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Biotransformation of sucrose into 5-hydroxy-2-hydroxymethyl-γ-pirone by *Aspergillus flavus*

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ABSTRACT

The sucrose hydrolysis and the preference of consumption of glucose instead of fructose were investigated for the production of 5-hydroxy-2-hydroxymethyl-γ-pyrone (HHMP) in the presence of *Aspergillus flavus* IOC 3974 cultivated in liquid Czapeck medium. Standardized 0.5g of pellets were transferred as inoculum into twelve conical flasks of 250 ml containing 100 ml of medium with different sucrose concentration, which was kept at 120 rpm and 28°C for 16 days without pH adjustment. Aliquots of 500μl of the broth culture were withdrawn at 24 h intervals and analyzed. The major yield of HHMP was 26g l^{-1} in 120g l^{-1} of sucrose. At these conditions, *A. flavus* produced an invertase capable of hydrolyzing 65% of total sucrose concentration in 24h, and an isomerase capable of converting fructose into glucose. In this work, it focused the preference for glucose and, then, of fructose by *A. flavus* and the strategy used to produce HHMP.

Key words: *Aspergillus flavus*, biotransformation, kojic acid, secondary metabolite, fructose-isomerase.

INTRODUCTION

Fungi are lower eukariotes microorganisms that have been important in both ancient and modern biotechnological processes. They are known as excellent metabolites producer agents of antibiotics, alcohols, enzymes, organic acids, pharmaceuticals and several organic compounds (Wang et al. 2005). Fungi present a different way of nutrition. They secrete in the environment a wide range of secondary metabolites and powerful enzymes, such as peroxidase and hydrolases, among others, which oxidize and hydrolase lignins, cellulose and polysaccharides into micro molecules, such as phenyl alcane and glucose, and absorb them as foodstuff (Papagianni 2003). Based on this mode of action, fungi are classified in the fifth kingdom (Bennett 1998), and can be found as yeasts, molds and mushrooms that produce metabolites and enzymes with biological activities. In this aspect, the genera *Aspergillus*, *Penicillium*, *Peacylomyces* and *Fusarium* present high potential in producing active metabolites and enzymes that must be screened in a great range of strain to select a great producer (Mercier et al. 1998). This kind of work developed with fungi is known as Mycotechnology, which is one part of Biotechnology operating at the scientific frontier approaching medicine, food industry, agriculture and cosmetic by the forefront of molecular biotechnology (Bennett 1998).

In this paper, it is described that 5-hydroxy-2-hydroxymethyl-γ-pyrone, which is known as kojic acid,

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is a secondary metabolite produced from carbohydrate sources, mainly from those with pyranosidic structures, by aerobic fermentation (Ariff et al. 1997). However, other fungi that belong to *Aspergillus genera* were described to produce the same metabolites: *A. oryzae* (Wakisaka et al. 1998), *A. tamarii* (Rosfarizan et al. 1998) and some strains of *A. parasiticus* (Varga et al. 2003).

This substance is industrially interesting (Park et al. 2003) for presenting an inhibitory activity against tyrosinase (Kim et al. 2004) and other several correlated enzymes, such as the polyphenol oxidases (PPO) (İyidoğan and Bayindirli 2004). Previous studies showed that this substance presented antibiotic activity. It also presents an potential application as a precursor of flavor enhancer and as an antioxidant agent by inhibiting oxidations of polyphenol (Ariff et al. 1996). Several works have been developed to discover new microorganisms and substrates that can be used in the production of HHMP (5-hydroxy-2-hydroxymethyl-γ-pyrone) (Burdock et al. 2001). However little is known about the mechanisms of HHMP formation, and only biosynthesis discussions have been published without characterizing the enzymes, the biotransformation of different sources of carbohydrate to HHMP, and the kinetic parameters for glucose and sucrose (Mohamad and Ariff 2007).

During sucrose fermentation by *A. flavus*, the HHMP is synthesized by the direct conversion of glucose through multistep enzyme reactions. Although the enzyme system involved in HHMP biosynthesis was found to be very stable under a chemically defined resuspended cell system, the action of fructose-isomerase was never observed before.

The present study was undertaken to investigate the different sucrose concentrations on the biotransformation of this disaccharide to HHMP and monosaccharides preference of consumption during filamentous fungus cultivation after the hydrolysis of the glucose by an invertase produced fungus.

