Improvement of Red Pigment Production during Biomembrane Surface Cultivation of *Penicillium novae-zelandiae* by Supplementing with Corn Straw

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Corn straw was used to improve the productivity of a red pigment during the biomembrane surface liquid cultivation of *Penicillium novae-zelandiae*. Both the dosage and particle size of corn straw powder had a significant effect on the fermentation period and pigment yield. After the optimization, the maximum yield of synthesised red pigment reached 0.43 g/L per day on day 9, which was 2.3 times higher than the initial productivity obtained by biomembrane surface cultivation without corn straw. An analysis on the mechanism suggested that corn straw shortened the fermentation period by providing the support for the growth of *P. novae-zelandiae* spores and biomembrane formation. Amino acids, including phenylalanine and tyrosine, released by corn straw, were the key reason for the improvement in the pigment yield. In addition, the increase of reducing sugars in the fermentation broth, due to the hydrolysis of cellulose and hemicellulose by the hydrolytic enzymes secreted by *P. novae-zelandiae,* provided a carbon source for fungal growth that might also be beneficial to pigment production.

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INTRODUCTION

Pigments, including synthetic pigments and natural pigments, are widely used in food, brewing, cosmetics, and other industries. In the past, synthetic pigments were extensively used because of their bright color, high stability, and low cost. However, most synthetic pigments cause harm to human health to varying degrees, and almost none can provide nutrients to the human body (Downham and Collins 2000). Therefore, synthetic pigments have been prohibited or strictly limited in many countries. For example, in the United States, the number of synthetic pigments allowed for food applications in 1960 was 37, but only seven are allowed at present. Natural pigments hold an advantage over synthetic pigments in terms of safety. Some natural pigments, including carotenoid and betanin, are nutritious and contribute human health. With continuous improvements in quality of life, the demand for natural pigments is growing rapidly; in Japan, China, and the United States, the numbers of natural pigments allowed for use total 97, 48, and 30, respectively (Mapari *et al.* 2005, 2010).

The color red represents romance and passion. Red pigment is widely used in food-contact industries globally. The demand for natural red pigment is increasing. In 1998, the

market demand of natural red pigment in China reached 15,000 tons, whereas the annual growth rate has remained at a level of more than 10%. Natural red pigment can be produced through two methods (Schmimidt-Dannert 2000; Mortensen 2006; Zhang *et al.* 2006): One is extraction from plant and animal materials, such as skins, seeds, roots, and insects; this method is characterized by high production cost and serious environmental pollution. Microbial fermentation is another method of pigment production, and it prevails due to its advantages, including high productivity, no season limit, and easily industrialized production.

Monascus pigment produced by *Monascus purpureus* or other *Aspergillus* strains has been studied extensively. This pigment, with both nutritional and medicinal value, can be applied for the coloration of meat, cheese, and beer, besides its use as dyes for the textile, cosmetic, and pharmaceutical industries. In addition, it also presents antimicrobial activity against pathogenic microorganisms and various beneficial effects to human health by acting as an antioxidant and exhibiting anti-cholesterol activities (Vendruscolo *et al.* 2016). Many bacteria, microalgae, and yeast can produce a variety of red pigments (Arad and Yaron 1992; Cai and Corke 2000; Frengova *et al*. 2004; Zhang *et al*. 2006).

The low productivity of most bacteria and microalgae restricts commercialization of their pigments. The yeast *Rhodotorula* also produces red pigments, including carotenoids. However, carotenoids from yeast are unsuitable for industrial production due to their high extraction cost. Therefore, screening of new strains and novel pigments has become a hot spot in this field, whereas *Penicillium* genus has caught the attention of many researchers. *Penicillium* strains, including *P. persicinum* and *P. oxalicum*, produce various types of red pigments (Ogihara *et al.* 2001; Mapari *et al.* 2009; Valéria *et al.* 2013). Han (1998) has reported that *P*. sp. PT95 produces carotenoid by solid fermentation of corn meal. Mapari *et al.* (2008) screened red pigments produced by *Penicillium* strains using computer technology.

