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Use of different carbon sources in cultivation of baker's yeast for production of glycerol-3-phosphate dehydrogenase

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Abstract

The physiological state of yeast cells changes during culture growth as a consequence of environmental changes (nutrient limitations, pH and metabolic products). Cultures that grow exponentially are heterogeneous cell populations made up of cells regulated by different metabolic and/or genetic control systems. The strain of baker's yeast selected by plating commercial compressed yeast was used for the production of glycerol-3-phosphate dehydrogenase. Glycerol-3-phosphate dehydrogenase (GPD) has been widely used in the enzyme assays with diverse compounds of industrial interest, such as glycerol or glycerol phosphate, as well as a number of important bioanalytical applications. Each cell state determines the level of key enzymes (genetic control), fluxes through metabolic pathways (metabolic control), cell morphology and size. The present study was carried out to determine the effects of environmental conditions and carbon source on GPD production from baker's yeast. Glucose, glycerol, galactose and ethanol were used as carbon sources. Glycerol and ethanol assimilations required agitation, which was dependent on the medium volume in the fermentation flask for the greatest accumulation of intracellular GPD. Enzyme synthesis was also affected by the initial pH of the medium and inoculum size. The fermentation time required for a high level of enzyme formation decreased with the inoculum size. The greatest amount of enzyme (0.45 U/ml) was obtained with an initial pH of 4.5 in the medium containing ethanol or glycerol. The final pH was maintained in YP-ethanol, but in the YP-glycerol the final pH increased to 6.9 during growth.

Key words: Baker's yeast, glycerol-3-phosphate dehydrogenase, induction.

Introduction

Glycerol is a by-product of the biodiesel industry and its production has increased exponentially in recent years [15]. A large number of studies have been conducted and innovative uses of glycerol are currently under investigation [13]. Glycerol is produced or used by cells in microorganisms [14]. In *Saccharomyces cerevisiae*, for instance, glycerol is produced from dihydroxyacetone phosphate by glycerol-3-phophate dehydrogenase, followed by dephosphorylation by glycerol-3-phosphatase [6, 7]. The two physiological roles of glycerol production in *Saccharomyces cerevisiae* have inspired a number of detailed investigations into the regulation of *GPD* and *GPP* genes, encoding glycerol-3-phosphate dehydrogenase and glycerol-3-phosphatase [10]. Despite the similar physical and catalytic properties of their gene products, *GPD1* and *GPD2* genes are differentially regulated on the transcriptional level [3, 7, 19]. Remize *et al.* [16] describe the overexpression of *GPD*1 in a strain of *Saccharomyces cerevisiae* that encodes glycerol-3-phosphate dehydrogenase and enhances glycerol formation.

The physiological state of yeast cells changes during culture growth as a consequence of environmental changes (nutrient limitations, pH and metabolic products). Cultures that grow exponentially are heterogeneous cell populations made up of cells regulated by different metabolic and/or genetic control systems [1, 2]. Each cell state determines the level of key enzymes (genetic control), fluxes through metabolic pathways (metabolic control), cell morphology and size [1, 10]. Cytoplasmic glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) is one of the metabolic enzymes for which the intracellular level of activity is affected by the physiological state of the cell. An increase in glycerol-3-phosphate dehydrogenase levels has been described when cells are grown in non-fermentable carbon sources (ethanol, glycerol, acetate and lactate) [17]. Glycerol-3-phosphate dehydrogenase may be used for analyses in industrial bioanalytical applications [11]. Moreover, this enzyme, together with lipase and glycerol kinase, is an important parameter in the clinical determination of blood triglyceride level [5].

The purpose of the present study was to investigate the effects of substrates (glycerol, ethanol, glucose and galactose), initial pH of the medium, inoculum size and agitation on glycerol-3-phosphate dehydrogenase production from baker's yeast.

