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Riboflavin fermentation of molasses with agro-industrial nitrogenous by-products

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Abstract

Riboflavin production with *Eremothecium ashbyii* was investigated with molasses as the carbon source. Pretreatment of molasses by steam, chemical and active carbon methods improved yield, while ion-exchange and inversion were inhibitory. Of the low-grade nitrogen sources, lentil, oil seed cake and beef washings favoured flavinogenesis.

1. Introduction

In spite of the competition from a number of highly efficient chemical syntheses, a considerable quantity of pure riboflavin or riboflavin concentrates is produced by fermentation[1]. Nearly 20% of total production of riboflavin in U.S. is by microbial synthesis[2]. Most of this is consumed in the form of crude concentrate for animal feed supplements. Pure crystalline therapeutic grade is made by chemical syntheses starting from *o*-xylene, and at present only the chemical route is adopted in our country[3].

We report here our investigations on the production of riboflavin by fermentation with molasses as carbon source and various inorganic and organic indigenous and low-grade nitrogenous waste/by-product resources.

2. Methods

Organism

Eremothecium ashbyii and *Ashbya gossypii* were obtained from Central Bureau Voor Schimmelcultures, Baarn (Netherlands), Czechoslovakian collection of yeasts, and

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Northern Regional Research Centre, Illinois, U.S.A. The cultures were maintained on YMPG medium composed of yeast extract 3 g, malt extract 3 g, peptone 5 g, glucose 10 g, agar 15 g per litre water, pH 5·8.

Cultural conditions

Combinations of 3·0% w/v molasses with 1·5% malt extract (M_1); or malt extract plus 0·3% yeast extract (M_2); or 1·5% beef extract plus 1·5% peptone (M_3); or 0·3% yeast extract plus 1·5% beef extract plus 0·5% peptone (M_4); sterilized at 121° C for 20 min were tested for growth of *E. ashbyii*. The media and cultural conditions are indicated under appropriate tables. A loopful of 48–72 hr old cultures from YMPG slants was used as inoculum.

Molasses treatment

Molasses was procured from the Madurandakam Cooperative Sugar Factory, Tamil Nadu. After solid sediments from suitably diluted samples were removed, they were treated as follows to remove pigmentation and inhibitory factors.

(*a*) *Chemical treatment*[4,5] : To 250 ml molasses containing 37·5 g, 75 ml of 100 gpl neutral lead acetate and 175 ml water was added and sample filtered. To 200 ml of filtrate, 150 ml of sodium phosphate (70 g)—potassium oxalate (30 g) and 150 ml water were added to remove excess heavy metal ions. Samples were filtered and diluted for use.

(*b*) *Steam treatment*[6] : Diluted molasses (25% w/v) was steamed for 30–60 min and allowed to settle overnight for sedimentation.

(*c*) *Ion exchange*[7] : Quarternary amine type Amberlite IRA-400 anion exchange resin was used at 3 g/100 ml syrup and cation exchanger sulfonated coal type Zeocarb resin at 6·6 g/100 ml syrup.

(*d*) *Activated carbon treatment*[8,9] : Diluted molasses (25% w/v or 15% w/v) at 80° C (pH 5·0) was passed through 1 part/wt. activated carbon.

(*e*) *Inversion*[5]: After removal of solid sediments 40 ml molasses containing 3 g was inverted by ISI method[5] and used directly.

Sugar analyses of molasses

After inversion according to ISI method[5], total carbohydrate sugar was estimated by the anthrone method[10].

Riboflavin assay

Riboflavin was assayed fluorimetrically by the method of Radhakrishnamurthy and Sarma[11] in the culture filtrate after washing the cells. Fluorescence was measured with 365 nm excitation filter and 530 nm emission wavelength in a Carl Zeiss PMQ II spectrophotometer with fluorescence attachment. Values were compared with riboflavin (USP grade) standards of 1–10 μg/ml.

Dry weight

Culture samples (8 or 10 ml) were centrifuged, washed once with distilled water and dried at 80–100° C for 48 hr.

3. Results and discussion

The total sugar content in molasses was 64·2% by the anthrone method. Preliminary studies with various combinations of media constituents showed M_4 medium to be a good basic medium for further studies. Of the strains tested *E. ashbyii* (CBS 269·75) was the best choice for fermentation as with less biomass the production of riboflavin was maximum indicating high efficiency of conversion (Table I).

The riboflavin production on M_4 medium with 1·5, 3·0 and 7·5% w/v activated carbon treated molasses (50 ml medium in 500 ml flasks sterilized at 121° C), incubated under stationary conditions for 16 days was 33·0, 21·0 and 21·3 μg/ml respectively. The riboflavin yield on 1·5% molasses—M_4 medium sterilized at 110° C for 25 min was only 21·0 μg/ml indicating no improvement of yield. Riboflavin production is reported to be affected by sterilization temperatures[12–15].

Table I

Growth and yield of riboflavin by *E. ashbyii* and *A. gossypii* strains from different culture collections[a]

Organism	Final[b] pH	Dry wt. mg/ml	Riboflavin μg/ml
Eremothecium ashbyii CBS 269·75	7·5	2·11	33·0
E. ashbyii NRRL Y-1363	7·0	3·83	15·0
E. Ashbyii CCY 24-1-1	7·0	2·86	13·3
Ashbya gossypii NRRL Y-1056	7·2	3·93	9·0

[a] 50 ml of M_4 medium with 1·5% w/v carbon treated molasses in 500 ml Erlenmeyer flasks incubated for 16 days at room temperature under stationary conditions.

