

Microbial Conversion of Lignin-Based Compounds Into Carotenoids By Rhodococci

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Research Article

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Abstract

Lignin valorization is considered an integral part for an economically viable biorefinery. However, heterogenous nature of lignin imposes a big challenge for upgrading diverse lignin-derived intermediates and subsequent downstream processing. To overcome this challenge, we proposed to explore unique convergent pathways in *Rhodococcus* strains to funnel lignin-derived compounds into single target products. A feasible bioprocess for co-production of lipids and carotenoids from lignin by *Rhodococci* was developed. This process would potentially extract more values from lignin via biological upgrading of lignin.

Introduction

Escalating environmental problems and global warming arising from overexploitation of petroleum sources is the main driving force in the quest for renewable sources. Lignocellulosic biomass stands out to be a promising renewable feedstock to produce biofuels and bio-based chemicals for the replacement of petroleum-based counterparts. Lignocellulosic biomass is composed primarily of polysaccharides (i.e., cellulose, hemicellulose) and aromatic polymer (i.e., lignin). Great endeavors have been made to convert cellulose and hemicellulose into various fermentation products [1]. However, less attention has been paid to lignin upgrading. As a matter of fact, value-added use of lignin has been recognized essential for an economically viable biorefinery. Although different upgrading pathways have been proposed for lignin upgrading, heterogeneous nature of lignin imposes inherent obstacles to the generation of well-defined product streams. Consequently, diverse lignin-derived intermediates and end products entail extensive separation and purification to obtain target products.

Microbial systems for lignin valorization could overcome the problems with converting heterogenous products since their convergent metabolic pathways could funnel diverse lignin-derived compounds into single products [2]. Among a number of bacteria capable of utilizing lignin as carbon source, *Rhodococcus* strains are appealing and promising due to their excellent efficiency in assimilation of lignin-derived compounds [3]. Previous studies have used wild type *Rhodococcus jostii* RHA1 and *Rhodococcus opacus* PD630 to convert lignin or lignin model compounds into lipids [4, 5]. These *Rhodococcus* strains have also been genetically engineered to produce *cis,cis*-muconate [3]. In addition to the above mentioned products, *Rhodococcus* has also been reported to accumulate carotenoids as their primary metabolites [6]. Carotenoids are high value products that can be used in food, cosmetics, pharmaceuticals, etc. [7]. The market for carotenoids market was about 1.4 billion in 2018 [8]. Given that some oleaginous *Rhodococcus* strains are capable of producing carotenoid using carbohydrates as carbon sources, it is worthy to explore their potentials to utilize lignin as a carbon source for co-production of lipids and carotenoid. Such work would be of significance to not only broaden the spectrum of lignin-derived products but also extract more values from lignin.

In this study, we evaluated the performance of four *Rhodococcus* strains in utilizing lignin model compounds for cellular growth and biosynthesis of lipids and carotenoids. The parameters influencing

the fermentation performance were also investigated.

Materials And Methods

Strains and media

R. opacus (NRRL B-3311), *Rhodococcus rhodochrous* (NRRL B-16536), and *Rhodococcus erythropolis* (NRRL B-16531) were kindly provided by Agricultural Research Service of the United State Department of Agriculture (Peoria, IL), and *R. jostii* RHA1 kindly provided by Lindsay D. Eltis at University of British Columbia. These strains were routinely maintained on LB agar plates. For seed preparation, one colony was inoculated to 50 ml of LB medium, and cultured at 30 °C and 200 rpm until the optical density (OD) reached ~1.5. Then cells were collected by centrifuging at 5,000 g for 3 min, washed twice with 0.9% saline solution, and further resuspended in 10 ml of 0.9% saline solution. The basal medium for carotenoid accumulation was as follows (g/L): ethylenediaminetetraacetic acid disodium salt dihydrate 0.018, $\text{FeSO}_4 \times 7\text{H}_2\text{O}$ 0.013, $\text{CaCl}_2 \times 2\text{H}_2\text{O}$ 0.013, $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, Na_2HPO_4 7.5, KH_2PO_4 5.