MATERIALS AND METHODS

Microorganism and Medium

Aspergillus flavus IOC 3974 used in this work was obtained from the laboratory of collection of fungi of the Oswald Cruz Institute in Rio de Janeiro (Brazil). The conidia were suspended in a sterile solution of NaCl (1% w/v) and used as the initial inoculum (S1). In this work, three types of culture media were used to investigate the adaptation and better mycelial development of *A. flavus*: Czapek Dox agar (CDA), potato dextrose agar (PDA), and Sabouraud agar (SBA) (Keller et al. 2003). All of the media were sterilized at 121°C (1kgf cm^{-2}) for 15 minutes. The sucrose used as a carbon source was added into the media in different concentrations, and the pH was adjusted to 5.5 with NaOH (1 mol l^{-1}) before sterilization.

Cultivation of *Aspergillus flavus* for Obtaining Conidia (Spores)

Amounts of 500μl of the initial spores suspension (S1) of *A. flavus* ($\pm$ 10^8 conidia ml^{-1}) were transferred to Petri dishes containing 20 ml of CDA with a concentration of sucrose of 30 g l^{-1} and incubated at 28°C for 10 days. A volume of 20 ml of a sterilized solution of NaCl (1% w/v) was used on the plates for obtaining a second suspension of spores (S2).

Evaluation of *Aspergillus flavus* Growth on Solid Media

Amounts of 50μl of the suspension (S2) were transferred to a 5 mm disk of cellulose, centralized on the plates containing CDA medium with different sucrose concentrations: 30, 60, 120, 240 and 360g l^{-1} and incubated at 28°C for 10 days. This procedure was made in triplicate and repeated for the PDA and SBA media. The mycelial growths were evaluated by the biometric orthogonal axes method. The culture media containing the different sucrose concentrations were evaluated at every 24 h intervals by the measurement of mycelial growth diameters in the two directions of the orthogonal axes. Statistical analysis was applied, as well as the formation of the conidia was evaluated qualitatively. The culture media that presented better mycelial growth and good spores formation was selected and used as a medium of *A. flavus* cultivation (E1).

Standardization Method for Obtaining *A. flavus* Inoculum in the Liquid Media Cultivation

An amount of 1 ml ($\pm$ 10^8 conidia ml^{-1}) of the spores suspension (S2) from the culture medium that presented

better growth (E1) was transferred to five conical flasks of 250 ml containing 100 ml of Czapek liquid medium (pH 5,5) with concentrations of 30, 60, 120, 240 and 360g l^{-1} of sucrose properly sterilized at 121°C for 15 min. Then they were incubated in a shaker at 120 rpm with the controlled temperature at 28°C for 72h. Each conical flask was submitted to a vacuum filtration with a Büchner funnel with a quantitative filter paper. After the filtration, mycelium as pellets was used as inoculum. Standardization was carried out by transferring 0.5 g of pellets amounts from a flask containing 6% of sucrose to 250 ml conical flasks containing 100 ml of Czapek culture medium with the addition of 30, 60, 120, 240 and 360g l^{-1} of sucrose. This experiment was prepared in triplicate and further incubated at 120 rpm and 28°C for 16 days.

Evaluation Method of Different Concentrations of Sucrose on the Mycelial Development of *A. flavus* and HHMP Production

Standardized 0.5 g amounts of pellet as inoculum were transferred from the flask containing 6% of sucrose to twelve conical flasks of 250 ml, each one containing 100 ml of Czapek medium with different sucrose concentrations: 60, 120, 240 and 360g l^{-1}. The cultivation was kept at 120 rpm and 28°C for 16 days without a pH adjustment. Aliquots of 500μl of the broth culture, without mycelium, were withdrawn at 24 h intervals, and transferred into glass vials of 10 ml. This experiment was prepared in triplicate and samples were analyzed for the quantification of HHMP and residual saccharides.

Metabolite Identification

The identification of the metabolite was performed by comparing the sample with the standard. We used carbon-13 nuclear magnetic resonance spectroscopy (VARIAN/MERCURY 300 MH_Z), and the dimethyl sulfoxide (DMSO) as solvent, and Infrared Spectroscopy (Spectrometer SHIMADZU – IR 740).

Quantification of Kojic Acid

The quantification curve was built by quantifying the absorbance for different HHMP concentrations. Thus, solutions were prepared at concentrations of 100, 200, 400, 600, 800, 1000 and 1200μg ml^{-1} standard. The absorbances were determined in triplicate by UV-Vis spectrophotometry (GBC 911 system) at 269 nm.

