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Riboflavin production from mutants of *Ashbya gossypii* utilising orange rind as a substrate

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Abstract - The use of agroindustrial wastes such as orange rind as an alternative in the production of riboflavin was evaluated in this study with *Ashbya gossypii* mutants. *Ashbya gossypii* mutants were obtained using 300 µg/mL of MNNG after an exposition period of 90 min with 2.3% of survival rate. A total of 11 mutant high yield strains of riboflavin were selected. Of these mutants, the most productive in YM medium were ASHLVII, ASHLX, ASHLXI and ASHLXV. Three additional different mutants were shown to be unsusceptible to inhibition by itaconate in the medium. When we used orange rind at 0.3% in YM medium without malt extract, the rate of riboflavin production was enhanced in the mutant strains producing 223 mg/L of this vitamin, an increase of 184% in the ASHLXI and ASHLXV mutants, as compared with the wild strain.

Key words: riboflavin, *Ashbya gossypii*, mutants MNNG, solid wastes, orange rind.

INTRODUCTION

Riboflavin is a member of the water soluble B complex family of vitamins and is an essential component of basic cellular metabolism since it is the precursor of the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (Bigelis, 1989; Vitreschak *et al.*, 2002; Burgess *et al.*, 2004). Riboflavin (vitamin B_2) can be produced by many microorganisms including bacteria and ascomycetes, of which the most important are *Eremothecium ashbyii* and *Ashbya gossypii* (Vandamme, 1992; Burgess *et al.*, 2004). Chemical riboflavin production, successfully used for decades, is in the course of being replaced by microbial processes. These promise to save half the costs, reduce waste and energy requirements and use renewable resources such as sugar or plant oil. (Vandamme, 1992; Stahnmann *et al.*, 2000). In fermentation processes, the best riboflavin producers are the ascomycetes *Eremothecium ashbyii* and *Ashbya gossypii* (Yoneda, 1984); however, *A. gossypii* is preferred due to its genetic stability (Wickerman *et al.*, 1946; Demain, 1972) and it is considered an important biotechnological producer of riboflavin. Biosynthesis, regulation parameters and production of riboflavin by this microorganism and others have been widely studied (Demain, 1972; Lago and Kaplan, 1981; Bigelis, 1989; Bacher, 1991; Sauer *et al.*, 1996; Monschau *et al.*, 1998), resulting in well established fermentation processes with a maximum yield reported of 15 g/l using mutants of the wild strain (Lago and Kaplan, 1981; Bigelis, 1989). In fungi, when vegetable oils are used as the only carbon source, the glyoxylate cycle, which is an anaplerotic route (Kornberg, 1965a, 1965b), plays an important role in the growth and synthesis of riboflavin (Schmidt *et al.*, 1996b). The isocitrate lyase, the regulating enzyme of this cycle, catalyses the conversion of isocitrate to glyoxylate and succinate, which are administered to the tricarboxylic acid cycle for the conversion of fats to carbohydrates. Therefore, since the precursors of riboflavin, GTP and ribose-5-phosphate are formed from the metabolic route of carbohydrates, the glyoxylate cycle plays an important role when vegetable oils are used, these being the preferred substrates in the production of riboflavin, since they increase the production of this vitamin (Schmidt *et al.*, 1996a, 1996b). In this work it was possible to obtain large yields of mutant strains of *Ashbya gossypii,* which also have the advantage of being unsusceptible to inhibition by itaconate and capable of producing riboflavin from orange rind, an agroindustrial waste product readily available in the state of Yucatán, México.

MATERIALS AND METHODS

Microorganism and culture medium. *Ashbya gossypii* NRRL-Y 1056, obtained from the microbial collection at the CINVESTAV-México, was grown and maintained in solid YM medium with 2% agar in Petri dishes. The composition of the medium consisted of yeast extract 0.3%, malt extract 0.3%, gelatin peptone 0.5%, and glucose 1%; the initial

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pH of the medium was adjusted to 6.5 with 0.5 M H_2SO_4. In producing media for riboflavin, either orange rind or wheat bran at 1% w/v were used as the only source of carbon and nitrogen or added to YM medium using different combinations.

