activity (IU/mg protein) in the crude enzyme.
[d] Total activity (IU/L fermentation medium).
ND, not detectable.

ensuring a constant concentration of 1.26 g/L total N in each medium. Results for growth and P2O formation are summarized in Table 3. All of the complex, organic nutrients tested sustained good growth of the organism. Addition of these organic N sources seems to be necessary, since they could not be substituted by NH_4Cl, which gave only poor results. Clear differences pertaining to P2O formation in *T. multicolor* exist for the different N sources examined in this experiment. Especially, various enzymatic digests of casein, e.g., peptone from casein, tryptone, or casein hydrolysate, significantly stimulate formation of total P2O activity. Furthermore, increasing the concentration of peptone from casein to 9.1 g/L, compared to the initial experiments, enhanced both growth and P2O formation considerably.

Table 2
Effect of Various Carbon Sources on Growth and Formation of Pyranose 2-Oxidase in *T. multicolor*

Growth substrate	Biomass[a] (g/L)	Enzyme activity (IU/mL)[b]	(IU/mg)[c]	(IU/L)[d]
Medium B	48.5	0.57	0.76	166
L-Arabinose	21.4	0.01	0.02	1.3
D-Xylose	294.7	0.21	0.88	362
D-Galactose	51.6	0.49	0.96	152
D-Glucose	195.9	0.04	0.22	41
L-Sorbose	189.2	0.01	0.03	11
Mannitol	112.3	0.30	0.68	199
Xylitol	29.1	1.06	1.07	184
Cellobiose	147.2	0.29	0.36	261
Lactose	234.9	0.22	0.76	315
Maltose	145.1	0.10	0.32	89
Sucrose	199.1	0.04	0.57	48
Whey powder	101.2	0.81	0.59	494
Sunflower oil	341.9	0.01	0.12	29
Xylan from beechwood	NA	0.96	2.31	158
Cellulose microcrystalline	NA	0.11	0.33	23

Substrates were added in equal concentrations (20 g/L) to a medium that further contained peptone from casein (3.0 g/L), yeast extract (2.0 g/L), malt extract (1.0 g/L), and KH_2PO_4 (1.0 g/L). Medium B contained D-glucose, D-galactose, D-xylose, L-sorbose (each at a concentration of 5.0 g/L).

[a] Mycelial wet wt.

[b] Volumetric activity determined in the crude enzyme preparation.

[c] Specific activity (IU/mg protein).

[d] Total activity (IU/L fermentation medium).

NA, not available.

Bioprocess Experiments

Production of P2O was studied in a 20-L laboratory fermenter; the working volume was 15 L of a medium based on whey powder (final lactose concentration, 25 g/L) and peptone from casein, which were both found to be optimal for enhanced P2O formation in previous experiments. The time-course of this bioprocess experiment is shown in Fig. 1. The stationary phase of growth was reached after approx 150 h. At this time, 6.7 g/L of dry wt biomass, corresponding to a mycelial wet wt of 42 g/L, was formed. It is interesting to note that lactose was not depleted at the beginning of the stationary phase, but still could be found in concentrations of 8.8 g/L. Extracellular β-galactosidase activity could not be detected at any time in this cultivation. The pH value, which was initially 5.8, and was allowed to float, continuously dropped during growth of the organism, to

Table 3
Effect of Various Nitrogen Sources on the Formation of Pyranose 2-Oxidase in *T. multicolor* When Grown in Shaken Flask Cultures

N source	Concentration (g/L)	Biomass[a] (g/L)	Enzyme activity (IU/mL)[b]	(IU/mg)[c]	(IU/L)[d]
Peptone from casein	9.1	289.4	0.89	1.24	1550
Tryptone	9.9	354.7	0.54	1.01	1150
Casein hydrolysate	10.0	370.5	0.47	0.78	1040
Casamino acid	10.9	62.0	1.15	0.73	429
Yeast extract	12.0	112.2	0.59	0.57	402
Peptone from meat	10.7	97.5	0.57	0.71	332
Soybean meal	15.7	118.7	0.37	1.07	262
Peptone from soybean	13.1	139.0	0.31	0.91	257
Cornsteep liquor	38.2	92.8	0.44	0.28	243
Casein	10.0	334.3	0.11	0.21	228
Meat extract	10.5	108.4	0.18	0.28	118
Skim milk	40% (v/v)	233.3	0.05	0.13	73.3
NH_4Cl	4.8	29.5	0.34	0.91	60.7
None	–	37.1	0.03	0.27	5.5

The N sources were added on the basis of an equivalent N concentration of 1.26 g/L total N. Whey powder (20 g/L) was used as growth substrate.

