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Full Length Research Paper

Laboratory scale production of the human recombinant iduronate 2-sulfate sulfatase-*Like* from *Pichia pastoris*

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Clone IDS28 of the yeast *Pichia pastoris* expressing the human iduronate 2-sulfate sulfatase-*Like* (hIDS-*Like*) was employed for low-scale production of the recombinant enzyme in a saline culture media without phosphate. The biological activity found was between 7.3 and 29.5 nmol h^{-1} mg^{-1} of total protein. It is about 1.73 to 7 times higher than the result obtained with the same clone in shake flask culture.

Key words: *Pichia pastoris*, iduronate 2-sulfate sulfatase, monod model, Hunter syndrome, methanol induction.

INTRODUCTION

The Mucopolysacaridosis II or Hunter Syndrome (MPSII) (MIN 309900) belongs to a group of lysosomal storage disorders caused by the deficiency of the enzyme that catalyzes the degradation of some glycosaminoglicans (GAG). Hunter Syndrome is caused by the deficiency of the iduronate 2-sulphate-sulphatase enzyme (E.C. 3.1.6.13) (Parkinson et al., 2004).

Pichia pastoris is yeast with the ability to use methanol as a sole source of carbon and energy; an adaptation that is related to the induction of the alcohol oxidase gene (*AOX*), along with dihydroxy acetone synthase (DAS) and other enzymes involved in methanol metabolism. Alcohol oxidase is absent during growth in dextrose, but can increase to up to 30% of the total protein of the cell when it is grown in methanol (Wolf, 1996). *P. pastoris* has been used since 1984 to produce around 500 heterologous proteins (Córdoba et al., 2003; Cos et al., 2006).

Different methanol feeding strategies have been used to express proteins in *P. pastoris.* In many cases, a strategy was used in the culture processes to maintain the cell under methanol-limiting conditions, thus avoiding methanol accumulation in the culture media (Cregg, 1993). Since methanol is highly toxic to *P. pastoris*, it is necessary to maintain its concentration as low as possible (~0.5 %v/v), (Invitrogen 1996). The capacity of methanol degradation of a recombinant cell can be affected by the phenotype obtained after integrating the expression cassette into the microorganism genome. Thus, Mut^+ clones are able to use methanol at higher concentrations than other clones (Mut^S or Mut^-) (Jungo et al., 2006). This shows the need for independent studies on methanol supply (Rodríguez et al., 1997; Poutou et al., 2005b). The three main purposes of this paper were to improve the total activity of hrIDS-*Like* at 3L bioreactor, to study the growth behaviour in glycerol as carbon source, and to start the chromatographic purification.

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MATERIALS AND METHODS

Strains

One strain of *P. pastoris,* GS115: His$^-$, transformed with the integrative plasmid pPIC9-hIDS-*Like* (GS115/pPIC9-hIDS-*Like*, His$^+$, Mut$^+$), identified as IDS28, was used to produce human recombinant hrIDS-*Like*. The hIDS-*Like* expression is induced by methanol addition because of the *AOX*1 promoter present in the genetic construction (Invitrogen, 1996; Landázuri, 2002; Córdoba, 2004).

Culture procedures

All pre-inoculums were obtained in a 15 ml tube with 3 ml (air relation 1/5) of YPD [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose] inoculated with 300 µl from the Master Cell Bank stock (MCB) at 30ºC, 250 rpm, for 42 h. Inoculums of the batch culture were developed in a 50 ml shake flask with 100 ml of MG (1.34 % (w/v) YNB, 1% (v/v) glycerol, 4x10^{-5}% (w/v) biotin), for 24 h at 30ºC and 250 rpm (Amador et al., 1994; Poutou et al., 1994; Invitrogen, 1996).

A Cole-Palmer® 3L bioreactor was used to develop the batch culture; the working volume of basic saline media was 1 L without phosphate (FM21-wP) [0.15 g L^{-1} $CaSO_4 \cdot 2H_2O$, 2.40 g L^{-1} K_2SO_4, 1.95 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.65 g L^{-1} KOH, supplemented with 4 ml of a mineral stock solution (PTM1) composed of (0.5 %v/v H_2SO_4, 6 g L^{-1} $CuSO_4 \cdot 5H_2O$, 3 g L^{-1} $MnSO_4 \cdot H_2O$, 65 g L^{-1} $FeSO_4 \cdot 7H_2O$, 20 g L^{-1} $ZnSO_4 \cdot 7H_2O$, 0.1 g L^{-1} KI, 0.2 g L^{-1} HBO_3, 0.5 g L^{-1} $CaSO_4 \cdot 2H_2O$, 0.5 g L^{-1} $CoCl_2 \cdot 6H_2O$, 0.2 g L^{-1} $Na_2MoO_4 \cdot 2H_2O$)]; a glycerol concentration between 1 and 8% (w/v) was studied at 30ºC, pH 6.4 ± 0.2 (controlled by NH_4OH addition), between 250 and 1100 rpm, 0.5-10 vvm., for 84 to 170 h; foam was controlled by adding oleic acid. Between 4.5 and 28 ml of methanol was added every 12 or 24 h to induce the *AOX* 1 promoter (Invitrogen, 1996; Córdoba et al., 2003; Córdoba 2004).