In other test, we substituted components of the growth medium: in medium A we substituted malt extract with orange rind in a concentration of 0.3%, in medium B glucose was substituted with orange rind in a concentration of 1% and in medium C both glucose and malt extract were substituted using 1.3% of orange rind.

Growth conditions. The experiments were carried out in 250 mL Erlenmeyer flasks with a working volume of 100 mL. Before inoculation, medium was autoclaved for 15 min at 121 ºC and 15 psi pressure. The medium was inoculated with vegetative mycelia or spore suspension. Flasks were incubated (30 ºC, 200 rpm) in a rotary shaker. Samples were withdrawn aseptically at regular time intervals, during 7 days of incubation and centrifuged at 14500 rpm. The concentration of riboflavin was determined in the supernatant.

Mutagenesis. Three aliquots of 5 mL each one were taken from a suspension of *Ashbya gossypii* cells grown in YM medium for 48 h. These aliquots were mixed with a solution of the mutagenic agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Three concentrations of the mutagenic agent were used: 100, 200 and 300 µg/mL. The mutagenic agent solution was prepared by weighing 0.01 g of MNNG and dissolving it in 10 mL of a phosphate buffer solution (0.1 M, pH 7). This solution was sterilised by passing it through a Millipore membrane (0.22 µm). Subsequently, the mutagenic agent was allowed to act onto each concentration by a period of 90 min, while 1 mL aliquots were taken every 30 min from each sample. One millilitre of phosphate buffer was then added and the cells were centrifuged for 10 min at 14000 rpm, changing the buffer solution 3 times to eliminate the mutagenic agent. The cells were resuspended in 2 mL of sterile medium which contained (g/l) glucose 10, peptone 5, yeast extract 3, and malt extract 3. Immediately, 1 mL was taken from each time of action of the mutagenic agent in order to make a series of dilutions, which were poured on Petri dishes containing solid YM medium, and incubated at 30 °C for 96 h.

Survival control was carried out on the *Ashbya gossypii* strain grown in the YM medium; after an incubation period of 48 h, a 1 mL sample was taken and dilutions were made which were subsequently poured in a solid YM medium. The number of non-mutated cells were counted obtaining the number of colony formed units (CFUs) initial (100% survival).

Isolation of high yield mutants and mutants unsusceptible to itaconate. The strains were first grown in liquid YM medium and later in solid medium. High yield strains were selected on the basis of the intensity and diffusion of the yellow colouring present in the medium, the yellow colouring being an indicator of the presence of riboflavin (Stahnmann *et al.*, 2000). The stability of the mutant strains was determined by cultivating them in YM medium, changing the medium every 15 days, and monitoring the riboflavin production. The method for selecting unsusceptible mutants to itaconate was as described by Schmidt *et al.* (1996a).

Enzymatic action of the isocitrate lyase. The reaction catalysed by the isocitrate lyase was carried out according to the method described by Dixon and Korhberg (1959) and modified by Schmidt *et al.* (1996b). One unit of enzyme activity was defined as the amount of enzyme required to produce 1 µmol glyoxylate phenylhydrazone min^{-1}. The protein content was determined using the Bradford method.

Determination of riboflavin. The concentration of riboflavin was determined by reading absorbance at 445 nm in a Spectronic 20 spectrophotometer (Bausch and Lomb, Sc. Co.). The data were referred to standard plot ($y = 0.0344x + 0.0011$; $R^2 = 0.995$) previously prepared using known riboflavin concentrations between 0-25 mg/L.

RESULTS

Obtainment of mutants of *Ashbya gossypii*

From exposition periods used; as well as from different doses of the mutagenic agent (MNNG); the following results were obtained: with a concentration of 100 µg/mL, no effect was observed on the wild strain which yielded 130000 CFU/mL, corresponding to 100% survival. With an increase in the concentration of 200 and 300 µg/mL and an increase in the exposition periods, the number of survivors diminished. A total of 3000 CFU/mL was obtained, corresponding to 2.3% of survival using a concentration of 300 µg/mL and an exposition period of 90 min (Fig. 1).