Amounts of 50μl of the samples were transferred to a volumetric bottle of 50 ml, and the volume was completed with deionized water. A quantitative analysis was performed as described in (Gomara et al. 2004). Each sample was analyzed in triplicate.

Quantification of the Total Reducing Sugars

Amounts of 250μl of the samples and 200μl of HCl 2N were transferred to volumetric bottles of 50 ml and the residual non-reducing sugar (sucrose) was hydrolyzed at 70°C for 10 minutes in a water bath. After cooling, the solution samples were neutralized with 200μl of a solution of NaOH 1N. The bottles were completed with deionized water. Amounts of 1.5 ml of this solution were transferred to glass tubes and aliquots of 0.5 ml of an alkaline solution of 3,5-dinitrosalicilic acid (DNS) were added. The analysis was performed in a Quimis model Q798 spectrophotometer. Each sample was analyzed individually in triplicate. The pH was measured at the first and the 16^{th} days of incubation. Fructose was quantified by Saliwanoff, and glucose was quantified by difference.

Quantification of Fructose by Seliwanoff Reagent

The standard curve was built as follows: fructose was measured by the adapted Seliwanoff's method described by Souza et al. (2007). From a standard solution of fructose (150 mg/100 ml), aliquots were withdrawn and transferred to individual glass tubes and diluted with distilled water to reach 1, 10, 20, 30, 40-100mg/100 ml of fructose and ready to reach 200μl of individual solution. 4ml of Seliwanoff reagent were added, and the solutions were boiled for 3 minutes and analyzed by spectrophotometer at 486nm after reaching the room temperature. Samples were measured in the same way, replacing fructose solution by broth medium aliquots.

RESULTS AND DISCUSSION

Measurement of Mycelial Growth of *Aspergillus flavus* in Solid Media

A. flavus was cultivated in Petri dishes forming concentric halos of mycelial growth. The diameters in the orthogonal directions were measured. This procedure,

TABLE I
Comparison of mycelial diameter growth of *A. flavus* in different solid media.

Sucrose concentrations (g l^{-1})	Diameters of mycelial growth in (cm) 9 days of cultivation Different culture media		
	CDA	PDA	SBA
30	6.063 ± 0.294	5.150 ± 0.203	6.567 ± 0.341
60	5.550 ± 0.165	5.327 ± 0.228	6.900 ± 0.274
120	7.817 ± 0.305	7.500 ± 0.195	7.550 ± 0.445
240	5.433 ± 0.306	7.067 ± 0.318	6.800 ± 0.681
360	5.783 ± 0.289	7.163 ± 0.222	6.700 ± 0.376

which is called "biometric method of orthogonal axes", made possible to evaluate the mycelial growth speed in the culture media CDA, PDA and SBA in different sucrose concentrations: 30, 60, 120, 240 and 360g l^{-1}. The results showed that CDA presented the best mycelial growth (Table I). The sucrose concentrations above 120g l^{-1} of sucrose presented smaller mycelial growth. Several factors might have influenced the decrease of the metabolism, like the osmotic pressure and the high fructose concentration after sucrose hydrolysis. The analysis of the different concentrations of the substratum showed that sucrose 30g l^{-1} presented a smaller mycelial growth when compared with the concentrations of 60 and 120g l^{-1}.

The results were important to evaluate the mycelial growth profile of *A. flavus* in different media with different sucrose concentrations in a period of 9 days to select the one which presents the best conditions for *A. flavus* adaptation. In this case, CDA with 120 g l^{-1} of sucrose was the chosen medium for the cultivation of this microorganism, even in a solid to produce the conidia.

Standardization of the Inoculum with Pellets

The culture medium CDA with 60g l^{-1} of sucrose presented more spherical shape and an uniform pellet size with a diameter interval of 1.5 mm = D = 3 mm. The standardization of the inoculum was accomplished with fixed amounts of 0.5g (0.0169g of dry weight withdrawn from medium containing 6g l^{-1} cultivated at 120 rpm for 72 h), and of fresh mass of pellets, properly drained and transferred to liquid medium. This procedure allowed a better standardization and quantification of the inoculum in the biotechnological process for the production of HHMP. However, the use of conidia had a huge margin of error due to its fast sedimentation in the suspension. For this problem, a standardization of the inoculum was developed with pellets.

