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Use of cheese whey as a substrate to produce manganese peroxidase by *Bjerkandera* sp BOS55

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Manganese peroxidase, MnP, is one of the major ligninolytic enzymes produced by a number of white-rot fungi. The ability of this enzyme to degrade lignin by the fungus *Bjerkandera* sp BOS55 has opened its application to related bioprocesses such as recalcitrant-compound degradation and effluent decolorization. The medium reported to induce MnP production is composed of chemical grade reagents, all with relatively high costs for application to detoxification purposes. The use of inexpensive sources for MnP production can bring its implementation closer. For this purpose, dairy residues from cheese processing were considered. MnP production obtained using crude whey as the sole substrate reached appreciable levels, around 190 U L^{-1}, values comparable to those found with synthetic media (between 175–250 U L^{-1}). Thus, this cheese-processing byproduct can be used as an inexpensive alternative for the large-scale production of MnP.

Keywords: cheese whey; manganese peroxidase; *Bjerkandera* sp; white-rot fungi

Introduction

Whey, a liquid residue from cheese and casein manufacturing, is a by-product rich in nutrients: lactose, proteins, lipids and mineral salts, amongst others [11,12]. Its limited exploitation has led the industry to discard an important fraction of the total production. Efforts from an environmental point of view must focus on the utilization of cheese whey for new purposes in the biotechnological industry [3,6], rather than discarding its overproduction.

The ability of ligninolytic enzymes produced by white-rot fungi to decompose lignin have made them attractive for the degradation of highly toxic complex pollutants in wastewaters [1,15]. In order to make these processes feasible, large-scale production of these enzymes has to be developed. This can be achieved by maximizing the fermentation productivity and reducing operational investments, among which chemicals required for fungal growth are the most relevant. An integrated treatment system of effluent detoxification would comprise fungal enzyme-aided degradation of recalcitrant structures of pollutants and secondly, conventional biological treatment (aerobic-anaerobic) for the degradation of intermediates and the reduction of organic matter.

Manganese peroxidase (MnP) is a ligninolytic enzyme synthesized during secondary metabolism in response to nutrient limitation [5,10]. It was observed recently that some white-rot fungi produce high titers of MnP in stationary cultures in rich media [2,13,14]. Taking into account the nutritional value of cheese whey, these microorganisms can be proposed for developing a large-scale production system based on the use of this substrate. In this study, the production of MnP by *Bjerkandera* sp strain BOS55 using three types of cheese whey was evaluated and compared with the result obtained using synthetic media.

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Materials and methods

Microorganism and culture conditions

Bjerkandera sp BOS55 was maintained at 4°C on peptone yeast extract slants from which it was transferred to glucose malt extract plates [13]. The plates were incubated at 25°C for 5–6 days. Eight agar plugs (5-mm diameter) were punched from the leading edge and cultured in an Erlenmeyer flask (5-L) with 300 ml of medium containing 10 g glucose L^{-1} and 2 g peptone L^{-1} for 7 days at 30°C. The biofilm of growing mycelium was mixed in a Waring blender for 1 min and 10% (v/v) homogenized mycelium was used as inoculum in the experiments [7,17]. Erlenmeyer flasks (250-ml) containing 90 ml of culture medium, 10% homogenized mycelium and 0.5% Tween 80 were incubated in an orbital shaker (New Brunswick Scientific, NJ, USA) at 30°C and 150 rpm for 12 days.

