Original Article

MICROBIAL CONVERSION OF WASTE COOKING OIL INTO RIBOFLAVIN BY *Ashbya gossypii*

CONVERSÃO MICROBIANA DE ÓLEO DE COZINHA RECOLHIDO EM RIBOFLAVINA POR *Ashbya gossypii*

Shiping Wei[1], Wenjie Zheng[2], Fang Zhao[3], Zhenglong Jiang[1], Dongsheng Zhou[1]

1. School of Marine Sciences, China University of Geosciences, Beijing, China. weishiping@cugb.edu.cn ;
2. Animal Plant and Food Stuffs Inspect Center of Tianjin Entry-Exit Inspection and Quarantine Bureau, Tianjing, China;
3. Longkou Agricultural Technology Promotion Center, Shandong Province, China.

ABSTRACT: The conversion of waste cooking oil into riboflavin by *Ashbya gossypii* was investigated in this paper. The effect of initial pH and the original volume of added waste cooking oil in the medium were evaluated to optimize the fermentation efficiency. The results show that when the initial pH was adjusted to 6.5 and 40 g/L waste cooking oil was added in the medium, no residual waste cooking oil was observed and the riboflavin yield reached 4.78 g/L. During the fermentation process, pH, biomass, free amino nitrogen and reduced sugar were dynamically monitored to evaluate the efficient utilization of waste cooking oil for riboflavin yield. The results show that when pH was kept in the range of 6.5-6.8 during the fermentation process, the levels of free amino nitrogen and reduced sugar could be used more efficiently and the riboflavin yield increased to 6.76 g/L .

KEYWORDS: Riboflavin. *Ashbya gossypii*. Waste cooking oil. Microbial conversion.

INTRODUCTION

Considerable quantities of waste cooking oils and animal fats are available worldwide, especially in the developed countries (CHHETRI et al. 2008). They are generated in large quantities during food or semi-product preparation by frying in an industrial environment, e.g. in fast-food networks, large restaurants, dining rooms and cafeterias, etc. When poured down drains, they not only coat and eventual occlude drainage and sewage pipes (TAKENO et al. 2005), but the oxidation of their fatty acids and subsequent transformations may also cause undesirable and malodorous byproducts. Disposal of the used cooking oil creates a significant challenge because of possible contamination of water and land resources. From both an economical and ecological standpoint, the development of feasible ways for reutilization of waste cooking oils and fats would be highly desirable (ADAMCZAK et al. 2009). Some of the used cooking oil is used for soap preparation and as additives in domestic animals feedstock etc. (DOMÍNGUEZ et al. 2010), but a major part of it is discharged into sewer drains, landfills and rivers causing environmental pollution. In the past several decades, an alternative method of utilizing the waste cooking oil for producing biodiesel is attracting enormous interest as it is renewable resource (THANH et al. 2010, CANKCI, 2007, MATH et al. 2010). In this research work, we reported the use of *Ashbya gossypii* for conversion of waste cooking oil into the riboflavin. *A. gossypii*, a filamentous fungus closely related to yeast (PRILLINGER et al. 1997), was originally isolated from cotton as a pathogen causing stigmatomycosis (ASHBY; NOWELL, 1926) and later was recognized as a natural overproducer of riboflavin (vitamin B_2) (WICKERHAM et al. 1946, DEMAIN et al. 1972). Currently, except for *A. gossypii* is still used for large-scale riboflavin production (STAHMANN et al. 2000, LIM et al. 2001), it is also an attractive model to study biology of fungal development (WENDLAND; WALTHER, 2005).

Riboflavin is an essential compound in living organisms, including microorganisms, plants and mammals. It serves as the precursor of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which are required as electron-accepting oxidoreductases (STAHMANN et al. 2001, SUGIMOTO et al. 2009). It is widely used in the food enrichment, pharmaceutical and feed supplement industries. Riboflavin can be produced by many microorganisms, including bacteria, yeasts and molds (DEMAIN, 1972, STAHMANN et al. 2000, LIM et al. 2001). *A. gossypii* is able to utilize various carbon sources for riboflavin production, such as glucose, sucrose, fructose, maltose, mannose, or glycerol. Although addition of corn oil or soybean oil can stimulate riboflavin overproduction by *A. gossypii* (DEMAIN, 1972), the possibility of using waste cooking oil has not been investigated. If it proves feasible, it will both ameliorate the environmental problems and develop

Received: 15/05/12
Accepted: 15/12/12

a new method for further exploiting the waste cooking oil.