Fermentation process optimization

For the assessment of uptake of lignin model compounds, the above four *Rhodococci* were subjected to growth in basal medium containing 2 g/L sodium benzoate as the sole carbon source. The strains showing the best growth were further screened for their abilities to produce carotenoids. In addition to benzoate, the other three lignin-based substrates were also tested, including vanillic acid, 4-hydroxybenzoic acid, and alkaline lignin (TCI America™). The key parameters including carbon to nitrogen (C/N) ratio, osmotic pressure, and pH were optimized. For the C/N ratio, the best-performing *Rhodococci* strain was grown in a medium with a C/N ratio of 20, 50, 80, and 110. For testing osmotic pressure effects, an initial concentration of 0, 5, 15, and 30 g/L NaCl was used. For pH-mediated two-stage fermentation, the pH was kept at 7 till 72 h and then adjusted to either 6 or 8 till the end of fermentation. The pH was maintained at a certain level by adjusting it every 12 h using 5 M NaOH or 2 M H_2SO_4 .

A fed-batch fermentation mode was also adopted to maximize the uptake of lignin-derived compounds while alleviating substrate inhibition. In a typical run, the best-performing *Rhodococci* strain was inoculated to 25 mL of medium containing 2 g/L sodium benzoate, reaching an initial OD of 0.2. After 24 h, the liquid culture was added with 1 mL of a concentrated sodium benzoate solution (50 g/L) and cultivated for 12 h. Thereafter, 2 mL of the concentrated sodium benzoate solution was pulse-added at an interval of 12 h from 36 to 108 h. All the fermentation tests were conducted at 30 °C with an agitation rate of 200 rpm in an incubator shaker.

Analytical methods

Dry cell weight (DCW) and titers of lipids and carotenoids were determined following the methods described in our prior study [9]. The OD of cellular biomass was determined at 600 nm using a

spectrophotometer (GENESIS 10 UV-Vis). In the case of using alkaline lignin solution which appeared dark brownish, it was difficult to measure OD directly. Thus, the colony forming units mL⁻¹ (CFU mL⁻¹) was used to determine the cell growth when the strains were cultured in such lignin-containing medium.

Results And Discussion

Evaluation of carotenoid production potential by *Rhodococci*

Rhodococci strains are well known for their versatility to utilize various compounds as substrates, such as carbohydrates, aromatic compounds, and fatty acids. In this study, four *Rhodococci* strains, namely *R. opacus* (NRRL B-3311), *R. rhodochrous* (NRRL B-16536), *R. erythropolis* (NRRL B-16531), and *R. jostii* RHA1 were first tested for their capability of utilizing lignin model compounds. The four strains were challenged by growing in medium containing 2 g/L sodium benzoate as the sole carbon source. Benzoate was selected because it is commonly used as lignin model compound [10, 11]. As shown in Fig. 1, among the four tested strains, *R. jostii* RHA1 and *R. rhodochrous* showed fast growth, and both reached the stationary phase within 24 h of cultivation. In contrast, a 48 h lag phase was noticed for *R. opacus* and *R. erythropolis*, and *R. opacus* only showed a slight growth even after 96 h fermentation. *R. jostii* RHA1 has been reported by many studies for their high capability to utilize lignin or lignin rich liquor [4], but *R. rhodochrous* has rarely been reported to utilize lignin model compounds. Given that more robust growth was demonstrated by *R. jostii* RHA1 and *R. rhodochrous*, these two strains were selected for further carotenoid fermentation. After 96 h of fermentation in benzoate-containing medium, *R. rhodochrous* produced ~ 0.5 mg/L carotenoid, while *R. jostii* RHA1 only produced half of that. Taking into account the performance in both utilization of lignin model compounds and production of carotenoids, *R. rhodochrous* was chosen for the further fermentation study as discussed below.

Utilization of lignin model compounds and lignin by *R. rhodochrous*

Heterogenous nature of lignin often results in diverse monomers after its depolymerization. Thus, it is important to know if *R. rhodochrous* is also able to utilize a wide range of lignin derived compounds. To this end, we further evaluated its ability to utilize lignin model compounds other than benzoate. Vanillic acid and 4-hydroxybenzoic acid were selected as they are common compounds derived from lignin depolymerization [12, 13], and were reported to be used as lignin model compounds by prior studies [14]. *R. rhodochrous* was able to use all the three tested lignin model compounds, but different growth patterns were demonstrated. The strain grew faster in benzoate, and reached the stationary phase within 12 h (Fig. 3), while it reached the stationary phase after 24 h of cultivation with either vanillic acid or 4-hydroxybenzoic acid. In *Rhodococci*, benzoate was converted into catechol, which undergoes ring opening via either ortho-cleavage or meta-pathway [15]. Different from benzoate, 4-hydroxybenzoic acid and vanillic acid are transformed into protocatechuate, followed by ring cleavage via a series of enzymatic reactions [16]. The similarity of catabolic pathways for vanillic acid and 4-hydroxybenzoic acid may explain the similar growth trend of *R. rhodochrous* when fed with these two compounds. Lastly, we tested whether *R. rhodochrous* can grow in medium with lignin as the sole carbon source. With 10 g/L