Identification of HHMP

The identification of HHMP was performed using carbon-13 nuclear magnetic and Infrared Spectroscopy. The spectra of samples were compared with the spectra of standards HHMP. These spectra showed similar chemical shifts (Table II).

TABLE II
Chemical shifts of the sample and standard in C-13 NMR. The chemical shifts are reported in relation to tetramethylsilane (TMS).

Sample (ppm)	Standard (ppm)
141.61	139.34
145.77	144.34
176.27	174.19
110.19	109.92
168.18	168.14
59.55	59.56

Infrared spectroscopy was performed from KBr pellets at a ratio of 1:400 (sample/KBr) and 1:600 (standard/KBr). The spectra of infrared absorption bands showed a clear evidence that the sample has the same structural features of standard a substance. Overall, it was observed that the absorption at 3400 nm indicates the presence of hydroxyl, and 1611 nm refers to car-

bonyl. Absorption in the aromatic region was not observed.

These results confirm the authenticity of the identification of the metabolite produced by *A. flavus* as the 5-hydroxy-2-hydroxymethyl-γ-pirone.

Quantification of HHMP Production

The cultivation of *A. flavus* in solid medium showed that the level of sucrose concentrations is equal to 240g l^{-1}, and 360g l^{-1} do not permit an identical metabolism as that one for 120g l^{-1}. These results were the basis for the investigation of the development of mycelial growth in liquid medium. In this case high concentrations of sucrose increased the viscosity and the osmotic pressure. This aspect also influenced a smaller diffusion of the molecular oxygen, affecting the biosynthetic route of HHMP production. The maximum metabolite production was reached at the 15th day of cultivation, influenced by different concentrations of sucrose (Fig. 1).

This production has started from at the 6th day for all sucrose concentrations. However, 60g l^{-1} and 120g l^{-1} of sucrose presented a better production, reaching out 21g l^{-1} and 26g l^{-1} of kojic acid, respectively (Table III). The best yield coefficient was 0.367g of HHMP *per* gram of added sucrose. These results are indicative of an important information, so that they established better conditions of cultivations for high HHMP production in a biotechnological process.

TABLE III

Kojic acid data production related to different sucrose concentrations. Y: yield coefficient of product formation, So: initial concentration of substrate, t: time (day) and P: maximum production of metabolite.

So (g l^{-1})	t (day)	$Y_{P/So}$ (g g^{-1})	P (g l^{-1})
30	11	0.133	4 $\pm$ 0.11
60	15	0.367	21 $\pm$ 0.49
120	15	0.216	26 $\pm$ 0.76
240	15	0.038	9 $\pm$ 0.26
360	15	0.038	9 $\pm$ 0.22

The quantification of residual sugar concentration was developed to estimate the material balance between sugar converted to HHMP and sugar converted to mycelial biomass. At the 15th day, the yield of HHMP reached 26g l^{-1}, and the residual sugar measured was 3.8% (w/w) from the initial amount that was added into the medium (Table IV).

The smallest adaptation of the filamentous fungus in PDA could have happened due to the absence of mineral salts, mainly phosphate, which is a very important nutrient for glycolytic route and the Krebs Cycle. On the other hand, Sabouraud and Czapek media possess enough amounts of the nutrients that are necessary for the development of the mycelium. The difference was that, in this specific investigation, the standard HHMP presented pale yellow coloration in solution, so that it can be confused with the coloration of the own culture medium. Because of the hydroxyl and keto groups in the position C-4 and C-5, this structure presents potential to chelate transition metals, mainly iron, producing red coloration. A number of works proved the capacity of γ-pyrones to form complexes with metals such as iron (Marwaha and Sohi 1994). In this process, sucrose was used as a source of carbon and was hydrolyzed by invertase to form glucose and fructose in the culture medium by the microorganism. The glucose was consumed immediately and acted as a precursor for HHMP. Fructose was then isomerized to glucose and follows the same biotransformation. The production of fructose was measured by the method of Seliwanoff, which was adapted and described as Souza et al. (2007). HHMP presents a six-member ring and all the evidences indicate that it was produced by the biotransformation of glucose in few main steps, without a break of the monosaccharide chemical structure.