The batch cultures were followed by optical density (OD_{620nm}), dissolved oxygen (DO), pH, glycerol concentration (g L^{-1}), methanol concentration (%v/v), total extra-cellular proteins (g L^{-1}), proteolytic activity (PU ml^{-1} min^{-1}), and hrIDS-*Like* activity (nmol h^{-1} mg^{-1} of total protein). Samples were taken every 2 h during the growth phase (when glycerol was the carbon source, during the first 24 h), and every 12 h during the induction phase (when methanol was the carbon source). The cell density, X (g DCW L^{-1}) (DCW, Dry cell weight), was determined by a calibration curve (X vs. OD_{620nm}) represented by X = 1.1726 OD_{620nm} (R^2=0.987).

Glycerol quantification

The glycerol concentration (g L^{-1}) was determined using a chromatography method (HPLC) Waters®. To eliminate proteins, a volume of 300 µl of culture supernatant was purified through an ionic exchange column; the samples were then filtered through a 0.2 µm filter. 20 µl of this final filtrate was injected into the HPLC system by using deionised water with a flow of 1.2 ml min^{-1} in a pre-column KC-G as a running phase, followed by a column KC-811 Shodex® at 60ºC; a refraction detector at 40ºC was used to estimate the glycerol concentration in the culture supernatant through a calibration curve of glycerol vs. the curve area (A) below represented by the equation: Glycerol (g L^{-1}) = 1.31x10^{-4} A, R^2 = 0.9973 (Córdoba, 2004).

Methanol quantification

The methanol concentration was determined by using the gas chromatography method (GC) Varian®. An internal standard pattern of 2-propanol was used in a Nukol column from Supelco® at 40ºC coupled with a FID detector at 240ºC; He AGA® with a pressure of 15 psi at the injector's inlet at 220ºC (Córdoba, 2004) in order to estimate the methanol concentration in the culture supernatant through a calibration curve of methanol vs. the quotient under the curve of methanol and 2-propanol areas (A), represented by the equation: methanol (%v/v) = 1.8951 A, R^2 = 0.9994.

Proteolytic activity quantification

The proteolytic activity in the culture supernatant was measured as described by Hubner (1993); the incubation temperature of the reaction was 30^{o}C. The activity (PU ml^{-1} min^{-1}), calculated as 10 PU, was represented by 0.5 units $OD_{260\ nm}$.

Extra-cellular proteins quantification

To estimate the concentration of extra-cellular proteins, the Lowry method was employed. The calibration curve was defined by the equation µg ml^{-1} of extra-cellular proteins = [(0.0003 x A_{620nm}) + 0.0351] x Df, R^2 = 1. A was measured with a Spectronic® Model 21D (Lowry et al., 1951).

Enzyme activity (fluorometric assay)

The supernatant (10 µl) of fermentation was mixed every 24 h with 20 µl of substrate solution containing 1.25 nM of 4-methylumbelliferyl-α-iduronate 2-sulphate (MU-αIdoA-2S) dissolved in 0.1 M CH_3COONa/CH_3COO pH 5.0 ± 0.2 and 10 mM $Pb(CH_3COO)_2.3H_2O$. It incubated to 37ºC for 4 h, after which were added 40 µl of Pi/Ci buffer [NaH_2PO_4 0.4 M, $C_6H_5Na_3O_7.2H_2O$ 0.2 M pH 4.5 ± 0.2 and NaN_3 0.02% (w/v)] and 10 µl of LEBT. The solution was incubated to 37ºC for 37 h. The reaction was stopped with 650 µl of buffer of stop ($NaHCO_3/NaCO_3$ 0.5 M, pH 10.7 ± 0.2, with glycine 1.7 mM). The fluorescence was determined in a fluorometer Turner 450, with wave lengths of excitation and emission of 360 and 415 nm, respectively. The control was leukocytes or human plasma (Voznyi et al., 2001). Enzyme activity was expressed as nmol of substrate converted h^{-1} mg^{-1} of total protein.