Materials and Methods

Strain, growth media and culture conditions: Baker's yeast strains were selected by plating commercial compressed yeast (Mauri Brazil, Ind. Com. Imp. Ltda – Pederneiras, SP, Brazil, 2007) which were maintained at 4°C on slants of complete medium containing 20 g l^{-1} glucose, 1 g l^{-1} yeast extract (Difco), 20 g l^{-1} peptone (Difco) and 20 g l^{-1} agar (Difco). The inoculum was grown overnight from

two full loops of cells in 50 ml YP-glycerol (1% yeast extract, 2% peptone and 4% glycerol at pH 4.5 with agitation in a rotary shaker at 250 rpm for 24 h). Three different agitation rates (170, 250 and 420 rpm) were used. Culture growth in 50 ml of YP-carbon source in a 125-ml Erlenmeyer flask was inoculated for an initial cell concentration of 0.050 mg ml^{-1}. Cells were batch cultured at 30°C for 48 h (transition between log and stationary phase). Culture growth was monitored by turbidity measurements. A standard curve transformation of absorbance reading into dry weight was used according to the equation: Dry weight (mg ml^{-1}) = absorbance at 570 nm x factor x dilution.

Preparation of cell extracts: 100 mg of cell pellet (dry weight) were washed twice with Tris buffer (0.01 M Tris, 0.01 M NaF at pH 7.0) at 4°C and lyophilized. Disruption of the cells was obtained by shaking the dried pellet vigorously in 1.0 ml of the same buffer and equal volume of glass beads (0.05 mm diameter) six times (30-second periods with 30-second intervals). The tubes were maintained on ice during the intervals. The extracts were centrifuged for 10 min at 10,000 rpm and the cell debris was washed with 1 ml buffer. The two supernatants were blended to form the crude enzyme extract.

Glycerol-3-phosphate dehydrogenase assay: The reaction was performed in a final volume of 3.3 ml, containing the following reagents: 0.42 mM of NAD^+, 3.6 mM of glycerol phosphate, 0.11 M of glycine/NaOH buffer, 0.88 M of hydrazine at pH 9.8 and diluted extract. Formed NADH was monitored through spectrophotometry at 340 nm. The reaction rate was monitored (first minute of reaction) and an absorbance coefficient for NADH of 6.02 x 10^3 M^{-1} cm^{-1} was used (1 U = 1 mmol of NADH formed per min). The reaction was started with the addition of fresh extract (20-folds) and a blank was prepared with all the reagents except the substrate.

Protein assay: Layne's method and serum bovine albumin (as standard) were used [9].

Results and Discussion

The level of glycerol-3-phosphate dehydrogenase is dependent on the carbon source [2]. Under aerobic conditions, cytosolic redox equivalents can be oxidized by external NADH dehydrogenases or the glycerol 3-phosphate shuttle or can be indirectly transferred to the mitochondrial matrix through redox shuttles [6, 14]. In anaerobic cultures, the redox balance dictates that an increased specific rate of glycerol production must be balanced by an increased conversion of glucose into more oxidized metabolites [3].

Another way to control glycerol production is through oxygen-limited culturing. Weusthuis *et al.* [18] found that glycerol formation in a growth-limiting oxygen feed only sets in when the specific oxygen uptake rate of the biomass decreases below the rate that is required to reoxidize the assimilatory NADH formed in biosynthesis [3]. The present results revealed that the level of glycerol-3-phosphate dehydrogenase was dependent on the agitation of the medium (Table 1). Shaking of the medium increases the amount of dissolved oxygen. The highest amount of enzyme was found in the YP-ethanol medium, whereas the lowest amount of enzyme formation occurred in the medium containing galactose.

Table 1. Effect of agitation of culture and carbon source on intracellular levels of glycerol 3-phosphate dehydrogenase and biomass formation[(a)].