The authors' previous work reported observing a potential red pigment during co-culture of *Penicillium novae-zelandiae* HSD07B and *Candida tropicalis*, whereas toxicity trials showed that co-culture pigments are potentially acceptable for food applications (Wang *et al.* 2011). However, at the industrial scale, co-culture system will not be feasible, considering that *C. tropicalis* is a conditioned pathogen. Thus, a pure cultivation, *i.e.*, biomembrane surface cultivation, was developed to produce the red pigment (Wang *et al.* 2012a, 2013). Unfortunately, this pure cultivation is limited by a long fermentation period, which significantly reduces pigment productivity. Thus, in this study, a low-cost agricultural waste, corn straw (CS), was used to improve production of red pigment. The objectives of this work include the following: to (i) investigate the feasibility of shortening fermentation period by supplementing with CS and to (ii) analyze the mechanism underlying increasing pigment productivity caused by addition of CS.

EXPERIMENTAL

Materials

P. novae-zelandiae HSD07B (CCTCCM2012198)) was obtained from the Henan Province Engineering Laboratory for Bioconversion Technology of Functional Microbes, Henan Normal University, Xinxiang, China. All chemicals used were of spectral or analytical grade unless otherwise stated (Henan Pujin Biotechnology Co., Ltd., Zhengzhou,

China). Xylan, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonate) (ABTS), veratryl alcohol, and carboxymethyl cellulose were purchased from Sigma Corporation (St. Louis, MO, USA). Corn stalk (CS) used in this work was obtained from Sijiqing farmland in Xinxiang, Henan Province, China, and its composition is shown in Table 1. Prior to use, CS was dried under the sun and then shattered using a fodder grinder to a yellowish-brown powder approximately 100 mesh in size. Soluble substance and protein in CS were analyzed in accordance to the APHA standard method (APHA 1998). Cellulose, hemicelluloses, and lignin were determined according to traditional wet chemistry method (Lan *et al*. 2011).

Table 1. Composition of CS Used in this Work

Composition	Cellulose	Hemicellulose	Lignin	SS	Protein	Others
Content (%, dry matter)	38.2	23.5	12.7	15.1	2.8	8.7
SS: Soluble substance, includes soluble saccharides and starch						

Shake cultivation and biomembrane surface cultivation

Strain HSD07B was grown on potato dextrose agar for 3 days at 28 °C before harvesting spores using a camel hairbrush, and spore suspension was prepared in sterile water. Shake cultivation was performed according to the following procedure: four 500 mL Erlenmeyer flasks, each containing 200 mL of modified Czapek Dox liquid medium composed of 10.0 g/L of glucose, 3.0 g/L of $NaNO_3$, 1.0 g/L of K_2HPO_4, 0.5 g/L of $MgSO_4$, 0.5 g/L of KCl, and 0.01 g/L of $FeSO_4$ (Henan Pujin Biotechnology Co., Ltd., Zhengzhou, China), were inoculated with a 0.5 mL aliquot of spore suspension (3.7×10^7 spores/mL), and flasks were incubated in a thermostat shaker at 120 rpm and 28 °C. The procedure for biomembrane surface cultivation was the same as that of shake cultivation except that the rotary speed of thermostat shaker was at 50 rpm. Optical density (OD) value of red pigment solution was determined at maximum absorption wavelength of pigment solution (500 nm), and concentration of red pigment was calculated according to the regression Eq. 1,

$$Y = 1.4491X + 0.0130 \qquad (R^2 = 0.9998), \qquad (1)$$

where Y represents concentration of red pigment (g/L), and X is OD value of pigment solution at 500 nm.