[a] Mycelial wet wt.

[b] Volumetric activity determined in the crude enzyme preparation.

[c] Specific activity (IU/mg protein).

[d] Total activity (IU/L fermentation medium).

reach a minimum of 4.1. At the transition of the exponential to the stationary phase of growth, it started to increase slightly. As can be clearly seen in Fig. 1, formation of P2O is growth-associated in *T. multicolor*, but laccase

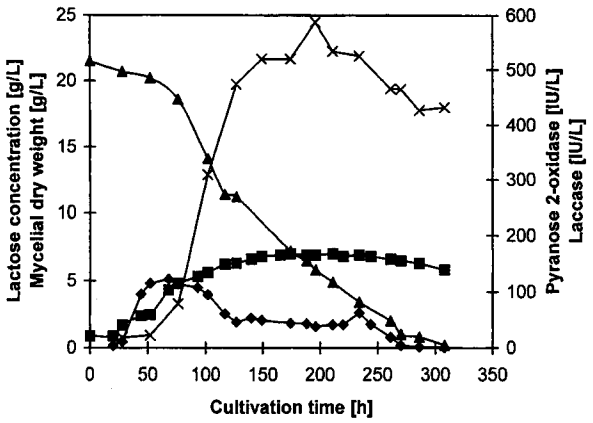

Fig. 1. Time-course of a batch fermentation of *T. multicolor* on a medium containing whey powder as the substrate. Symbols: (■), mycelial dry wt; (▲), lactose; (X), total pyranose 2-oxidase activity (IU/L medium); (♦), laccase activity.

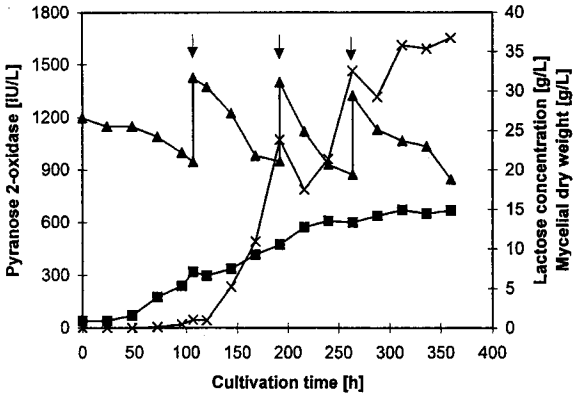

Fig. 2. Time-course of a fed-batch fermentation of *T. multicolor* on a whey-based medium. The arrows mark the addition of a 1 *M* solution of lactose, so that the lactose concentration in the medium was increased by approx 10 g/L. Symbols: (■), mycelial dry wt; (▲), lactose; (X), total pyranose 2-oxidase activity (IU/L medium).

activity peaked at the early growth phase and thereafter quickly decreased. The maximum of total P2O activity (588 IU/L of culture medium, corresponding to 87.8 IU/g dry biomass) was found after 200 h of growth; thereafter, P2O activity slightly decreased.

Since both growth of *T. multicolor* and formation of total P2O activity were only observed at elevated lactose concentrations in the batch cultivation, a fed-batch fermentation was performed. The initial growth medium was identical to the batch cultivation. At 107, 192, and 264 h cultivation time, a 1 *M* lactose solution was added by sterile filtration to increase the lactose concentration in the medium by approx 10 g/L, thus ensuring an elevated concentration of this carbohydrate during the entire course of the cultivation. Results are shown in Fig. 2. Contrary to the batch cultivation, an increase in biomass was observed for more than 300 h, reaching a maximum of 14.9 g/L dry wt (corresponding to 85.4 g/L wet wt). Simultaneously, total P2O activity increased to a maximum value of 1,650 IU/L, which corresponds to 111 IU/g dry biomass. Again, the initial culture pH of 5.8 slowly dropped to approx 4.2, and then stayed constant for the entire cultivation.