Culture media

Whey medium composition: Three different types of cheese whey were selected: crude whey, skimmed whey and concentrated whey, whose characteristics are given in Table 1. Crude and skimmed whey are the liquid fractions after precipitation and removal of milk casein during cheese making from whole or skimmed milk. Concentrated whey is produced from crude whey at a later stage by a three-stage evaporation system at a maximum temperature of 60°C. Prior to autoclaving them, all media were diluted to a concentration of 10 g L^{-1} of reducing sugars and 22 mM total Kjeldahl nitrogen (TKN) and adjusted to pH 4.5 with dilute HCl. In parallel experiments, a filter-sterilized min-

Table 1 Composition of different cheese wheys used as substrate

Component	Crude	Skimmed	Concentrated
Soluble Kjeldahl nitrogen (g L^{-1})	1.4 ± 0.04	0.5 ± 0.03	3.6 ± 0.03
Reducing sugars (g L^{-1})	45.7 ± 0.4	25.3 ± 0.4	153.0 ± 1.1
Total organic carbon (g L^{-1})	25.8 ± 1.3	15.2 ± 1.1	87.5 ± 1.9
Mn content (mg L^{-1})	0.15 ± 0.05	0.08 ± 0.04	0.32 ± 0.15
pH	6.6	6.7	6.3

eral BIII solution (100 ml L^{-1} [17] was added to study the effect of some essential mineral salts.

Synthetic medium composition: Four synthetic media were prepared containing combinations of different carbon and nitrogen sources: glucose or lactose (10 g L^{-1}) and diammonium tartrate or peptone (22 mM NH_4^+). A solution containing BIII mineral supplement (100 ml L^{-1}) and 20 mM acetate buffer (pH 4.5) was supplied in all cases.

Enzymatic and analytical assays

Manganese peroxidase activity was determined spectrophotometrically at 30°C. The reaction mixture contained 50 mM sodium malonate (pH 4.5), 1 mM 2,6-dimethoxyphenol (DMP), 1 mM $MnSO_4$, and up to 600 μl of supernatant in a total volume of 1 ml. The reaction was initiated by adding 0.4 mM H_2O_2 and corrected for laccase (activity prior to adding H_2O_2) and manganese-independent peroxidase (MIP) activity. MIP was measured in a reaction mixture which contained 50 mM sodium tartrate (pH 4.0), 1 mM DMP, 1 mM EDTA and up to 550 μl of supernatant in a total volume of 1 ml. The reaction was initiated by adding 0.4 mM H_2O_2 and corrected for background laccase activity. The formation of coerulignone was monitored at 469 nm ($\varepsilon = 49\,600\ M^{-1}\ cm^{-1}$). A unit of activity is defined as 1 μmol of coerulignone formed per min [18].

Reducing sugars (RS) were determined by the dinitrosalicylic acid method, using d-glucose as a standard [4]. Total organic carbon (TOC) was measured by a total organic carbon analyser (TOC-5000) (Shimadzu, Kyoto, Japan) and soluble total Kjeldahl nitrogen (TKN) was analysed in an organic nitrogen analyser DN-1900 (Rosemount, CA, USA). Mn was determined by atomic absorption spectrophotometry (Varian SpectrAA 300–400); specifications given in 3111 Method [16]. Enzymatic and analytical measurements were carried out in triplicate.

Results and discussion

MnP production with cheese whey

Glucose, ammonium tartrate, 2,2-dimethylsuccinate, mineral salts and vitamins were included in the formulation of a standard medium enabling the production of MnP by white-rot fungi [17]. One of the major drawbacks in the application of this enzyme to large-scale is the production cost. The use of other more inexpensive nutrient sources needs to be evaluated to find more favorable economic conditions.

The possibility of producing MnP from cheese whey was evaluated for three different cheese whey products (Figure 1). Due to the low levels of Mn originally present in cheese whey (only 0.15 mg L^{-1} in comparison with 1.82 mg L^{-1} in the standard medium), a mineral salts solution was supplemented in parallel assays. Results showed that the best medium included crude whey, with levels of MnP activity around 175 U L^{-1}. The supplementation of mineral salts to the cheese whey products did not improve MnP production with this medium. The lowest levels of MnP production (70 U L^{-1}) included the skimmed whey; the removal of fats and lipids is likely to negatively affect fungal growth, as was visually noted, and consequently MnP production. In the case of concentrated whey, the partial denaturation of proteins occurring during the concentration of whey could also explain the lower activity obtained with this medium (90 U L^{-1}).