Methods
Effect of CS dosage on pigment production

Fifteen flasks were used in this test. During biomembrane surface cultivation of strain HSD07B, twelve 500 mL flasks (containing 200 mL of modified Czapek Dox liquid medium) were divided into four groups and were seeded with 100-mesh CS powder at different doses (1.0, 3.0, 5.0, and 7.0 g/L), respectively. Additional three flasks were used as control, and no CS was seeded. Cultivation was conducted in a thermostat incubator at 28 °C (Donglian Electronic Technology Co., Ltd., Haerbin, China).

Effect of particle size of CS powder on pigment production

CS was powdered at different particle sizes (approximately 50, 100, 150, and 200 mesh). Portions (5.0 g/L) were seeded into three 500 mL flasks each containing 200 mL of modified Czapek Dox liquid medium. Biomembrane surface cultivation was conducted in

a thermostat incubator at 28 °C. During cultivation, fermentation period, biomembrane thickness, and red pigment production were determined. The test was then repeated thrice.

Enzymes in fermentation broth

Enzyme activity was determined by a spectrophotometer (Aucy Technology Instrument Co., Ltd., Shanghai, China). Lignin peroxidase (LiP) activity was measured as described by Roy and Archibald (1993), with 1 U defined as 1 µmol of veratryl alcohol oxidized to veratraldehyde per minute. Laccase (Lac) activity was determined as described by Bourbonnais and Paice (1990), with 1 U defined as 1 µmol of ABTS oxidized per minute. Manganese peroxidase (MnP) activity was measured as described by Martínez *et al.* (1996), with 1 U defined as 1 µmol of Mn^{2+} oxidized to Mn^{3+} per minute. Carboxymethyl cellulase was measured according to a previously described method (Kim *et al.* 2008), with 1 U defined as the formation of 1 µmol of glucose per hour. Xylanase was measured using xylan as substrate (Karaoglan *et al.* 2014), and 1 U of activity was defined as the amount of enzyme releasing 1 µmol of reducing sugar equivalent per minute. Reducing sugars were measured by 3,5-dinitrosalicylic acid colorimetric method.

Effect of amino acids on pigment production

Amino acid composition in CS was determined by using an amino acid autoanalyzer (L-8900, Hitachi Ltd., Tokyo, Japan). After identifying the composition, contents were calculated, and amino acids with different dosages were added to the modified Czapek Dox liquid medium to investigate their influence on pigment production. Dosages of amino acids were calculated according to Eq. 2,

$$Y = 5.0 \times \underline{X}, \tag{2}$$

where *Y* refers to dosages of amino acid (g/L), 5.0 is CS dosage in the modified Czapek Dox liquid medium (g/L), and *X* corresponds to content of amino acid in CS.

Component analysis of red pigment

Components of red pigment obtained by biomembrane surface cultivation with and without CS were analyzed by using a silica gel thin-layer chromatograph (TLC) (Anhui Liangchen Silicon Material Co. Ltd., Huoshan, China). Treatment of red pigment before analysis was as follows: fermentation broth was filtered using a filter paper (Grade 1:11 µm, Whatman, GE Healthcare Life Sciences, Buckinghamshire, UK). Cell-free filtrate was mixed with ethanol (filtrate: ethanol = 1:1.5), and the mixture was subjected to centrifugation at 2600 g for 10 min. The supernatant was dried in a rotary evaporator at 50 °C, and crude pigment was mixed with 100 mL petroleum to remove hydrophobic substances. The remaining red pigment was dissolved in distilled water and analyzed by TLC using 1-butanol:ethanol:water (3:5:2) as mobile phase.