This study was developed to optimize the process production of this metabolite, starting from sucrose. It presented innovative results from the biochemical point of view. In this study, it was observed that the sucrose had been 65% w/w of its total concentration in the hydrolyzed culture medium, being obtained fructose and glucose by an invertase produced fungus. During the sucrose fermentation in a submerged cultivation, the *Aspergillus flavus* initially consumed the glucose, which was observed by the concentration decline of this monosaccharide in the culture medium. However, the fructose that was produced in the same concentration remained unaffected until the total consumption of the glucose, which happened at 168h (Fig. 2). At this time, the microorganism started the fructose consumption. This hap-

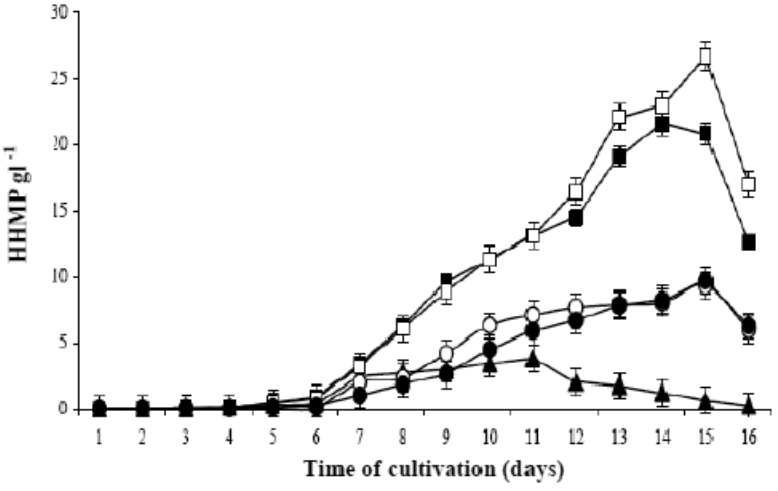

Fig. 1 – Profiles of HHMP production in liquid medium CDA with different sucrose concentrations. Sucrose 30g l^{-1} (▲), sucrose 60g l^{-1} (■), sucrose 120g l^{-1} (□), sucrose 240g l^{-1} (○), sucrose 360g l^{-1} (●).

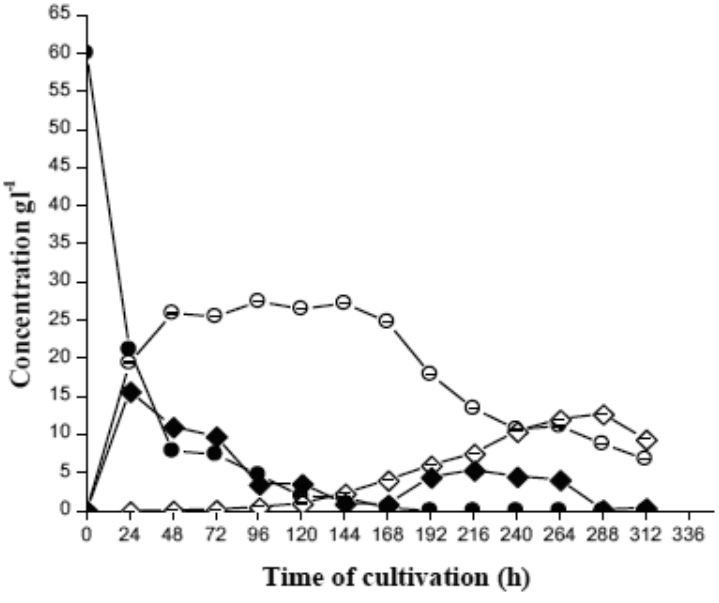

Fig. 2 – Profiles of sucrose hydrolyze, fructose isomerase into glucose and its consumption: Sucrose (●), HMPP (○), glucose (♦), fructose (◊).

pened because an isomerase converted the fructose into glucose, which could be evidenced by the increase of the glucose concentration starting from 168h, and remaining constant up to 264h in the culture medium. In fact, the sucrose was hydrolyzed totally in 168h. It is clearly evident that it could not produce glucose a different way and, then, convert the fructose. The increase of the concentration of this monosaccharide felt in function of the isomerase activity. This enzyme was not characterized in this study yet.

A residual sugar concentration was analyzed to verify the total sugar conversion and to estimate the material balance between sugar converted to HHMP and sugar converted to mycelial biomass. At the 15th day,

TABLE IV
Residual sugar concentration in a Czapek Dox liquid medium.