alkaline lignin as carbon source, *R. rhodochrous* also showed a 7-fold increase of cellular biomass, reaching $\sim 10^8$ CFU/mL after 72 h of cultivation. This corresponded to an OD of ~ 0.3 (OD of 0.05 corresponding to about 1.5×10^7 CFU/mL), which is much lower than that obtained from lignin model compounds. Prior studies have proved that the use of lignin depolymerization compounds generated instead of lignin as a polymeric substrate can favor the growth of *R. opacus*, which in turn increase lipid production [17, 18]. Hence, it would be necessary to depolymerize lignin via either chemical or biological methods to facilitate the growth of *R. rhodochrous* in lignin as carbon source and improve the production of target products.

Optimization of carotenoid production

Microbial production of carotenoid is dependent on various factors, including pH, temperature, C/N ratio, and osmotic pressure [19, 20]. Undoubtedly, understanding how these factors influences carotenoid production by *R. rhodochrous* is critical for maximizing carotenoid biosynthesis.

To investigate the effects of C/N ratio on carotenoid production, a C/N ratio ranging from 20 to 110 was tested. With a C/N ratio of 20, carotenoid production plateaued at 120 h and decreased thereafter, while carotenoid production showed a gradual increase with the extension of culture time at a higher C/N ratio (Fig. 5b). By increasing C/N ratio to 50 and 80, an increase of 40% was observed (Fig. 5b). It is known that a high C/N stimulates lipid accumulation in cells, and carotenoids are generally hydrophobic. Thus, increased lipid contents may provide more hydrophobic regions for carotenoids to accumulate [20]. Indeed, with the increase of C/N ratio, both lipid titer and lipid content demonstrated a concomitant increase (Fig. 5c). With a C/N ratio of 50 and 80, lipid contents were about 18% and 20%, which were 5% and 7% higher than that with a C/N ratio of 20. A recent study also indicated an engineered *Y. lipolytica* with high capability of producing carotenoid would store produced carotenoid in lipid droplets within cells [21]. In addition, Yamane et al. proposed that a high C/N ratio possibly reduces the consumption of NADPH for primary metabolism such as protein synthesis, leaving more NADPH available for carotenoids biosynthesis [22]. Therefore, increased carotenoid production at higher C/N ratio could be partially attributed to increased NADPH and lipid accumulation in cells. Further increasing the C/N ratio to 110 did not improve carotenoid production despite that it had the highest lipid content (Fig. 5b&c). Although biosynthetic pathways of fatty acids and carotenoids are suggested to share the same precursor (i.e., acetyl-CoA) [23], a high C/N ratio can flux more precursors toward lipid biosynthesis than carotenoid biosynthesis. Overall, these results indicated that an appropriate C/N ratio is critical for lipid synthesis and carotenoid accumulation. In the case of carotenoid production by *R. rhodochrous*, the optimum C/N ratio was 50, which was used for the latter sections.

To investigate the effects of osmotic pressure on carotenoid production, the fermentation media were supplemented with 0, 5, 15, and 30 g/L NaCl, respectively. With low concentration of NaCl (5 g/L), *R. rhodochrous* showed no difference in growth compared with the control where no salt was added (Fig. 6a). However, when NaCl concentration was elevated to 15 and 30 g/L, the growth rate was slowed down and less biomass was produced (Table 1 & Fig. 6a). Osmotic pressure imposed by high concentration of

salt can influence microbial metabolism and growth [24]. Although negative effects on microbial growth was incurred by osmotic pressure, carotenoid production was enhanced by ~30% when 15 g/L NaCl was supplemented compared with no NaCl addition. Further increase of salt concentration to 30 g/L retarded cell growth and had no positive effects on carotenoid accumulation. The strain's ability to not only tolerate high salt concentration but also use salts for stimulating carotenoid accumulation is important for using lignin-based substrates. For example, the pretreatment liquor generated from alkaline pretreatment contains both lignin and salts [25]. Such lignin-rich liquor could be directly used by *R. rhodochrous* to produce carotenoids, while the salts in the liquor could benefit carotenoid production. It would make the process more practical and economical while converting salt-containing lignin waste streams.