RESULTS AND DISCUSSION

Several batch cultures were developed, with varying initial substrate concentrations. The processes were randomly labeled as: M6 (11.34 g L^{-1} of glycerol), M13 (17.27 g L^{-1} of glycerol), M7 (25.53 g L^{-1} of glycerol), M8 (40.13 g L^{-1} of glycerol) and M9 (83.32 g L^{-1} of glycerol).

Monod in 1942 studied the influence of the essential compound concentration, as a component of a culture media free of inhibitor substances, on a particular growth rate. Similarly, Langmuir in 1918 used the isotherms of the enzymatic catalyzed reactions, and a unique substrate suggested by Henry in 1902 and Michelis-Menten in 1913. This model suggests the existence of a limiting substrate that affects the growth rate, in such way that a fixed quantity of this substance yields a fixed quantity of biomass; $Y_{x/s}$: yielding factor biomass-substrate; whereby:

$$\frac{1}{x}\frac{dx}{dt} = \mu_x = \mu_M \frac{s}{s + K_s} \quad (1)$$

Where K_s and μ_M are kinetic parameters that represent the saturation constant and the maximum specific growth rate, respectively, and "s" is the limiting substrate concentration in the exponential growth phase. Even if this equation revealed cause-effect relations, the physical meaning of the parameters is unknown or may not exist. In some cases, a meaning is assigned; one of them is the limitation of the growth rate because of the mediate permease transport trough membrane (Doran, 1998).

Jahic et al. (2002) reported $\mu_M = 0.26h^{-1}$ and $K_s = 0.1$ g l^{-1} for the production of a cellulose-lipase recombinant fusion protein in *P. pastoris* strain SMD1168. d'Anjou and Daugulis (1997) reported $\mu_M = 0.25$ h^{-1}, $K_S = 0.005$ g l^{-1} and $Y_{x/s} = 0.42$ g of dry biomass g^{-1} of glycerol for the production of antifreeze protein from cormorant in *P. pastoris* GS115. The mineral salt concentration in both reports was approximately 7 times higher than the concentration used in this project. The highest K_s value we obtained suggests a substrate inhibition effect.

Figure 1 shows a change in the glycerol consumption slope after nearly 35 h in the culture; a marked change is observed in the M9 culture, which could be due to the growth limitation caused by other nutrients, such as O_2, N_2, bivalent mineral salts, or the combination of all of them. In M8 there was no limitation of O_2 at any time. In M9, after nearly 35 h, O_2 levels were below 20% (Figure 1). In all the other cultures, the concentration levels of dissolved oxygen always remained above 20%. There was no evidence of exhaustion of the NH_4^+ used for pH control; the culture was permanently fed with NH_4OH, particularly when it grows with glycerol. When nitrogen is limited, yeasts tend to sporulate (Rose and Harrison, 1989); an effect that was not observed. Therefore, this seems to suggest that NH_4^+ is not the limiting nutrient.

Culture M6 showed an activity of IDS-*Like* 15 nmol h^{-1} mg^{-1} of total protein after 33h, while that of culture M4 was of 2.6 nmol h^{-1} mg^{-1} of total protein after 35 h (Córdoba, 2004). It is important to note that the IDS are rendered inactive in presence of PO_4^{3-}; therefore, cultures M6 to M13 were carried out in a saline medium without phosphates (FM21-sF).

With FM21-sF medium, the maximum cell density obtained expressed as LnX/Xo was around 4, with very little growth during the methanol addition stage. In the FM21 medium, the Ca^{2+}, Mg^{2+}, Mn^{2+} concentrations were measured (data not shown) after approximately 35 h, and the values found were: Ca^{2+}, 77% of the initial value; Mg^{2+}, 30% of the initial value, and Mn^{2+}, 36% of the initial value. Figure 1 shows a drastic change in the cell density of all five cultures, a fact which can be explained by the limitation of Mg^{2+} and Mn^{2+}, since these ions are co-factors in the synthesis of vitamins (growth factors). The growth speed in M7 was 54% slower than that found in the replica culture with phosphates (data not shown). All this seems to suggest that phosphate is crucial for growth since it participates in the metabolism of both glycerol and methanol, and in the general regulation of energy, among other things.