Culture				Cell Extracts		
Media	Agitation	pH Final	Biomass (mg/ml)	Protein (mg/ml)	Activity (U/ml)	Specific activity (U/mg protein)
YP-glycerol	with	6.1	7.3 ± 0.1	21.5 ± 0.5	0.45 ± 0.06	0.021
YP-ethanol	with	4.5	8.7 ± 0.3	17.9 ± 1.0	0.46 ± 0.03	0.026
YP-glucose	with	4.5	10.7 ± 0.0	17.9 ± 0.3	0.13 ± 0.05	0.007
	without	4.5	3.3 ± 0.1	30.4 ± 0.7	0.24 ± 0.06	0.008
YP-galactose	with	4.6	11.2 ± 0.0	19.7 ± 0.5	0.18 ± 0.01	0.009
	without	4.5	4.0 ± 0.1	25.1 ± 0.6	0.15 ± 0.03	0.006

(a): volume of medium in flask – 25 ml, with 0.050 mg/ml initial cell density.

Dissolved oxygen is dependent on the volume of the medium in the flask and the agitation rate. As illustrated in Fig. 1, biomass growth and glycerol-3-phosphate dehydrogenase activity using glycerol as the carbon source were dependent on the agitation rate and volume of the medium used in the fermentation flask. A small volume of medium (15 to 20 ml) was the best for enzyme formation at 170 rpm and a larger volume (50 to 60 ml) was the best at 250 rpm. The highest level of glycerol-3-phosphate dehydrogenase was obtained when the volume of medium was 30 ml and the agitation 420 rpm. Thus, an increase in the medium volume in the flask reduces aeration and the best agitation rate is dependent upon the medium volume/flask volume ratio (Fig. 1).

The baker's yeast was followed in the batch cultures for biomass production and enzyme accumulation for 72 to 96 h. Fig. 2 shows the effect of three different inoculum sizes on biomass and GPD activity. The best combination of inoculum size and growth time was 0.075 mg/ml and 60 h for biomass production and 0.050 mg/ml and 48 h for GPD synthesis.

Transmembrane proton flow was found to be very sensitive to external ethanol [8]. Low intracellular pH is a potent stimulator of cAMP synthesis in glucose-repressed or de-repressed cells of *Saccharomyces cerevisiae* [12]. Normal intracellular pH can be restored by H^+-ATPase of the plasma membrane. Moreover, acidic pH can inhibit components of the cytoplasmic translation

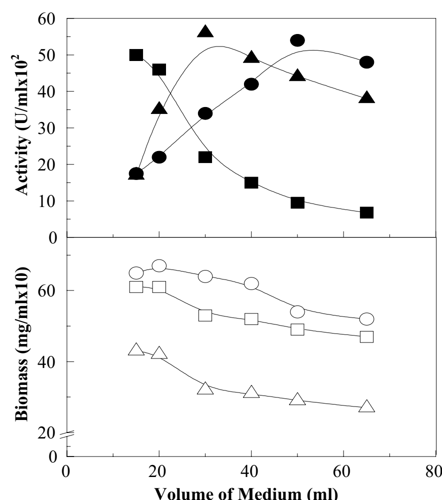

Figure 1. Effect of medium volume in flask and culture agitation on biomass and glycerol-3-phosphate dehydrogenase activity from baker's yeast; agitation: 170 rpm (Δ), 250 rpm (☐) and 420 rpm (O) for biomass assays; 170 rpm (■), 250 rpm (●) and 420 rpm (▲) for GPD activity assays. The medium volume /flask volume ratio are: 0.12, 0.16, 0.24, 0.32, 0.40 and 0.52 for 15, 20, 30, 40, 50 and 65 ml of medium, respectively on flask of 125 ml.