Statistical analysis

Statistical analysis of data was conducted using the software SPSS for Windows (Version 11.5, IBM Co., New York, USA), and significant difference in data was determined using the least significant difference test at the level of $p = 0.05$. A correlation analysis was also conducted using SPSS to investigate effects of different CS dosages on pigment production

RESULTS AND DISCUSSION

Comparison of Shake and Biomembrane Surface Cultivation

Both shake cultivation and biomembrane surface cultivation were used to produce the red pigment of *P. novae-zelandiae*. Pigment yield of shake cultivation was extremely low, indicating its unsuitability for the pigment production (Fig.1a-left). During biomembrane surface cultivation (Fig. 1a-right), the pigment concentration reached 2.4 g/L on day 12 (Fig. 1b), and this value was significantly higher than that of shake cultivation. However, biomembrane surface cultivation also presented limitations. The biomembrane was necessary for pigment production (Wang *et al*. 2012a), and approximately 7 days were required for its formation, leading to a 12-day fermentation period and low productivity of 0.2 g/L. day.
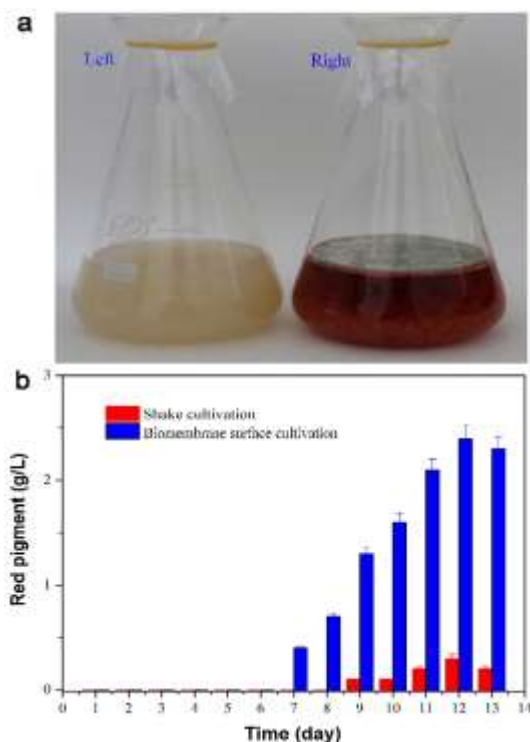

Fig. 1. Shake cultivation and biomembrane surface cultivation for production of red pigment: a. color comparison (Left, shake cultivation; Right, biomembrane surface cultivation); b. variation in concentration of red pigment with time

Effect of CS Dosage on Pigment Production

Although correlations were non-significant, CS dosage appeared to influence fermentation period and biomembrane thickness. When CS dosage reached 3.0 g/L, fermentation period was shortened to 9 days, and biomembrane thickness increased to 0.80 cm (Table 2). Using SPSS, the correlation between CS dosage and pigment yield was analyzed. Results suggest that CS dosage featured a significantly positive correlation with pigment concentration and productivity. A two-tailed test indicated that correlation coefficients reached 0.947 and 0.940 and were significant at the 0.05 level (Table 3). The 5.0 g/L dosage was selected as ideal additive dosage for pigment fermentation in consideration of both fermentation period and pigment productivity.

**Table 2.** Effect of Different CS Dosages on Pigment Production

Dosage (g/L)	Fermentation Period (day)	Biomembrane Thickness (cm)	Red Pigment (g/L)	Productivity (g/L.day)
0	12	0.50 ± 0.04	2.3 ± 0.11	0.19
1.0	11	0.61 ± 0.06	2.5 ± 0.26	0.25
3.0	9	0.80 ± 0.07	3.2 ± 0.21	0.35
5.0	9	0.78 ± 0.06	3.8 ± 0.34	0.42
7.0	9	0.79 ± 0.10	3.7 ± 0.42	0.41

Table 3. Correlations between CS Dosage and Fermentation Period, Biomembrane Thickness, and Productivity

Kendall's Tau-b		Fermentation Period (day)	Biomembrane Thickness (cm)	Red Pigment (g/L)	Productivity (g/L.day)
Dosage (g/L)	Correlation Coefficient	–0.864	0.845	0.947*	0.940*
	Sig. (two-tailed)	0.059	0.071	0.014	0.018
	N	5	5	5	5
Note: *Correlation is significant at the 0.05 level (two-tailed).					

