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Effect of carbon:nitrogen ratio (C:N) and substrate source on glucose-6-phosphate dehydrogenase (G6PDH) production by recombinant *Saccharomyces cerevisiae*

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Abstract

The use of lower cost components for the cultivation of a recombinant strain of *Saccharomyces cerevisiae* overexpressing G6PDH, recently obtained, was investigated. The utilization of yeast extract as nitrogen source and sugar cane blackstrap molasses or glucose as carbon sources was studied for G6PDH production, under three different C:N ratios: 7, 10, and 14. The results for cell concentration, enzymatic activity and total reducing sugar concentration, evaluated throughout the cultivations, demonstrated that the use of sugarcane blackstrap molasses and yeast extract, at a C:N ratio of 10, provided the highest G6PDH activity (P_{max} = 5180 U/L), with a three-fold increase in comparison to the original culture medium.

Keywords: Glucose-6-phosphate dehydrogenase; *Saccharomyces cerevisiae* W303-181; C:N ratio; Sugarcane blackstrap molasses; Yeast extract

1. Introduction

Glucose-6-phosphate dehydrogenase (G6PDH) (EC. 1.1.1.49) is the first enzyme of the pentose phosphate pathway. It catalyses the oxidation of glucose-6-phosphate using $NADP^+$, yielding pentose phosphates for nucleotides synthesis, as well as NADPH/NADH for reductive biosynthesis and protection from oxidative stress. The G6PDH deficiency is one of the most common enzymopathies in humans, resulting from more than 90 different genetic mutations (Au, Gover, Lam, & Adams, 2000). As a consequence, this enzyme finds numerous applications in medical and biochemical studies. The G6PDH presents also great interest as an analytical reagent, being used in various quantitative assays including the measurement of creatin-kinase and hexokinase activities, and the measurement of glucose, fructose, mannose, and ATP concentrations. In this respect, this enzyme can be considered one of the most profitable enzymes in the world market for diagnostics, and it is present in numerous analytical kits. The use of G6PDH for measuring glucose in the presence of fructose constitutes an important tool for the detection of illegal sugar addition in the final products of the wine and fruit juice industries, both highly developed in Brazil. Moreover, the entire amount of G6PD consumed in Brazil is imported, and its cost is very high (Abrahão-Neto, Infanti, & Vitolo, 1997; Godfrey & West, 1996).

Saccharomyces cerevisiae has been widely used for the expression of various genes for protein production (Chen, Kirk, & Piper, 1993; Lang, Gollnitz, Popovic,

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Nomenclature

Glu	glucose
YNB	yeast nitrogen base without amino acids
YE	yeast extract
SBM	sugar cane blackstrap molasses
X_{max}	cell concentration corresponding to the maximum enzymatic activity observed at t_f
P_{max}	maximum product (G6PD) concentration, expressed in activity, observed at t_f
t_f	time of cultivation corresponding to P_{max}
P_{spe}	specific enzymatic activity calculated according to Eq. (III)
Pr_X	cell productivity calculated according to Eq. (I)
Pr_P	enzyme productivity calculated according to Eq. (II)
μ_X or q_X	specific growth rate
q_P	specific enzyme production rate
μ_{Xmax} or q_X	maximum specific growth rate
q_{Pmax}	maximum specific enzyme production rate

& Stahl, 1997; Wang & Da Silva, 1993). This well-studied eukaryotic organism can be considered a suitable host for the production of both secreted and soluble cytosolic proteins at high productivity rates. In addition, *S. cerevisiae* is an attractive host for the production of food-grade recombinant enzymes, since it neither contains nor produces toxins, it is not pathogenic and generally recognized as safe (GRAS) (Lang et al., 1997). The establishment of optimal operational conditions for the cultivation of recombinant microorganism is difficult because of the additional complexity imposed by either segregational or structural plasmid instability. The rate of plasmid-free cells generation depends on the plasmid characteristics, cultivation conditions, cell growth rate (Ó'Kennedy & Patching, 1997; Wang & Da Silva, 1993).

Recently, a strain of genetically modified *S. cerevisiae* (W303-181) overexpressing G6PDH was obtained (Lojudice, Silva, Zanchin, Oliveira, & Pessoa, 2001). However, the cost of the culture medium utilized to grow this genetically modified microorganism is very high. The main reason for this drawback is the need of some particular micronutrients addition in the cultivation medium. Therefore, studies aiming to lower the cultivation cost, as well as scaling up studies, are necessary (Miguel, Neves, Vitolo, & Pessoa, 2003). It was shown that the type and concentration of carbon and nitrogen sources, as well as the C:N ratio of the medium for *S. cerevisiae* cultivation, influence cellular growth, metabolites biosynthesis and, as a consequence, the cost of the bioprocess (Thomas, Hynes, & Ingledew, 1996).

In this work, the use of low cost components in the *S. cerevisiae* (W303-181) culture medium was studied, aiming at increasing the enzyme productivity. Glucose and sugarcane blackstrap molasses were used as carbon sources, whereas Yeast extract was investigated as the nitrogen source at different C:N ratios. The sugarcane blackstrap molasses is a by-product of the Brazilian sugar/alcohol industry. Given the importance of sugar and alcohol industry in Brazil, this largely available substance is very cheap (Vitolo, Duranti, & Pellegrim, 1995).

2. Materials and methods

2.1. Chemicals

Uracil, adenine, L-tryptophan, L-histidine, β-nicotinamide adenine dinucleotide phosphate (β-NADP), phenylmethylsulfonyl fluoride (PMSF), β-mercaptoethanol, glucose-6-phosphate (G6P) and glucose were purchased from SIGMA (St. Louis, MO). Yeast nitrogen base without amino acids (YNB) and yeast extract (YE) were purchased from Difco (Detroit, MI). All other chemicals used were of analytical grade. Sugar cane blackstrap molasses was kindly provided by Using Açucareira Ester S.A. (Cosmópolis, Brazil).

2.2. Microorganism and inoculum preparation

The genetically modified strain of *S. cerevisiae* W303-181 was employed in batch cultivations. The stock culture was kept on agar slants at 4 °C. The composition of the stock culture and the genetic engineering techniques were previously described, resulting in a C:N ratio of 7 (Lojudice et al., 2001). The inoculum was prepared as described by Miguel et al. (2003). Accordingly, a loopful of the stock culture was transferred to test tubes containing 10 mL of culture medium and incubated on a rotary shaker (NBS Gyratory Shaker, New Brunswick, Edison, NJ) at 100 rpm and 30 °C for 24 h. Following, the culture medium was centrifuged (Jouan, Model BR4i, St. Herblain, France) at $3025 \times g$, 30 min, 6 °C and the pellet was resuspended in acetate buffer (0.1 M, pH 5.7) containing 30% glycerol (w/w). This suspension was stored at −70 °C. For the pre-inoculum preparation, the cells were thawed and incubated in 50 mL Erlenmeyer flasks containing 10 mL of culture medium, on a rotary shaker at 100 rpm and 30 °C for 24 h. To prepare