MATERIAL AND METHODS

Microorganisms and media

An UV mutant of *A. gossypii*, ATCC 10895-32, was generated in our lab and used in this study (WEI et al. 2012). The preliminary seed medium consisted of (per liter) 20 g glucose, 10 g corn steep liquor (North China Pharmaceutical Co. Ltd., China) and 5 g peptone (pH 6.5), and the second seed medium consisted of (per liter) 6 g glucose, 10 g corn steep liquor, 5 g gelatin (Beijing Chemical Reagent Co., China) and 10 g soybean oil (Shandong Luhua Co. Ltd., Laiyang, China) (pH 6.5).

The riboflavin production medium for kinetic analyses of riboflavin concentration and metabolic parameters contained (per liter): 20 g corn steep liquor, 25 g osseocolla (Beijing Zhijiao Plant), 40 g waste cooking oil (The oil was collected in local cafeteria and was mainly composed of stearic acid 1.2%, oleic acid 52% and linoleic acid 4.9%), 2 g NaCl and 1 g KH_2PO_4 (pH 6.5). Components of the optimized medium for analyses of utilization of metabolic intermediates were same as that of the riboflavin production medium except for the pH control at a constant 6.5.

Culture conditions

Both of the preliminary and the seed cultures were incubated at 28 °C on a rotary shaker (HNY-200B, Honour Instrument Factory, Jiangsu, China) at 150 rpm for 36 h in 250 mL Erlenmeyer flasks containing 15 mL medium. Flask cultures for investigation of waste oil utility, riboflavin yield and metabolic parameters were carried out in 500 ml Erlenmeyer flasks containing 100 mL of riboflavin production or optimized media. The second seed media were inoculated 2% v/v of the preliminary seed cultures, and the flasks containing of the production and the optimized media were inoculated 1% v/v of the second seed cultures. All of the flask cultures were incubated at 28 °C on a rotary shaker at 200 rpm for 7-9 days.

For determination of both the optimal initial pH and the mass of original waste cooking oil added, the experiments were carried out in different treatments with initial pH 6.0, pH6.5, pH 7.0, pH 7.5 and combined with 30 g/L, 40 g/L, 50 g/L waste cooking oil added respectively (the other components of the media were same as that of riboflavin production media).

Analytical methods

The riboflavin concentration was measured according to the method described by Tanner *et al.* (1949) with modifications. Two milliliters of culture broth was drawn and mixed well with 1 mL of HCl buffer (pH 2.0). The mixture was treated at 100 °C for 30 min, and then centrifuged at 10000 g for 5 min. A 0.5 mL aliquot of the supernatant was removed and diluted to 100 mL with sterilized water. The absorbance of the diluted supernatant was monitored by a Photofluorometer 930 (Kexiao Co., Ltd., Hangzhou, China), with riboflavin concentration being calculated from the calibration curve.

The contents of the entire flask or 10 mL aliquots of culture broth were centrifuged at 10000 g for 5 min, and the supernatant was used to analyze free amino nitrogen and reduced sugar (SHI et al. 2006). The pellet mycelia was weighed to determine the biomass by the ratio of mycelia to the volume of the culture,

Residual oil concentration was measured by the solvent extraction method (PARK; MING, 2004). The final culture was transferred to a screw-capped 250 mL Falcon tube and mixed with an equal volume of n-hexane. The tube was vigorously shaken for 5 min and then centrifuged at 5000 g for 10 min. The upper layer was removed and dried with a vacuum evaporator, and then weighed.