Productivity is an important factor influencing production cost of microbial products (Wang *et al.* 2012b). In this test, the authors observed that many insolubles from the CS powder added to liquid medium constantly floated on liquid surface. These floating materials provided the support for inoculated *P. novae-zelandiae* spores. This phenomenon led to high spore growth above the liquid surface and easy formation of biomembrane. These insolubles also act as substrate and aid growth of *Penicillium* strains (El-Gendy 2010). This finding can be used to explain the significant correlation of pigment productivity with CS dosages.
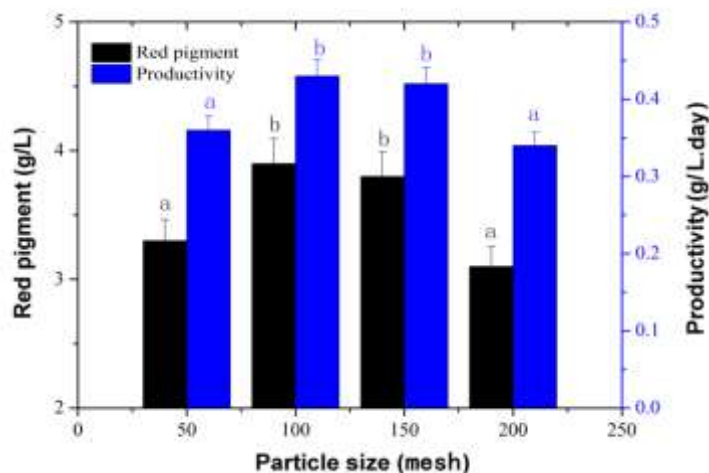
Fig. 2. Effect of particle size of CS powder on pigment production; mean values ± standard deviation (SD) are given (n = 4), and different letters on top of data bars indicate significant differences between mean values (p < 0.05)

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Effect of Particle Size of CS Powder on Pigment Production

Particle size of CS powder also significantly influenced pigment production. When powders with different particle sizes were added to fermentation medium, both 100- and 150-mesh CS powders aided pigment production, and on day 9, productivities of red pigment reached 0.43 and 0.42 g/L.day, respectively. Therefore, particle size of CS powder is crucial for red pigment production, and 100- to 150-mesh particle sizes suit pigment fermentation. The influence of particle size of CS powder on pigment fermentation remains unclear. However, this phenomenon may also be closely related with floatability and degradation of CS powder.

Enzyme Hydrolysis during Pigment Fermentation

As an agricultural waste, the main components of CS are lignin, cellulose, and hemicellulose (Lv and Wu 2012). Thus, improvement of pigment productivity may be attributed to (i) appearance of CS in fermentation broth as it quickens formation of biomembrane and reduces fermentation period and (ii) nutrients from CS that can stimulate or induce production of red pigment. To validate the latter, enzymes, including ligninolytic enzymes (Lac, MnP, and LiP) and hydrolytic enzymes (cellulase and xylanase), were tested (Fig. 3a). Results showed that in this work, *P. novae-zelandiae* used was not a MnP, LiP, or Lac producer, though other *Penicillium* strains have been reported to be capable of degrading lignin using these enzymes (Rodríguez *et al*. 1996; Zeng *et al*. 2006). However, *P. novae-zelandiae* can secrete both carboxymethyl cellulase and xylanase.
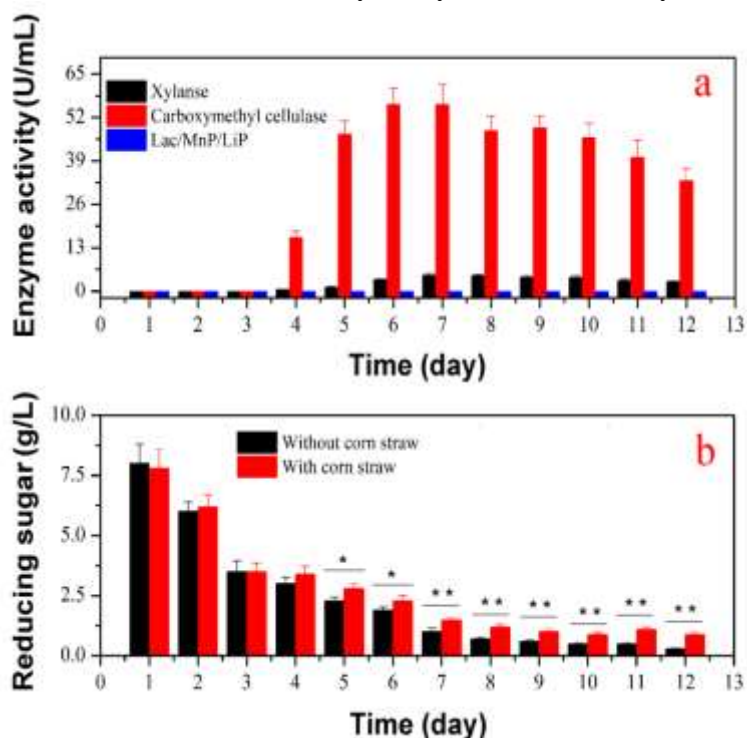