[b] Initial pH was 5·5 in all the flasks.

The time course of riboflavin production is shown in Fig. 1. Periodic pH correction did not result in increased yield. Aeration by incubation on shaker increases yield as seen in Fig. 1 compared with Table I.

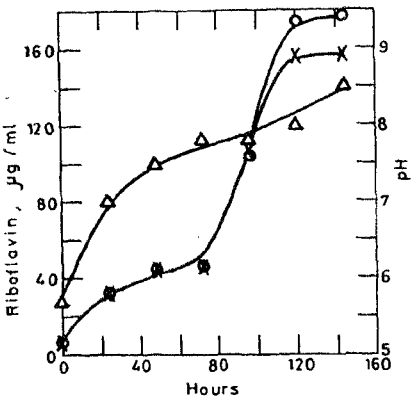

FIG. 1. Time course of riboflavin production by *E. ashbyii*[a].

[a] 30 ml of M_4 medium with 1·5% w/v carbon treated molasses in 150 ml flasks incubated on a shaker with 106 strokes/min.

⊙—⊙ A = Riboflavain in pH uncorrected medium.

× — × *B* = Riboflavin in medium where pH was corrected to 7·0 whenever it rose to 7·5 or above with sterile 1N HCl.

△—△ *C* = pH changes in uncorrected medium.

Effects of various pre-treatments of molasses on riboflavin production is shown in Table II. Pretreatment appears necessary for better yield. Active charcoal absorbs a large amount of pigments, some ions and organic toxic materials, whereas ion exchangers remove only charged ions. Inverted molasses gave low yield. A comparative study of the pretreatments of molasses for riboflavin production is not reported.

At the concentration of nitrogen tested urea and ammonium salts were inhibitory to flavogenesis (Table III), even in the presence of vitamins. The vitamins, thiamine, biotin and inositol are essential in the synthetic glucose medium[11]. Natural organic sources of nitrogen like sesame oil seed cake, ground lentils, beef washings and boiled beef extract enhanced yield. Higher concentrations of boiled extract tried, gave better yield of riboflavin.

Table II

Effect of various pretreatments of molasses on growth and riboflavin production of *E. Ashbyii*

Treatment[a]	Final pH[b]	Dry wt. mg/ml	Riboflavin μg/ml	
			Uninoculated control	Experimental
Untreated	8·5	3·10	5·00	68·50
Chemical	9·0	2·16	5·00	81·75
Steam	7·8	5·92	6·25	78·00
Anion exchanged	5·5	0·74	3·75	17·50
Anion + Cation exchanged	5·5	0·13	3·75	5·00
Activated carbon	9·0	5·09	4·37	88·50
Inverted	5·5	..	5·00	8·75

[a] The treatment procedures are indicated in Methods. 30 ml of 1·5 per cent w/v molasses M_4 medium in 150 ml flasks incubated for 108 hours on a shaker with 106 strokes/min.

[b] Initial pH in all cases was 5·5.

With complex carbon and nitrogenous materials different media have been tested for flavinogenesis[16] and are of industrial importance. The nutritional requirements of *E. ashbyii* for growth and riboflavin production has been studied with regard to carbon source[10,11,13] growth factors[10,11,17] nitrogen source[18,13,12,19,20] surface active agents, amino acids[21], lipids[16] and pH optimum[22] and yet several inconsistencies still remain in reported literature. Mathematical modelling of riboflavin fermentation is also reported[23]. Sanchez-Marroquin[25] used black strap molasses and obtained riboflavin concentrates using a mixture of *A. gossypii* and *E. ashbyii* of 20% protein in dry product and 2·8% riboflavin. The yield of riboflavin on various media are reported to range from 100 μg/ml to 2,480 μg/ml[1,11,16]. Variations in riboflavin yield even under similar conditions of experimentation are noted[11].

Our preliminary observations on riboflavin production with molasses and a few indigenous wastes[26,27] are encouraging. Further work on improvement of yield by strain improvement, media and cultural optimization is under progress.

Table III

Effect of various nitrogen sources on riboflavin production with *E. ashbyii*

Nitrogen[a] source	Riboflavin μg/ml	
	Uninoculated control	Experimental
No added nitrogen source	0	18·50
Ammonium sulphate	0	3·30
Ammonium sulphate + Vitamins[b]	2·5	3·75
Ammonium acetate	0	11·50
Urea	2·5	2·50
Sodium nitrate	0	18·50
Lentils[c]	1	75·40
Oil seed cake	3	58·00
Beef washings (45 per cent)	0	56·00
Boiled extract (90 per cent)	0	83·00
Boiled extract (45 per cent)	0	46·00

[a] Molasses concentration was 1·5 per cent w/v and was active carbon treated. All ammonia compounds, urea and sodium nitrate were added at nitrogen equivalence of 0·106 g nitrogen per 100 ml medium. Ground lentils (−200 mesh) and ground sesame oil seed cake (−60 mesh) were used at 4 per cent w/v as is basis. Urea and ammonium acetate alone were filter sterilized and added aseptically. 250 g beef was washed with 750 ml water and filtered washings used at 45 per cent w/v basis. Boiled extract was prepared by boiling 50 g beef in 120 ml water for 2 hours, filtered and used at 90 per cent w/v and 45 per cent v/v basis. Cultural conditions as in Table II.

[b] Vitamins *viz.* thiamine, biotin and inositol were 0·05, 0·6 and 5 mg/100 ml respectively.

[c] The protein content of dry lentil is reported to be 24 g/100 g[24].

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