It has been reported that shifting the pH could promote the production of carotenoids [26]. *R. rhodochrous* were usually reported to grow well at the neutral pH [27, 28]. Thus, the strain was cultured at the pH of 7 during the first 72 h, and then the pH was shifted to either 6 or 8 for the rest of fermentation. It was found that shifting pH did not change the cell growth, but affected the carotenoid production. A slightly alkaline condition with the pH to 8 increased the carotenoid production. However, an opposite effect was observed when the pH was shifted to 6.

Conclusions

Lignin valorization via bacteria can funnel multiple lignin derived compounds into single target product. Among the four *Rhodococci* strains, *R. rhodochrous* performed the best. The strain was able to uptake various lignin model compounds and lignin as carbon source to support its growth and production of valuable compounds. Different lignin-based substrates showed varying assimilability based on molecular weight and chemistry. The C/N ratio, high osmotic pressure, and pH can significantly affect the carotenoid accumulation. This study demonstrated a feasible process for co-production of carotenoid and lipid from lignin. This process would further expand the realm of products generated from lignin valorization by bacterial systems.

Declarations

Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to Participate

The authors agreed to participate in this work.

Consent to Publish

The authors agreed to publish this work.

Authors Contributions

The authors contributed to the ideation and writing of this work.

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Not applicable.

Conflicts of interest/Competing interests

The authors declare that they have no conflict of interest.

Availability of data and material (data transparency)

All authors declare that all data and materials support their published claims and comply with field standards.

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Table

Table 1. Dry cell weight and carotenoid content under different conditions

Parameters		DCW * (g/L)	Carotenoid (mg/g)
C/N	20	7.68±0.31	0.07±0.00
	50	8.27±0.13	0.10±0.00
	80	7.03±0.08	0.12±0.00
	110	6.51±0.04	0.06±0.00
Osmotic pressure	0 g/L	7.09±0.09	0.13±0.01
	5 g/L	6.95±0.06	0.13±0.01
	15 g/L	6.45±0.06	0.18±0.02
	30 g/L	6.14±0.03	0.15±0.01
pH	6	7.47±0.25	0.11±0.01
	7	7.78±0.33	0.16±0.01
	8	8.00±0.06	0.22±0.00

* DCW: Dry cell weight.