Fig. 3. Variations in enzyme activities and reducing sugars with time during biomembrane surface cultivation with and without CS: a. enzyme activities of xylanase, carboxymethyl cellulase, Lac, MnP, and LiP in fermentation broth obtained by biomembrane surface cultivation with 150-mesh CS; b. reducing sugars in fermentation broth obtained by biomembrane surface cultivation with and without 150-mesh CS. Mean values ± SD are given, and * represents significant level at 0.05.

Figure 3b shows that addition of CS increased concentration of reducing sugars in fermentation broth during biomembrane surface cultivation. In co-culture systems, production of *Penicillium* pigment requires a glucose starvation condition (Wang *et al.* 2013), whereas increasing reducing sugars in fermentation broth does not benefit red pigment production. During biomembrane surface cultivation, glucose starvation occurred in the biomembrane, and pigment production was not sensitively affected by concentration of reducing sugars in fermentation broth. CS contains cellulose and hemicellulose, and both can be hydrolyzed to reducing sugars, including glucose and xylan. Theoretically, these reducing sugars from CS hydrolysis can improve pigment production by providing a carbon source for pigment synthesis (Araújo *et al*. 2017).

Effects of Amino Acids on Pigment Production

After measurement, CS contained 2.2% of total amino acids. Seventeen amino acids were detected in CS (Table 4). Among these amino acids, 5.5% to 16.6% comprised Glu, Asp, Arg, Pro, Val, Gly, and Ala. However, none of these main amino acids can improve pigment production during biomembrane surface cultivation, whereas both Arg and Gly resulted in a decrease in pigment yield compared with that of control (Fig. 4). Interestingly, microscale amino acids, including Tyr and Pro, significantly improved pigment production ($p < 0.05$). The authors speculated that these amino acids may be precursors of red pigments secreted by *P. novae-zelandiae* because the two amino acids are involved in synthetic pathways of several red pigments, including betalains (Stintzing and Carle 2004; Jain and Gould 2015).

Components in Red Pigment

Alteration of cultivation methods and medium components generally results in changes in microbial metabolites. In the report of Wang *et al.* (2012a), components of red pigments obtained by monoculture and co-culture differed. Thus, red pigments produced by biomembrane surface cultivation with and without CS were analyzed by TLC. Figure 5 shows that addition of CS did not change components of the red pigment, and red pigments from biomembrane surface cultivation without CS mainly consisted of four components, which were the same as the red pigment from cultivation supplemented with CS. Therefore, the results indicate that addition of CS did not induce production of new pigments nor change characteristics and safety of red pigments.