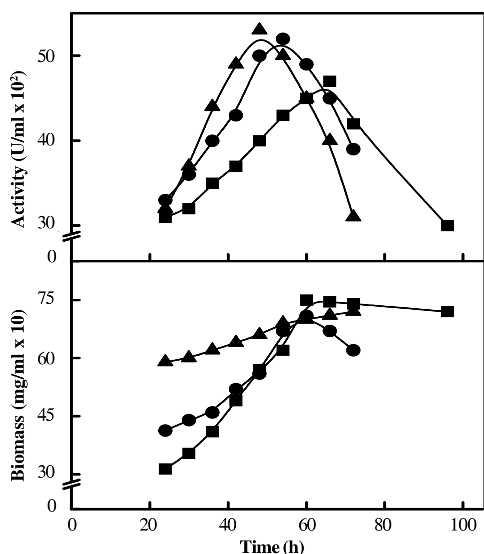

Figure 2. Effect of time and inoculum size on biomass and glycerol-3-phosphate dehydrogenase activity from baker's yeast; inoculum sizes: 0.025 mg/ml (●), 0.050 mg/ml (▲) and 0.075 mg/ml (■).

machinery [4]. However, hydrogen ion concentrations in the media employed in the present study (ethanol and glycerol as carbon sources) appear to regulate the synthesis of glycerol-3-phosphate dehydrogenase and 4.5 was the best initial pH for the accumulation of the enzyme with greatest activity (Tables 2 and 3). In fact a 53% and 62% increase in GPD relative activity was observed at pH 4.5 compared respectively, to 3.5 and 6.5 in cultures with YP-ethanol medium. Moreover the cultures with YP-glycerol medium at pH 4.5 allowed a 55% increase in GPD relative activity compared to 3.5.

Table 2. Effect of initial pH on glycerol-3-phosphate dehydrogenase formation in cultures with YP-ethanol medium.

pH		Biomass	Activity		Protein	Specific activity
Initial	Final	(mg/ml)	U/ml	Relative	(mg/ml)	(U/mg protein)
3.5	3.8	7.3 ± 0.2	0.24 ± 0.06	0.5	22.3 ± 0.4	0.011
4.5	4.5	8.0 ± 0.5	0.45 ± 0.04	1.0	17.9 ± 0.2	0.025
5.5	5.4	6.5 ± 0.7	0.37 ± 0.02	0.8	19.2 ± 0.5	0.019
6.5	5.9	4.3 ± 0.4	0.28 ± 0.03	0.7	16.8 ± 0.8	0.017

Table 3. Effect of initial pH on glycerol-3-phosphate dehydrogenase formation in cultures with YP-glycerol medium.

pH		Biomass	Activity		Protein	Specific activity
Initial	Final	(mg/ml)	U/ml	Relative	(mg/ml)	(U/mg protein)
3.5	4.0	7.0 ± 0.1	0.19 ± 0.03	0.45	10.9 ± 0.8	0.018
4.5	6.9	6.4 ± 0.4	0.45 ± 0.04	1.00	17.0 ± 0.2	0.025
5.5	7.7	5.4 ± 0.3	0.27 ± 0.06	0.62	13.3 ± 0.5	0.020

The optimization of fermentation parameters (oxygen, pH of the medium and inoculum size) for yeast growth and GPD production may result in the application of diagnostic kits for the determination of glycerol levels. The standardization of conditions in assays employing this enzyme may also be useful for further studies on its physicochemical properties.

Conclusions

The strain of baker's yeast selected by plating commercial compressed yeast was used for the production of glycerol-3-phosphate dehydrogenase. The use of glycerol and ethanol as the sole carbon source in the culture medium produced GPD with higher activity. The optimal pH of the culture medium used for cell production was 4.5. GPD activity was dependent on the agitation rate of the culture and the medium volume. Agitation of the growth medium was required for the assimilation of glycerol and ethanol by baker's yeast. The greatest GPD activity (0.46 U/ml) was reached with the inoculum size of 0.050 mg/ml of baker's yeast at 30 ml of YP-ethanol medium with an initial pH 4.5, after 48 h growth and agitation of 420 rpm.

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