Figures

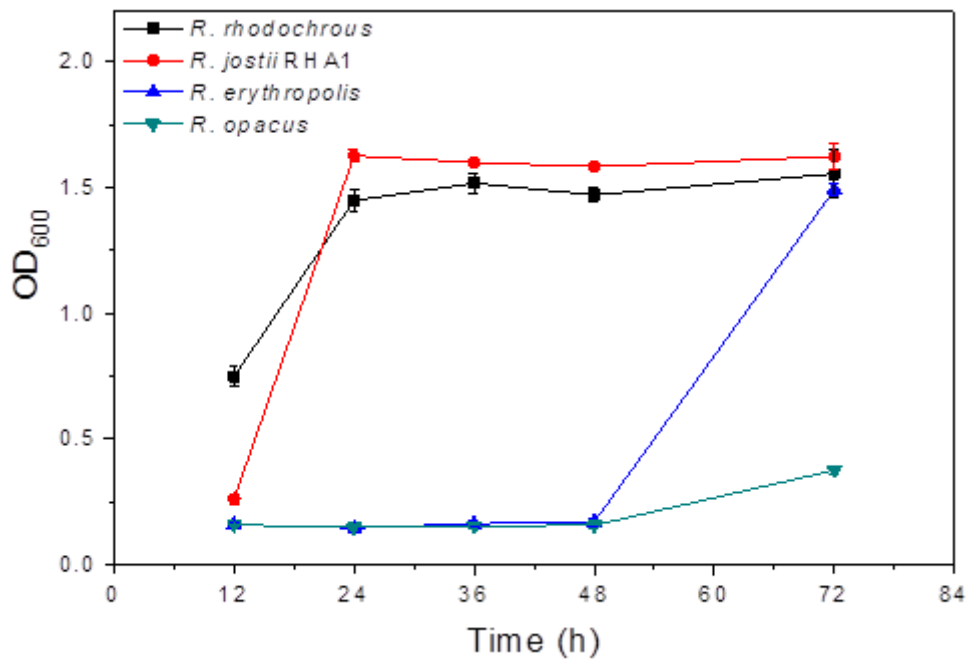


Figure 1

Growth kinetics of Rhodococci strains in 2 g/L benzoate

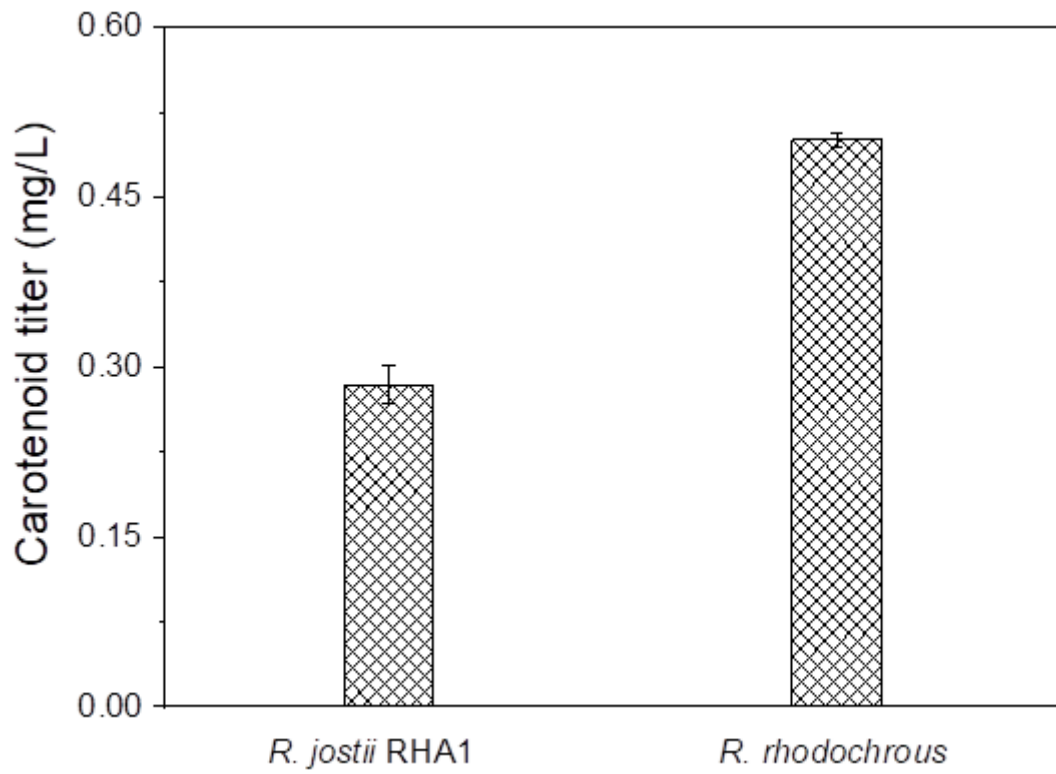


Figure 2

Carotenoid production by *R. jostii* RHA1 and *R. rhodochrous*