Physiological events leading to MnP production were investigated in combination with the analysis of substrate consumption, referred to as reducing sugars (RS) and total Kjeldahl nitrogen (TKN). MnP production generally began when TKN and RS reached levels ranging from 75 to 150 mg L^{-1} and 3–6 g L^{-1}, respectively (Figure 2). The initial peroxidase expression may be related to limitation of nitrogen, once the MnP began to decline quickly, a rapid consumption of reducing sugars was observed with levels diminishing to zero, with a subsequent limitation in carbon. This carbon limitation may be responsible for the later increase in MnP activity, as was found for the skimmed and condensed whey (Figures 2b and c). At the end of the experiment and in conditions corresponding to nitrogen and

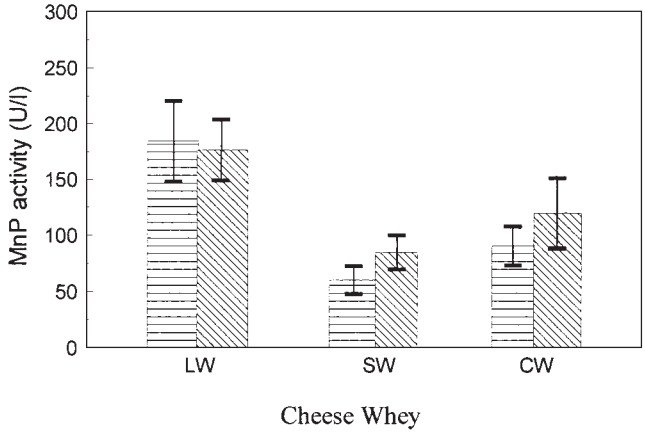

Figure 1 MnP titers achieved in cultures with different wheys as the only nitrogen and carbon source. Symbols: crude whey (LW), skimmed whey (SW) and concentrated whey (CW), without (horizontal bars) and with (slanted bars) BIII-mineral medium. Error bars represent standard deviation of the average value.