In figure 2 we can observe a change in the phenotype of the colonies, which could indicate a change in the genotype as a result of the treatment with MNNG.

Selection of high yield strains of *Ashbya gossypii*

The selection of high yield strains was realised from the suspension of mutants with 2.3% survival. The effects in the formation of riboflavin were easily observed in agarised YM medium (Fig. 2), since this vitamin is a yellow pigment produced by the colonies (Stahnmann *et al.*, 2000). Eleven strains capable of producing riboflavin in solid medium were selected and their capacity for producing riboflavin in a liquid medium was evaluated (Table 1). Strains of ASHLVII, ASHLX, ASHLXI and ASHLXV increased riboflavin production in 44, 88, 43, and 138% respectively in the YM medium.

Tests on stability in the production of riboflavin were also carried out. In figure 3 we observe that ASHLXI and ASHLXV strains did not undergo a reversion in the phenotype of riboflavin, while strains such as ASHLIII, ASHLVI and ASHLXX lost their capacity to produce riboflavin in YM medium.

Selection of mutants unsusceptible to itaconate and determination of isocitrate lyase

Of the 11 strains selected, ASHL0, ASHLIII and ASHLIV were not inhibited by the presence of itaconate in the medium. Itaconate is an efficient inhibitor of isocitrate lyase which was used as antimetobolite to screen mutants with enhanced isocitrate lyase activity. The enzymatic activity of isocitrate lyase of some strains (ASHL0, ASHLIII, ASHLIV, ASHLXI, ASHLXV and wild type) was measured and it could be noted that these strains, including wild strain, showed

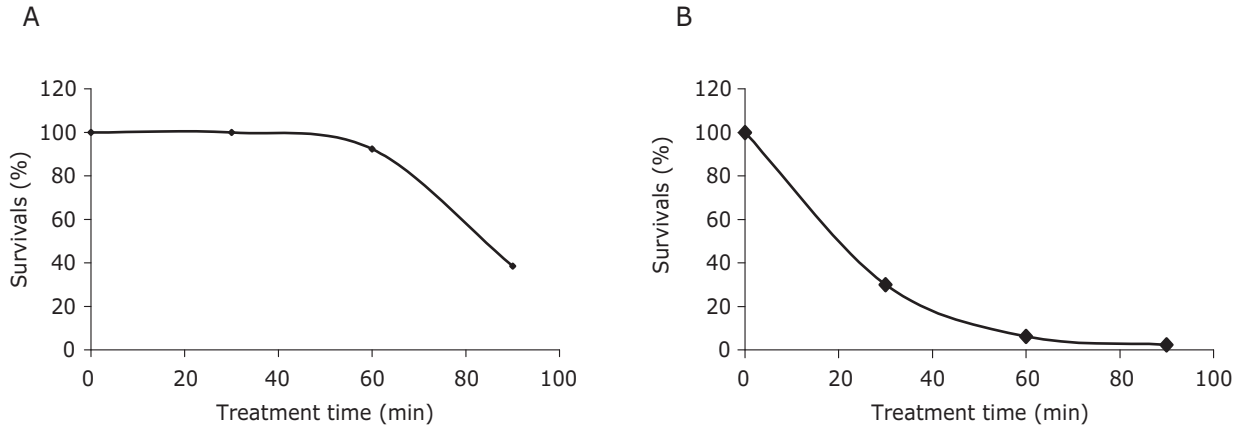

FIG. 1 - Curves of survival in the treatment of *Ashbya gossypii* with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). A: 200 µg/mL, B: 300 µg/mL.

similar values of 12 to 15 U of total activity and 0.05 to 0.07 (U/mg protein) of specific activity. The mutant-itaconate-resistant strains did not present isocitrate lyase activity greater than mutant itaconate non-resistant strains. Based on the results of riboflavin production in YM medium and the stability tests, experimental work continued with strains ASHL0, ASHLXI and ASHLXV.