DISCUSSION

In an initial screening procedure, which was done on agar plates containing the chromophore ABTS and peroxidase, it was found that carbohydrate-oxidizing enzyme activities are widespread among basidiomycetes. This confirms the results of previous screenings for these enzyme activities *(10,19,23,24)*. Out of 38 different species belonging to 24 genera, 25 organisms that formed a carbohydrate oxidase under the growth condi-

tions selected for this experiment were detected by a characteristic dye formation. The employed screening method proved to be very efficient and convenient. However, since the agar medium used in this screening contained the carbohydrates D-galactose, D-glucose, L-sorbose and D-xylose, several other sugar oxidases would be detected by this method, in addition to P2O (EC 1.1.3.10). These could include glucose oxidase (EC 1.1.3.4), hexose oxidase (EC 1.1.3.5), galactose oxidase (EC 1.1.3.9), or L-sorbose oxidase (EC 1.1.3.11).

To unequivocally identify suitable P2O-producing strains, several of the carbohydrate–oxidase-positive organisms were cultivated in shaken flasks, and P2O activity was then assayed in the mycelial extracts. Formation of P2O activity was commonly found in these selected basidiomycetes. Eleven out of 19 organisms formed P2O activity under the chosen growth conditions. Because of this frequent occurrence, it seems likely that this enzyme activity has a more general importance in the metabolism of basidiomycetous fungi than has been suggested before *(24)*. The effects of the two different media used for the cultivation of the basidiomycetes on the formation of P2O activity are not unambiguous. Especially with *P. chrysosporium* MB 56, this enzyme activity could not be detected when the organism was cultivated on medium B, but growth on medium A clearly resulted in the formation of P2O. It can be concluded that the growth conditions used for the screening were certainly not optimal for P2O synthesis, and that, because of this fact, detection of several P2O-positive strains could have been missed. As a result of the screening, *T. multicolor* MB 49 was identified as a promising, not-yet-described producing strain of intracellular P2O activity, and was selected for further investigations.

The most suitable substrate for the efficient production of P2O by *T. multicolor* was found to be whey powder, which contains approx 80% lactose. Although several more readily metabolized carbohydrates, when employed as C and energy source, resulted in higher biomass formation, levels of P2O activities were found to be significantly lower with these substrates than those obtained with whey powder or lactose. It seems likely that the relatively slow utilization of lactose by this basidiomycetous fungus, which does not secrete β-galactosidase activity into the extracellular environment under these growth conditions, is favorable for P2O synthesis, since higher concentrations of monosaccharides, i.e., glucose or galactose, which typically exert catabolite repression in microbial cells, are avoided. This is further corroborated by the fact that an appropriate feeding, which ensured a certain lactose concentration during the entire cultivation, greatly enhanced not only growth of the organism, but also the formation of total P2O activity, as well as of the units of P2O formed per g biomass.

Synthesis of P2O under apparently derepressed conditions is in agreement with several other reports on different fungal organisms. Its synthesis is growth-associated in an unidentified basidiomycete (no. 52), when

lactose is used as a substrate that was also poorly utilized *(22)*. P2O is only formed in the idiophase after the depletion of the C source, when using glucose- or polyol-based media by several organisms, including *Coriolopsis occidentalis, P. chrysosporium,* or *P. gigantea (6,24,25)*. Contrarily, its formation by *Polyporus obtusus* and *Oudemansiella mucida* was reported to be associated with growth, even when glucose was employed as the C source. With these two organisms, the maximum P2O activity coincided with the depletion of the carbohydrate substrate *(2,26)*. However, these physiological aspects of P2O synthesis in fungi have not so far been studied in detail.

Both growth of *T. multicolor* and P2O formation considerably decreased when the organism was grown in a stirred-tank laboratory fermenter, compared to the cultivations in shaken flasks. A possible explanation for this could be damage to the mycelium caused by high shear stress, which probably will occur near the tips of the impellers. These negative effects of shear stress and mechanical forces on filamentous fungi, which can cause breakage of the hyphae and leakage of intracellular material, have recently been reviewed *(27)*. Moreover, a characteristic change in the morphology was observed when comparing growth in shaken flasks and stirred-tank reactors. *T. multicolor* formed small pellets (2–3 mm diameter) in shaken flask cultures, but it grew in filamentous form in the fermenter cultivations. Presumably, conditions of low shear, e.g., as characteristic for air lift fermenters, will be favorable for the enhanced production of P2O in *T. multicolor*. Further investigations in this respect will be carried out in the authors' laboratory.

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