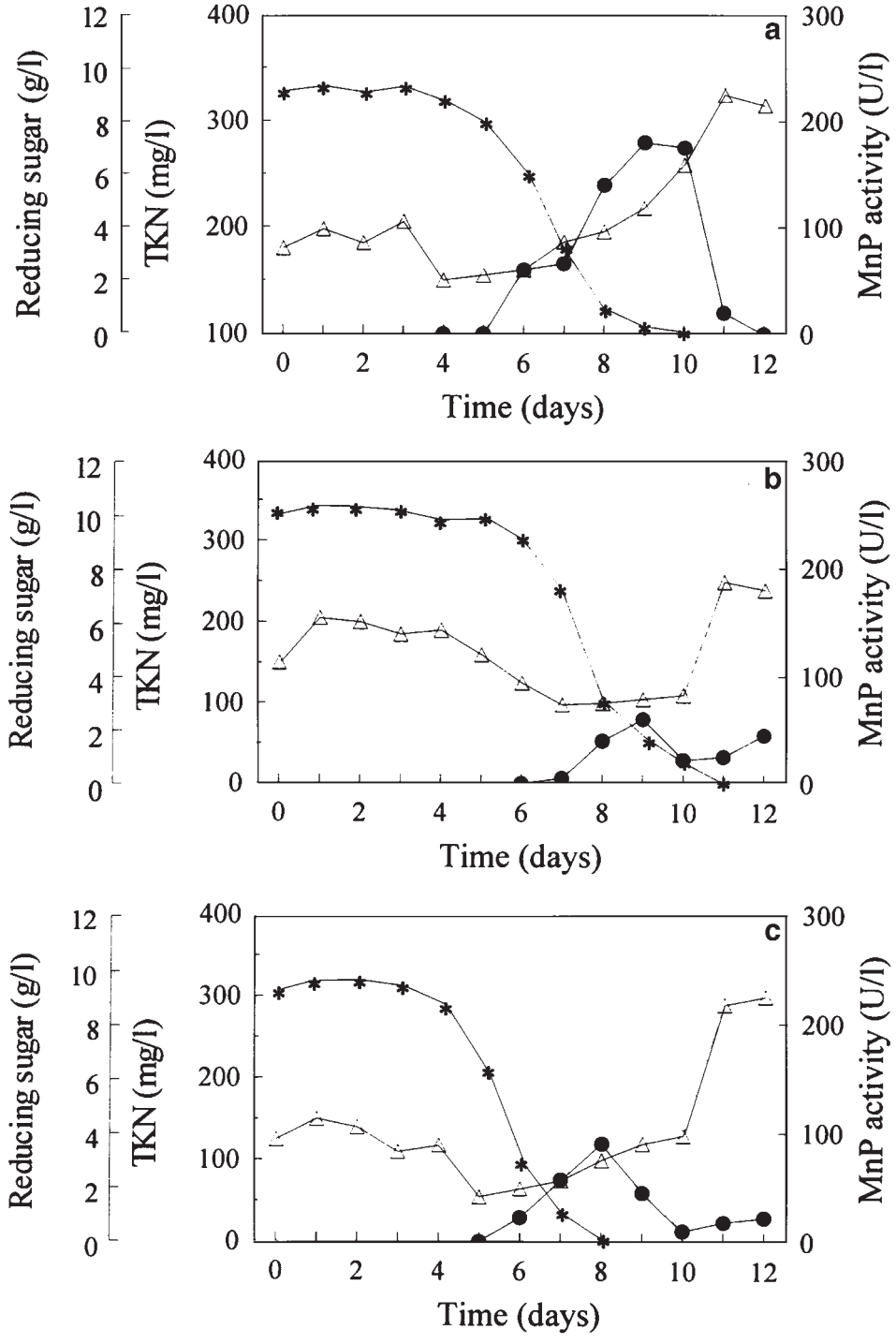

Figure 2 Trends of MnP activity and depletion of nutrients in cultures supplemented with crude whey (a), skimmed whey (b) and condensed whey (c). Reducing sugars (RS) (*); Kjeldahl nitrogen (TKN) (Δ); MnP activity (●).

carbon limitation, the concentration of TKN increased in the culture medium, indicating cell lysis involving proteases.

Comparison of MnP production: cheese whey vs synthetic media

The utilization of cheese whey as a substrate to produce MnP can constitute an alternative only if MnP productivity is comparable to that found with the standard medium. The uptake of glucose and ammonium from the standard medium may be easier than from cheese whey, and peroxidase productivity may differ. For comparison purposes, four different synthetic media were assayed at the same C/N ratio as that found in cheese whey. Carbon was added as glucose or lactose and nitrogen as diammonium tartrate or peptone. These results will also provide information