RESULTS AND DISCUSSION

Optimal initial pH and added waste cooking oil

To determine the optimal initial pH and original volume of added waste cooking oil on the growth of *A. gossypii* ATCC10895-32, different treatments were performed as shown in Table 1. The riboflavin yields were higher and much greater quantities of waste cooking oil were consumed when the initial pH was adjusted to 6.0-6.5. The riboflavin concentration reached 4.78 g/L when the initial pH was adjusted to 6.5 and 40 g/L original waste cooking oil was used. When the initial pH was adjusted to 7.0-7.5, the riboflavin yields dropped significantly and less waste cooking oil was consumed, especially when 50 g/L waste cooking oil was added in the original medium. We inferred that more oil being added to the medium in the beginning may have affected the oxygen uptake by *A. gossypii* and result in less biomass. In all the treatments, the final pH increased above 8.09, which was a signal of cessation of riboflavin synthesis (TANNER et al. 1949, ÖZBAS; KUTSAL, 1991). In order to clarify of how the pH levels and original volume oil affected the final pH and biomass and

contributed to the riboflavin yield, we chose the optimal initial pH at 6.5 and the original waste cooking oil concentration as 40 g/L to carry out a dynamic analysis of the fermentation process.

Table 1. Effect of initial pH and original added oil on fermentation

Treatments	Initial pH	Original Oil	Final pH	Final Biomass	Residual Oil	Riboflavin Concentration
		g/L		g/L	g/L	g/L
1	6.0	30	8.21±0.23	13.24±0.12	no	4.25±0.23
2	6.0	40	8.15±0.15	12.38±0.21	no	4.46±0.16
3	6.0	50	8.09±0.08	13.45±0.30	7.34±0.24	4.11±0.18
4	6.5	30	8.17±0.12	13.32±0.13	no	4.34±0.09
5	6.5	40	8.02±0.13	13.46±0.07	no	4.78±0.15
6	6.5	50	8.19±0.21	13.01±0.23	7.52±0.14	4.13±0.21
7	7.0	30	8.34±0.32	12.68±0.32	no	2.45±0.13
8	7.0	40	8.31±0.26	12.78±0.18	3.32±0.21	2.87±0.16
9	7.0	50	8.18±0.18	11.87±0.26	13.4±0.23	2.36±0.08
10	7.5	30	8.37±0.29	10.24±0.06	1.26±0.16	1.88±0.10
11	7.5	40	8.34±0.18	11.12±0.16	6.28±0.19	2.17±0.12
12	7.5	50	8.21±0.16	11.08±0.27	18.2±0.25	2.02±0.07

Notes: Each treatment was independently performed with three replicates and the incubation time was 7 days.

Kinetics of *A. gossypii* ATCC 10895-32 growing on waste cooking oil

The parameters, pH, biomass, free amino nitrogen and reduced sugar, were monitored daily during the fermentation process. The pH quickly dropped from the initial pH of 6.50 to 5.25 on the first day after inoculation. Relatively less riboflavin was synthesized during this period. After the first day, the pH was gradually increasing up with vigorous growth of *A. gossypii* and the riboflavin yield increased. When the pH was increasing in the range of pH 6.50 to 6.92, *A. gossypii* was in the stationary phase and riboflavin was oversynthesized (DEMAIN, 1972). When the pH increased from 6.92 to 8.19, the biomass of *A. gossypii* decreased and riboflavin synthesis tended to cease (Figure 1A and 1B).

Tanner et al. (1949) and Özbas et al. (1991) reported that the pH was as low as 4.5 in the first 24 to 36 hours, and that the best riboflavin yields were obtained when the pH was in the range of 6.0 to 7.0 when using sunflower oil as the carbon source. With the continuous consumption of nutrients in the medium, the pH can reach levels as high as 8.5, and subsequent riboflavin synthesis is negligible or absent.

The metabolic intermediates, free amino nitrogen and reduced sugar were also assayed to evaluate the efficient utilization of them during the fermentation process, as both of them were essential compounds for promoting the growth of riboflavin-synthesizing *A. gossypii*. They are generated in the pathway of riboflavin synthesis through of triglyceride (a constituent of oil) oxidized. It was first broken down by the enzymes released by *A. gossypii* and converted into fatty acid. The fatty acid was then oxidized through β-oxidation, the TCA cycle or the Glyoxylate cycle to synthesize riboflavin (STAHMANN et al. 2000, LIM et al. 2001, PARK et al. 2007).