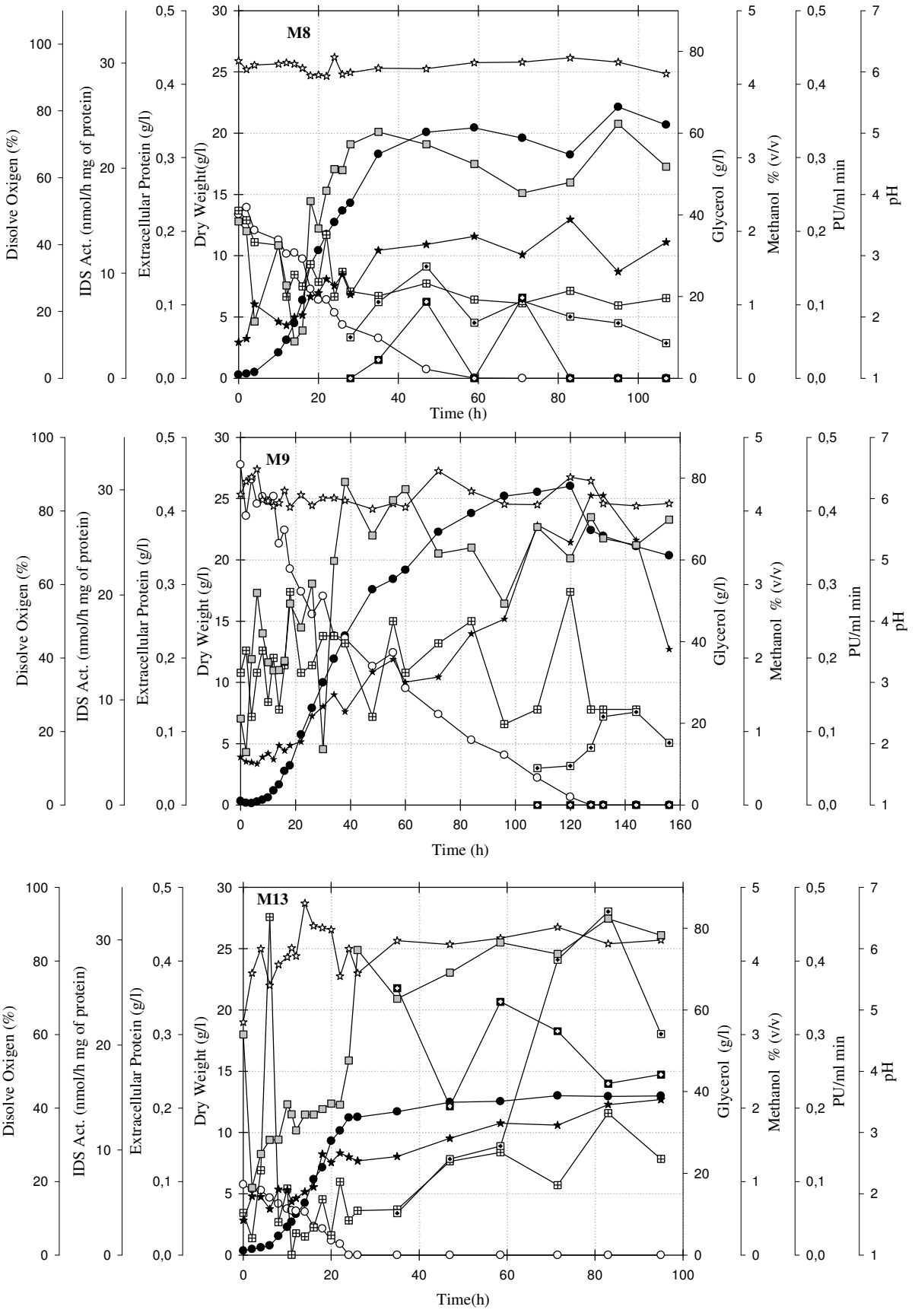
Without phosphoric acid in the medium, the initial concentration of salts and traces could not be increased due to their low solubility, particularly of Ca^{2+}. Besides, in the basic medium, Mg^{2+} becomes a non-soluble form and this effect reduces the availability of the element.

The pH in M6 was below 5.0 ± 0.2 after 70 h (Figure 1). At exactly the same time, as we can see in Figure 1, there is a decrease in the proteolytic activity, as opposed to what happened after 58 h when the pH was 6 ± 0.2. In all the other cultures, the pH remained at more or less 6 ± 0.2. In some experiments, pH values of 5.0 ± 0.2 have been appropriate for the expression of several recombinant proteins (Inan et al., 2005). Nevertheless, it is important to examine the influence of this parameter on the expression and the proteolysis and biological activities of the hrIDS-*Like*.