Production of riboflavin with ASHL0, ASHLXI and ASHLXV strains, using orange rind as a substrate

Previous to using orange rind, wheat bran was employed as a substrate. However, the production of riboflavin was rather poor and the decision to use only orange rind was taken. The data obtained using orange rind as the only source of carbon and nitrogen are shown in Table 2, where we found that the wild strain produced the greater quantity of riboflavin. From results obtained using orange rind, we decided to conduct three tests using different media describe previously. In all three media both the peptone and the yeast extract were conserved as sources of nitrogen and the results are shown in Table 3 where we can observe that the riboflavin production was increased several times in the medium A with ASHLXV and ASHLXI strains. The riboflavin production did not increase when we used other media with any strain.

DISCUSSION

The action of the mutagenic agent was observed to be different for each strain. Excessive production or loss in the production of riboflavin could be related to changes in the enzymes that regulate this biosynthetic route. This mutagenic agent has been used in the production of riboflavin from *Arthrobacter* sp. (Yamane *et al.*, 1994), *Bacillus subtilis* AJ12643 (Usui *et al.*, 1994) and *Candida famata* (Heefner *et al.*, 1992).

Some mutants lost their capacity to produce riboflavin; these changes could be due to repair mechanisms which appear in the DNA during replication (de Laat *et al.*, 1999). The activity of the isocitrate lyase detected in the strains was similar even though some mutant strains were non-

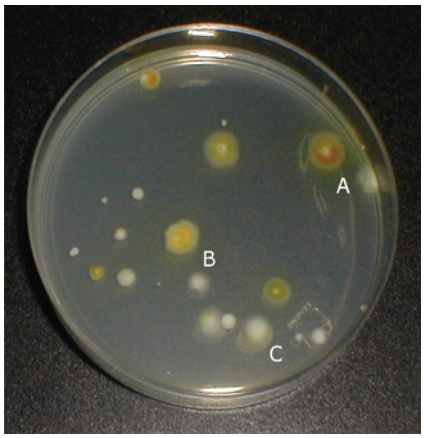

FIG. 2 - Strains of *Ashbya gossypii* mutated by treatment with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), grown in YM medium for 72 h. A and B: mutant strains with riboflavin overproduction, C: mutant strain which lost its riboflavin production ability.

TABLE 1 - Comparison of riboflavin production in the mutated strains of *Ashbya gossypii* in YM medium, the samples were taken after 7 days

Strains	Concentration of riboflavin (mg/L)*	Increase compared to the wild strain (%)
Wild	77.09 ± 3.9	–
ASHL0	41.31 ± 1.5	0
ASHLI	70.23 ± 2.4	0
ASHLII	58.62 ± 2.4	0
ASHLIII	27.40 ± 1.2	0
ASHLIV	23.49 ± 1.5	0
ASHLVI	8.27 ± 0.44	0
ASHLVII	111.75 ± 2.44	44.95
ASHLX	145.30 ± 3.67	88.46
ASHLXI	110.62 ± 2.5	43.49
ASHLXV	184.23 ± 4.72	138.9
ASHLXX	2.88 ± 0.61	0

* 3 replicates

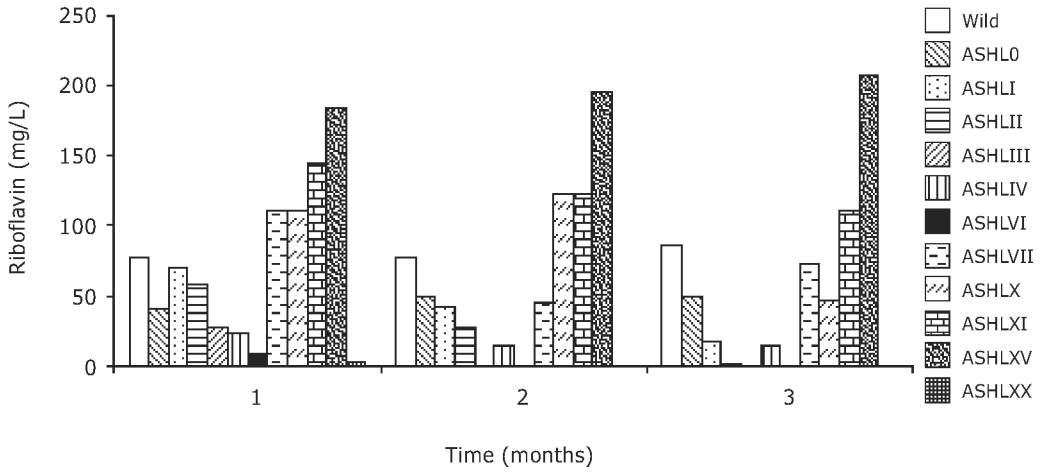

FIG. 3 - Stability in the production of riboflavin of the mutated strains, grown in YM medium.