Table 4. Contents of Amino Acids in CS

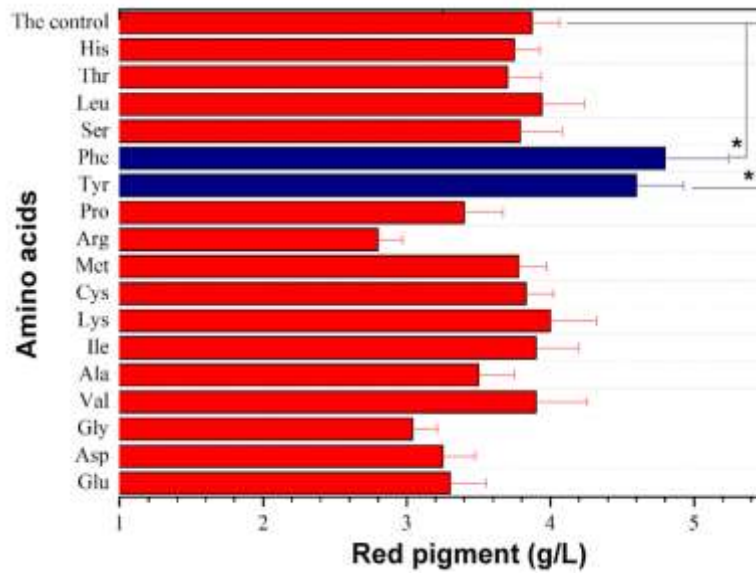| Amino Acid	Content (%)	Amino Acid	Content (%)
Glu	15.3	Arg	16.6
Asp	10.2	Pro	12.5
Gly	6.1	Tyr	3.3
Val	7.3	Phe	4.6
Ala	5.5	Ser	3.7
Ile	2.4	Leu	3.6
Lys	2.2	Thr	3.0
Cys	1.2	His	2.1
Met	0.5		

Fig. 4. Effects of different amino acids on production of red pigment; mean values ± SD are given as n = 4); * represents significance at 0.05 level compared with that of control.
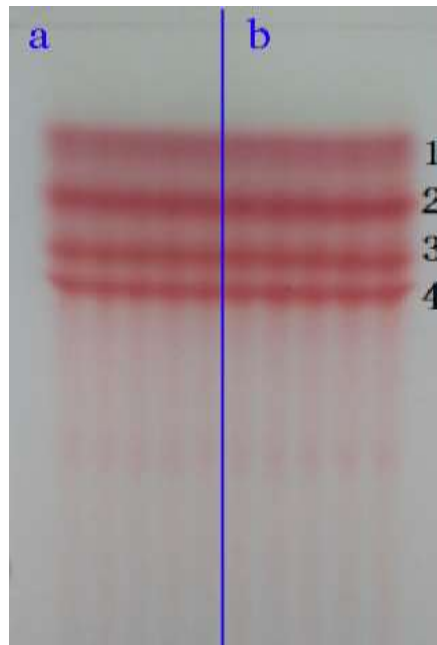

Fig. 5. TLC fingerprint in red pigments from biomembrane surface cultivation with and without CS. a. with CS; b. without CS.

CONCLUSIONS

1. Corn straw (CS) powder was used to improve productivity of *P. novae-zelandiae* in the production of red pigment during biomembrane surface cultivation. Maximum productivity of red pigment increased to 0.43 g/L.day, which was 2.3 times the initial

productivity obtained by cultivation without CS.

2. CS shortened the fermentation period by providing support for fungal growth and biomembrane formation. The amino acids phenylalanine (Phe) and tyrosine (Tyr) released by CS are crucial factors for improvement of pigment productivity.

3. Fungal growth was benefitted by increase in reducing sugars in fermentation broth caused by hydrolysis of cellulose and hemicellulose by hydrolytic enzymes produced by *P. novae-zelandiae*. This condition may also benefit production of red pigment.

ACKNOWLEDGMENTS

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