16 days of cultivations	Concentrations of residual sugar Analysis performed at every 24h, starting from the initial amount to the 16th day of cultivation				
Hours	30g l^{-1} Initial amount	60g l^{-1} Initial amount	120g l^{-1} Initial amount	240g l^{-1} Initial amount	360g l^{-1} Initial amount
24	29.40 ± 0.31	58.10 ± 0.33	116.16 ± 0.20	237.48 ± 0.32	345.62 ± 0.32
48	27.14 ± 0.28	56.08 ± 0.34	112.08 ± 0.38	217.75 ± 0.30	320.65 ± 0.29
72	24.54 ± 0.35	54.50 ± 0.17	104.74 ± 0.35	197.99 ± 0.33	283.41 ± 0.29
96	22.28 ± 0.54	36.01 ± 0.21	97.04 ± 0.36	178.23 ± 0.27	264.17 ± 0.31
120	20.02 ± 0.46	29.40 ± 0.15	90.60 ± 0.33	152.24 ± 0.30	241.93 ± 0.32
144	17.76 ± 0.30	20.30 ± 0.35	83.26 ± 0.61	138.48 ± 0.29	218.69 ± 0.32
168	15.55 ± 0.26	17.40 ± 0.23	76.46 ± 0.26	118.72 ± 0.30	190.45 ± 0.29
192	9.33 ± 0.15	12.05 ± 0.89	69.12 ± 0.13	71.23 ± 0.30	164.21 ± 0.25
216	7.48 ± 0.27	11.50 ± 0.74	48.38 ± 0.32	60.06 ± 0.32	138.97 ± 0.33
240	6.39 ± 0.28	10.08 ± 0.26	38.46 ± 0.26	48.36 ± 0.30	85.73 ± 0.30
264	4.42 ± 0.23	7.58 ± 0.23	28.54 ± 0.17	37.19 ± 0.31	66.49 ± 0.29
288	3.45 ± 0.27	6.35 ± 0.14	18.74 ± 0.18	26.02 ± 0.33	47.25 ± 0.30
312	1.98 ± 0.26	5.20 ± 0.14	9.24 ± 0.15	14.85 ± 0.31	28.01 ± 0.33
336	0.51 ± 0.18	3.50 ± 0.15	6.69 ± 0.36	10.93 ± 0.32	8.77 ± 0.26
360	0.50 ± 0.18	3.20 ± 0.13	4.56 ± 0.11	9.34 ± 0.29	8.74 ± 0.27
384	0.41 ± 0.14	3.20 ± 0.20	3.70 ± 0.36	8.92 ± 0.32	7.53 ± 0.19

the yield of HHMP reached 26g l^{-1}, and the residual sugar measured was 3.8% (w/w) from the initial amount (Table III). In the same way, the percentage of HHMP measured in relation to initial sugar concentration was 21.67% (w/w). It was demonstrated that 78.4% (w/w) of sugar were converted to mycelial biomass, carbon dioxide and other macromolecules not quantified.

CONCLUSION

The following conclusions can be drawn from the above results:

A. flavus could hydrolyze sucrose, isomerize fructose into glucose, and biotransform this monosaccharides to HHMP. In this case, it was not possible to quantify the yield of isomerization due to microorganism just consuming the produced glucose. However, the maximum concentration of fructose was 15g l^{-1} in the culture medium. This phenomenon showed that this microorganism produced an invertase and an isomerase capable to hydrolyzed glucose and convert fructose to glucose in a dynamic process.

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RESUMO

Foram investigadas a hidrólise da sacarose e a preferência pela glicose frente à frutose no processo de produção do 5-hidroxi-2-hidroximetil-γ-pirona (HHMP) na presença de *Aspergillus flavus* IOC 3974 cultivado em meio líquido Czapeck. Quantidades de 0,5g de pelletes foram utilizadas como inóculo. Doze frascos cônicos de 250 ml contendo 100 ml de meio de cultura com diferentes concentrações de sacarose foram utilizados. Os microrganismos foram cultivados a 120 rpm e 28°C por 16 dias sem ajuste do pH. O maior rendimento do HHMP foi 26g l^{-1} em 120g l^{-1} de sacarose. Nestas condições, *A. flavus*, foi capaz de produzir uma invertase possibilitando a hidrólise de 65% da concentração total de sacarose em 24 horas, conjuntamente com a produção de uma isomerase que foi capaz de converter a frutose em glicose. Este trabalho está focalizado

preferencialmente no consumo da glicose frente à frutose por *A. flavus* e na estratégia de produção do HHMP.

Palavras-chave: *Aspergillus flavus*, biotransformação, ácido kójico, metabólito secundário, frutose-isomerase.

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