The results of free amino nitrogen and reduced sugar were shown in Figure 1B. The free amino nitrogen dramatically decreased in the first two days presumably because that *A. gossypii* quickly used the free amino nitrogen to synthesize its own structure proteins given its sustained growth of *A. gossypii*. Subsequently, the released free amino nitrogen levels rose rapidly along with the

reduced sugar levels. Theoretically, the increased free amino nitrogen and reduced sugar can be used further for a higher riboflavin yield, but the alkaline pH causing mycelia lyses limited the further utilization of them and riboflavin synthesis was terminated.

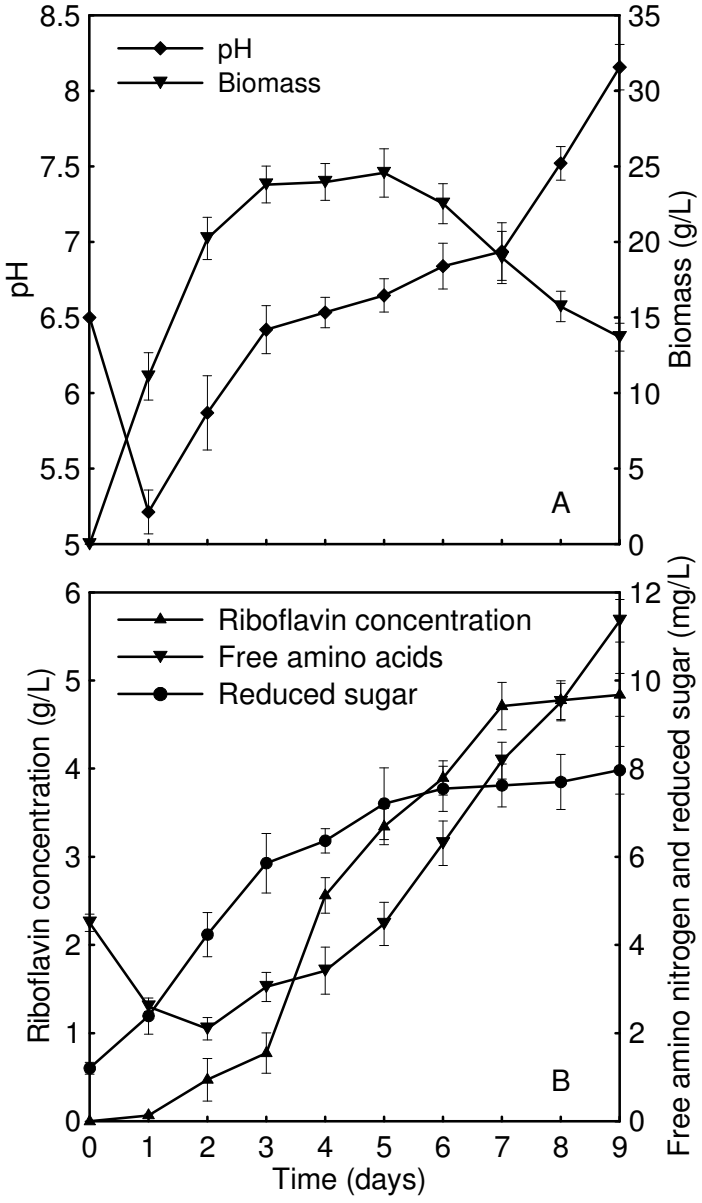

Figure 1. Kinetic analyses of riboflavin concentration and metabolic parameters by *A. gossypii* in the batch culture.