TABLE 2 - Production of riboflavin using orange rind at 1% as the only source of carbon and nitrogen, initial medium pH 3.7

Strains	Concentration of riboflavin (mg/L)
Wild	20.70
ASHL0	2.60
ASHLXI	8.70
ASHLXV	5.00

TABLE 3 - Comparison in the production of riboflavin in three different fermentation media

Media	Strains	Concentration of riboflavin (mg/L)*
A	Wild	78.9 ± 3.1
	ASHL0	27.4 ± 1.28
	ASHLXI	223.3 ± 1.3
	ASHLXV	222.8 ± 3.8
B	Wild	73.1 ± 1.77
	ASHL0	7.1 ± 0.2
	ASHLXI	89.4 ± 0.2
	ASHLXV	66.8 ± 2.4
C	Wild	90.0 ± 1.2
	ASHL0	16.6 ± 0.2
	ASHLXI	39.6 ± 1.2
	ASHLXV	83.2 ± 1.9

Fermentation media: Growth medium components were substituted as follows: (A) orange rind 0.3%; instead of malt extract (pH 6); (B) orange rind 1%; instead of glucose (pH 5.6); (C) orange rind 1.3% instead of glucose and malt extract (pH 5.3). In all media, peptone and yeast extract were used. *3 replicates.

resistant to the presence of itaconate, a potent inhibitor of isocitrate lyase, this could be due to the fact that this enzyme is found in the glioxylate cycle which takes place in the glioxysomes (van der Klei and Veenhuis, 1997). Therefore an increase in the number of these organelles would increase the activity of this enzyme.

The fact that the mutants did not produce large quantities of riboflavin in orange rind medium, such as in the YM medium, might be due to the pH of the medium. Using orange rind as the only substrate, we have observed that pH diminishes to ranges between of 4.5-5.5, and it has also been noted that with a decrease in pH, *Ashbya gossypii* does not produce riboflavin. This inhibitory effect of the pH on the production of riboflavin was observed in mutants of *Arthrobacter* sp. (Yamane *et al.*, 1993) and in strains of *E. ashbyii* and *A. gossypii* (Kolonne *et al.*, 1995).

When we added orange rind to the culture medium used for producing riboflavin, we could observe a larger production of riboflavin with an increase of 184% more for the ASHLXI and ASHLXV strains, as compared with the wild strain. It has been reported that mutants of *E. ashbyii* in a medium of molasses achieved an increase of 35% yield (Venugopal and Chandra, 2000). This may be due to that fact this medium provides optimum for the production of riboflavin and that some components of malt extract could provoke some kind of inhibitory effect the production. It is important to mention that the substitution of orange rind instead glucose, or both glucose and malt extract, did not beneficially increase riboflavin production. On the other hand, orange rind provides growth factors such as thiamin and biotin which are necessary for the growth of the microorganism, as reported by Ózbas and Kutsal (1991) and essential oils which could increase production. These production levels are higher than those obtained from mutated strains of *Bacillus subtilis* AJ12643 with values of 120 mg/L (Usui *et al.*, 1994), they are also higher than those reported by Ozbas and Kutsal (Ózbas and Kutsal, 1991) in media employing whey as the only source of carbon and nitrogen, giving values of 190 mg/L, and by the same investigators, in media containing whey plus thiamin,

giving a concentration of 210 mg/l. More recently, Jimenez *et al.* (2005) reported an increase of 10 fold in a triple mutant strain of *Ashbya gossypii* obtaining 228 mg/L of riboflavin production, similar to the results obtained by own investigations.

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