Methanol concentrations in cultures M6 and M7 at the time of maximum hrIDS-*Like* activity were below 1% (v/v). For cultures M8 and M9, this concentration reached values above 1% (v/v) (Figure 1). Moreover, the scant growth in methanol seen in cultures M6 to M9 does suggest a limitation in energy. With this in mind, different methanol feeding strategies in the production of recombinant proteins have been examined (Zhang et al., 2000; Trinh et al., 2003). Growth in methanol was scarce in M8 (Figure 1) after 36 h, and in M9 after 130 h. Waldron and Lacroute in 1975 demonstrated that the speed of proteins synthesis is directly proportional to the speed of growth (cited in Rose and Harrisonm, 1989).

Methylotrophic yeasts oxidize short-chain primary alcohols (C1- C5), including unsaturated and substituted alcohols. Maximum speed and Ks increase as the length of the chain increases. Some studies have been done regarding O_2 being a second limiting substrate in the kinetics of alcohol oxidase. It has been proved that AOX has little affinity for O_2, that the Km value lies between 0.24 and 0.4 mM, and that it depends on methanol concentration. Since O_2 concentration in humid air is ~0.2 mM, the AOX works outside of the concentration range making it a poor catalyst, a fact which is in turn compensated by an "enormous" production of AOX (Poutou et al., 2005b). The key factors behind the control of carbon flow through the de-assimilation (breathing, oxidation) and assimilation (anabolism) pathways are substrate availability, product elimination, availability of co-factors and their synthesis, and the specific activation and inhibition of the regulatory enzymes. Methanol metabolism is even more complex, since it also requires the transportation of substrates and products through the peroxisome's membrane, the regulation of the enzyme activity and the synthesis process, and the replacement of the peroxisome's enzymes.

The peroxisome's enzyme expression is strongly in-


M8
M9
M13
Disolve Oxigen (%)
IDS Act. (nmol/h mg of protein)
Extracellular Protein (g/l)
Dry Weight(g/l)
Dry Weight (g/l)
Glycerol (g/l)
Methanol % (v/v)
PU/ml min
pH
Time (h)
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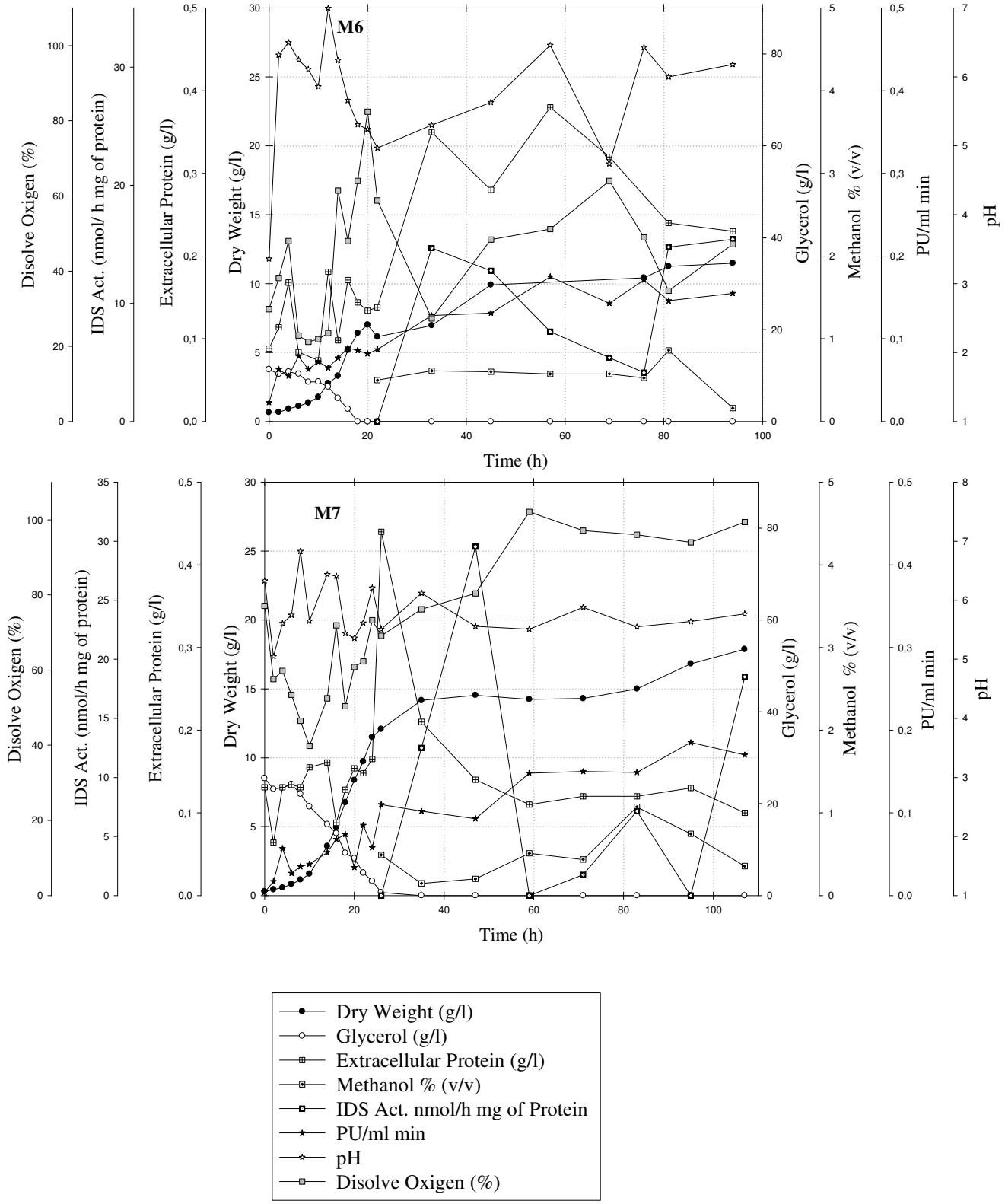