Based on the analyses of the kinetics of *A. gossypii* 10895-32 growing in the batch culture, we inferred that keeping the pH in the range of 6.5-6.8 was very critical for its better growth, more efficient conversion of the oil by depleting of the metabolic intermediates, leading to a further improvement of riboflavin yield. In a new batch culture, when the pH rose over 6.5 on the fifth day after inoculation, an autoclaved solution of KH_2PO_4 was added to maintain the pH at 6.5. Figure 2 shows that *A. gossypii* kept vigorous growth after the fifth day and the stationary phase was prolonged to sustain riboflavin oversynthesized. Levels of both of free amino nitrogen and reduced sugar dropped dramatically which indicated the metabolic intermediates were used more efficient and the riboflavin concentration was improved to 6.76 g/L.

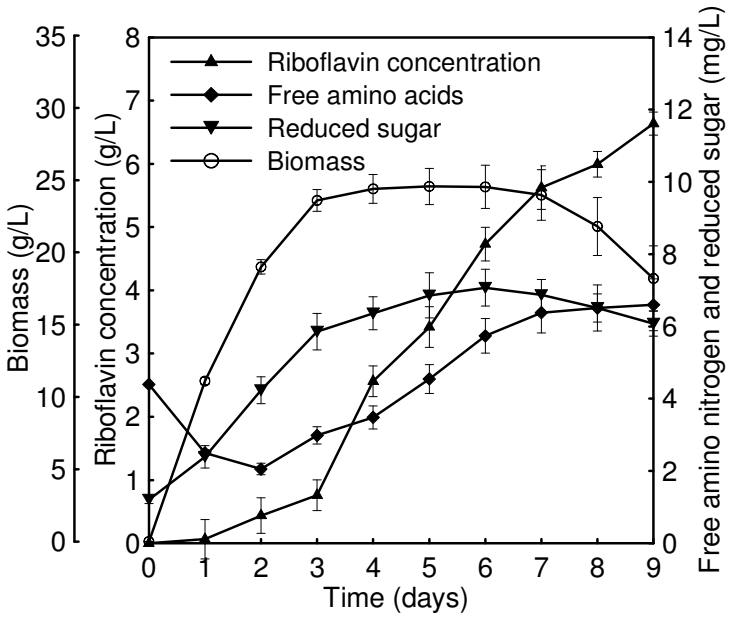

Figure 2. Riboflavin concentration increased by depletion of more free amino nitrogen and reduced sugar. When pH of the culture increased to over 6.5, a solution of KH_2PO_4 was added to maintain a constant pH 6.5.

CONCLUSIONS

Waste cooking oil can be converted into riboflavin by *A. gossypii.*

Higher riboflavin yields can be obtained by setting the initial pH to 6.5 and the volume of original added oil at 40 g/L in the batch culture.

Maintaining the pH in the range of 6.5-6.8 can sustain vigorous growth of *A. gossypii* and convert the waste cooking oil efficiently to achieve the maximal riboflavin concentration at 6.76 g/L.

ACKNOWLEDGEMENTS

The authors acknowledge James Hurley of Department of Plant Pathology and Microbiology, Texas A & M University, for making a critical reading and revision of this paper. This research was supported by "the Fundamental Research Funds for the Central Universities (2-9-2012-138)".

RESUMO: A conversão microbiana de óleo de cozinha recolhido em riboflavina por *Ashbya gossypii* foi investigada nesse estudo. O efeito inicial do pH e o volume original de óleo de cozinha recolhido foram avaliados para otimizar a eficiência de fermentação. Os resultados mostraram que quando o pH inicial foi ajustado para 6.5 e 0g/l de óleo de cozinha adicionado ao meio, nenhum óleo residual foi observado e a riboflavina pura atingiu 4.78g/L. Durante o processo de fermentação, pH, biomassa, amino nitrogênio livre e açúcar reduzido foram monitorados dinamicamente para avaliar a utilização eficiente do óleo de cozinha recolhido por riboflavina. Os resultados mostram que quando o pH é mantido numa amplitude de 6.5-6-8 durante o processo de fermentação, os níveis de amino nitrogênio livres e açúcar reduzido podem ser usados mais eficientemente e a riboflavina pura chega a 6.76 g/L.

PALAVRAS-CHAVE: Riboflavina. *Ashbya gossypii*. Desperdício de óleo de cozinha. Conversão microbiana

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