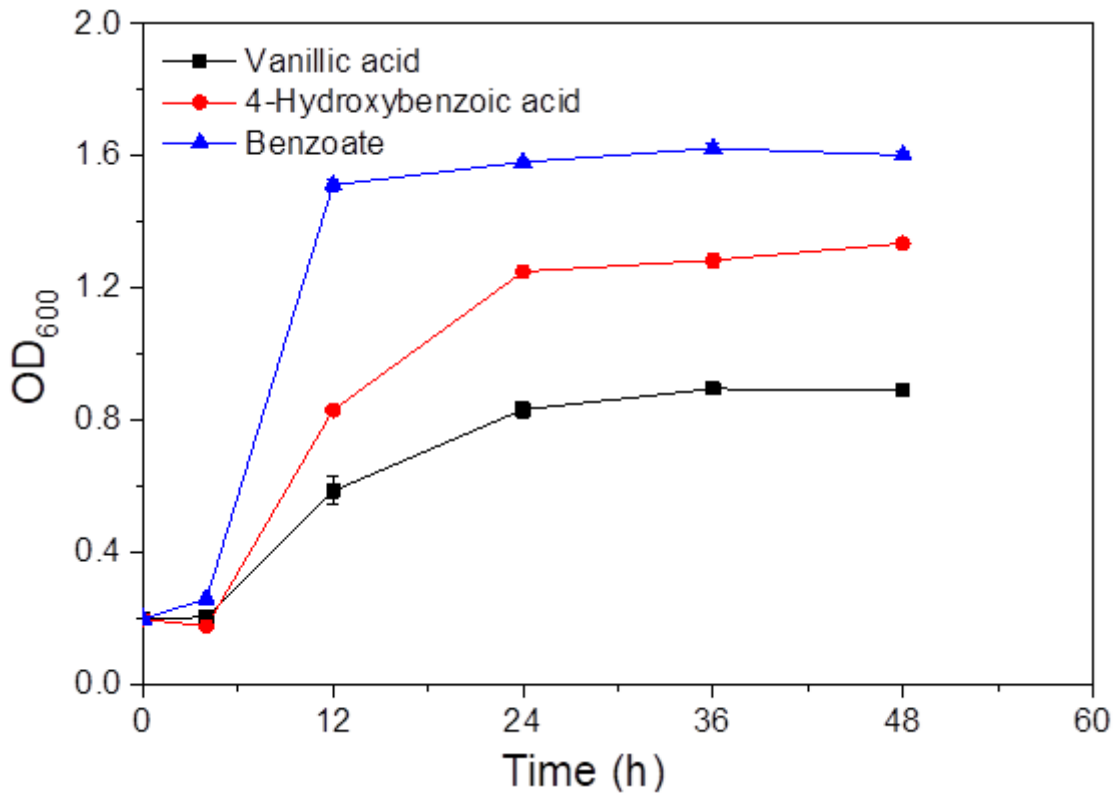


Figure 3

Growth of *R. rhodochrous* in lignin model compounds

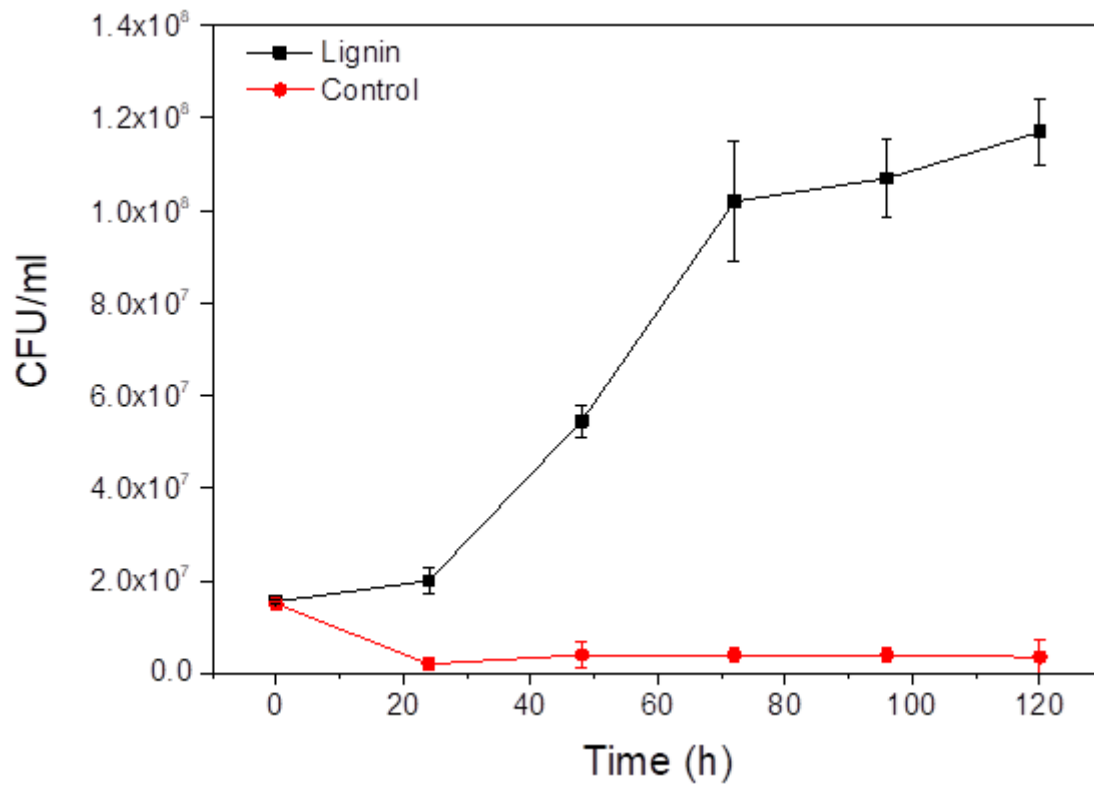


Figure 4

Growth of *R. rhodochrous* in alkaline lignin.