Figure 1. Kinetic behavior of IDS28 in different initial concentration of glycerol: M6 (11.34 g L^{-1} of glycerol, μ_x 0.144 h^{-1}), M13 (17.27 g L^{-1} of glycerol, μ_x 0.182 h^{-1}), M7 (25.53 g L^{-1} of glycerol, μ_x 0.199 h^{-1}), M8 (40.13 g L^{-1} of glycerol, μ_x 0.214 h^{-1}) and M9 (83.32 g L^{-1} of glycerol, μ_x 0.247 h^{-1}).

fluenced by the growth substrate; in cells grown in glycerol, xylose, ribose or sorbitol, the specific activity is 60% of that observed in methanol. The enzymatic machinery required to oxidize methanol suffers catabolite repression and methanol induction. Glucose and ethanol are repressors that are more effective; in cultures containing glycerol, the AOX in *P. pastoris* is not detected. It de-represses from the system as the specific speed decreases. Mixtures of methanol with other repressive sources indicate that both the repressor's and the inductor's effects compete with each other. Whenever a culture grown in methanol turns into glucose, or when there is a limitation in ammonium ions, it has been observed that peroxisome fuses with the vacuole's vesicles; this fusion being followed by proteolysis, a phenomenon also called degrading inactivation (Poutou et al., 2005b).

The hrIDS-*Like* activity increased when passed from culture M6 to M7, from 14.7 to 29.5 nmol h^{-1} mg^{-1} of total protein, between the 33 and 47 h of culture. In M8, the hrIDS-*Like* activity was 7.3 nmol h^{-1} mg^{-1} of total protein at 47h of culture. In M9, no hrIDS-*Like* activity was detected (Figure 1). Even when cell density increased with substrate concentration, the effect observed was not the same as when methanol was used as substrate. It is important to note that hrIDS-*Like* activity was lower when extra-cellular proteolytic activity increased (Figure 1), suggesting a proteolytic degradation.

In this culture strategy oleic acid (18:1) was use as antifoam; the direct use of fatty acids from C_6 to C_{14} may inhibit growth as it decreases with longer chains from C_{12} to C_{18}. Acid metabolism requires ATP and is trapped by a CoA-SH molecule due to the presence of a type 2 synthetase found in the peroxisome to initiate the β-oxidation pathway. Oleic acid has been used in other works as antifoam because of its chemical nature (Córdoba 2004; Poutou et al., 2005a) and taking this into account, the biogenesis of peroxisomes that is generated by this fatty acid (Cereghino et al., 2001; Schuller 2003) could behave as stimulating agent for peroxisomal enzymes production. The major finding of this work was that all this extracellular data collected were use to develop a simple structured mathematical model for the expression of hrIDS-*Like* based on the oxidative stress phenomenon (Mendoza et al., 2008), with a great probability to be useful to express other human sulfatases.

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