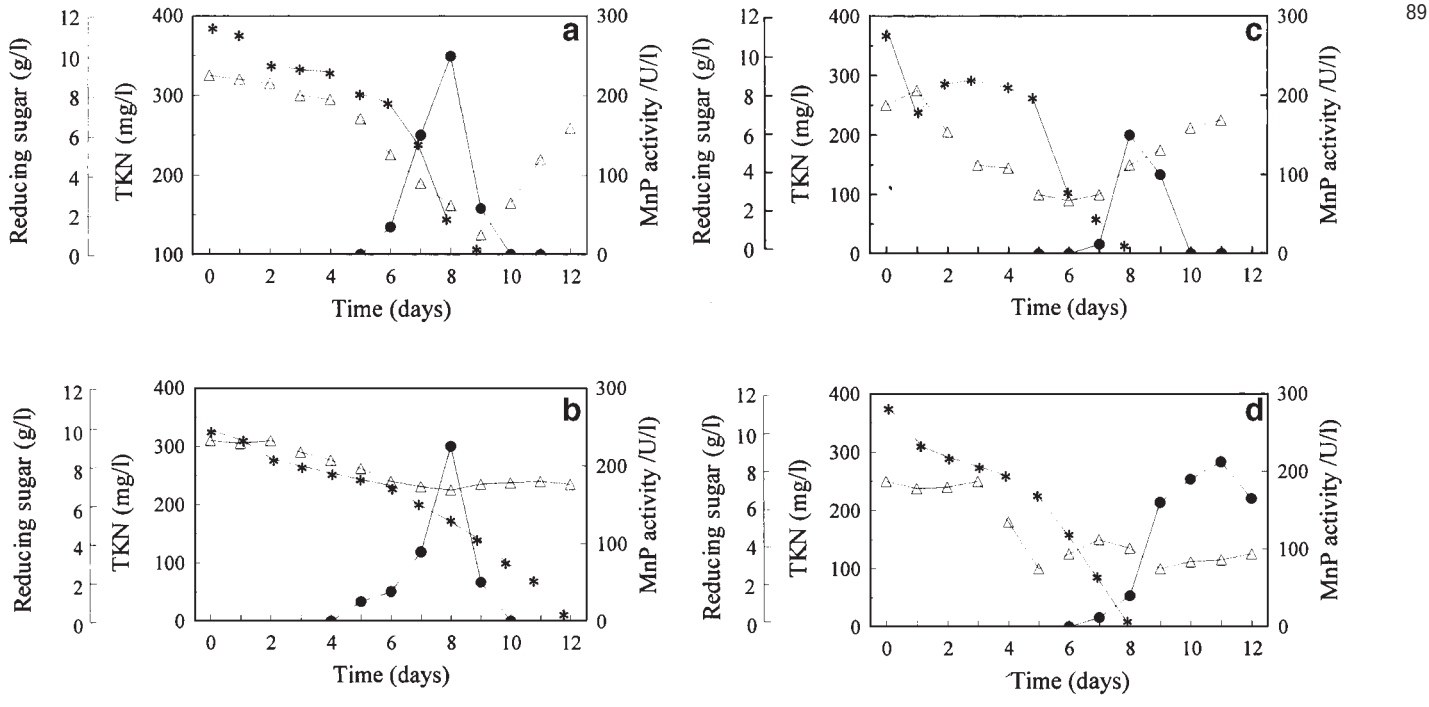

Figure 3 The time course of MnP production in nitrogen-sufficient conditions. (a) Glucose and 22 mM N-NH_4^+; (b) lactose and 22 mM N-NH_4^+; (c) glucose and 22 mM nitrogen as peptone; (d) lactose and 22 mM nitrogen as peptone. Symbols: reducing sugars (RS) (*); total Kjeldahl nitrogen (TKN) (Δ); MnP activity (●).

about the assimilation of lactose, a major component of cheese whey, by *Bjerkandera* sp BOS55.

The time course of MnP production was monitored in parallel with the depletion of nutrients, RS and TKN. All media provided detectable levels of MnP activity with values ranging between 175–275 U L^{-1}. These values are comparable to those found for other wild strains such as the model fungus *Phanerochaete chrysosporium* [8,9]. Under these conditions, the appearance of peroxidase activity appears to coincide with a minimum concentration of nitrogen: approximately 225 mg TKN L^{-1} for ammonium tartrate (Figure 3a, b) and 100 mg TKN L^{-1} for peptone (Figure 3c, d). The first appearance of MnP did not involve a carbon-limited medium according to levels in the medium 3–6 g L^{-1} at the onset of ligninolytic activity. These results are in agreement with those previously found for cheese whey. The use of other carbon and nitrogen sources (lactose and peptone instead of glucose and ammonium) had no significant effect on MnP production; however, the nutrient consumption rates were slightly different. Lactose was assimilated more slowly than glucose and peptone faster than ammonium.

Glucose and diammonium tartate have been used extensively as growth substrates for white-rot fungi to induce ligninolytic activity. The potential application of these enzymes relies on an effective and inexpensive production system, in which these chemicals can be substituted with industrial residues. Cheese whey products were as good carbon and nitrogen sources as was obtained with synthetic media. The fact that the best results were with crude whey with no addition of nutrients or mineral salts greatly simplifies the process, diminishing considerably the operational costs and encouraging its use in large-scale production.

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