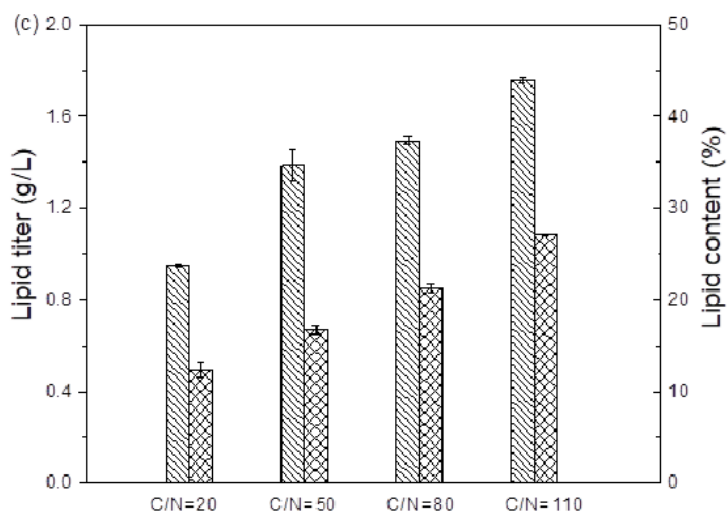
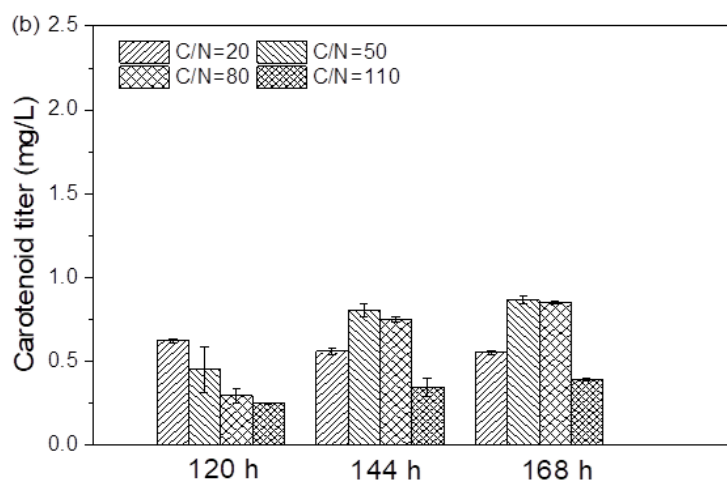
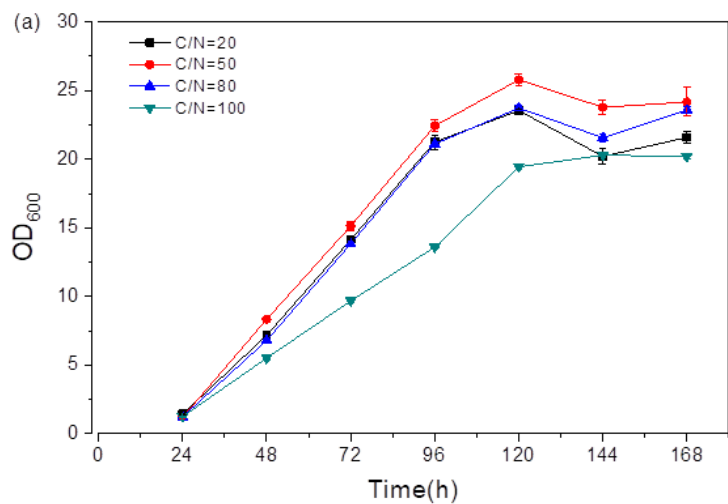


Figure 5

(a) Growth profile, (b) carotenoid production, and (c) lipid production by *R. rhodochrous* under different C/N ratios.

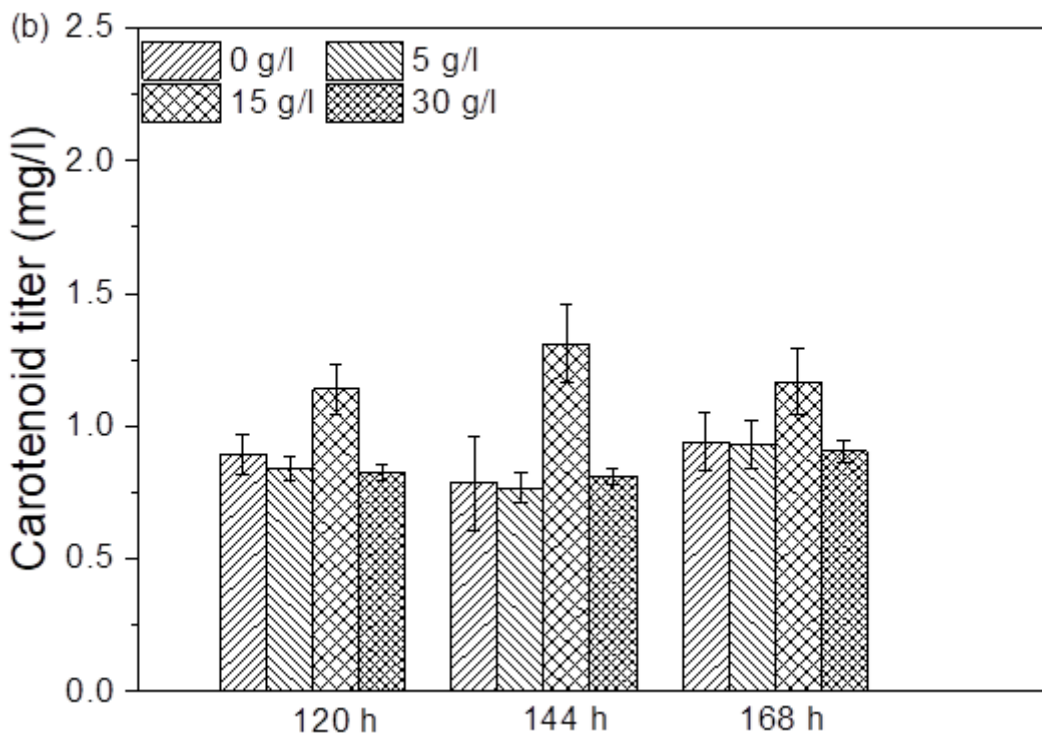
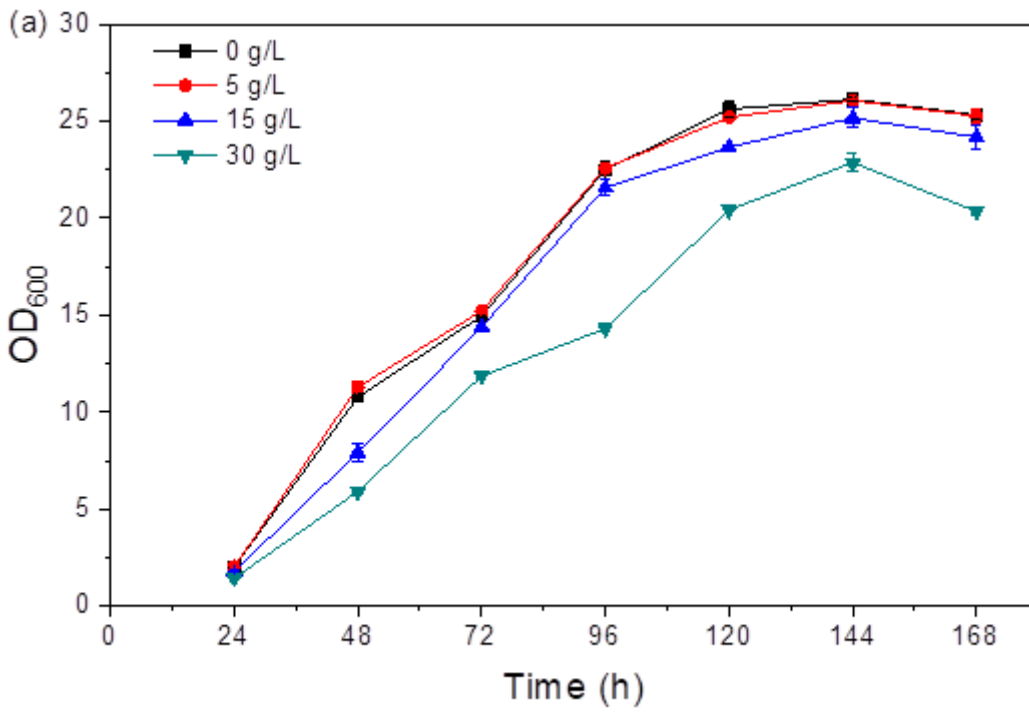


Figure 6

(a) Growth profile and (b) carotenoid production by *R. rhodochrous* under different osmotic pressure.

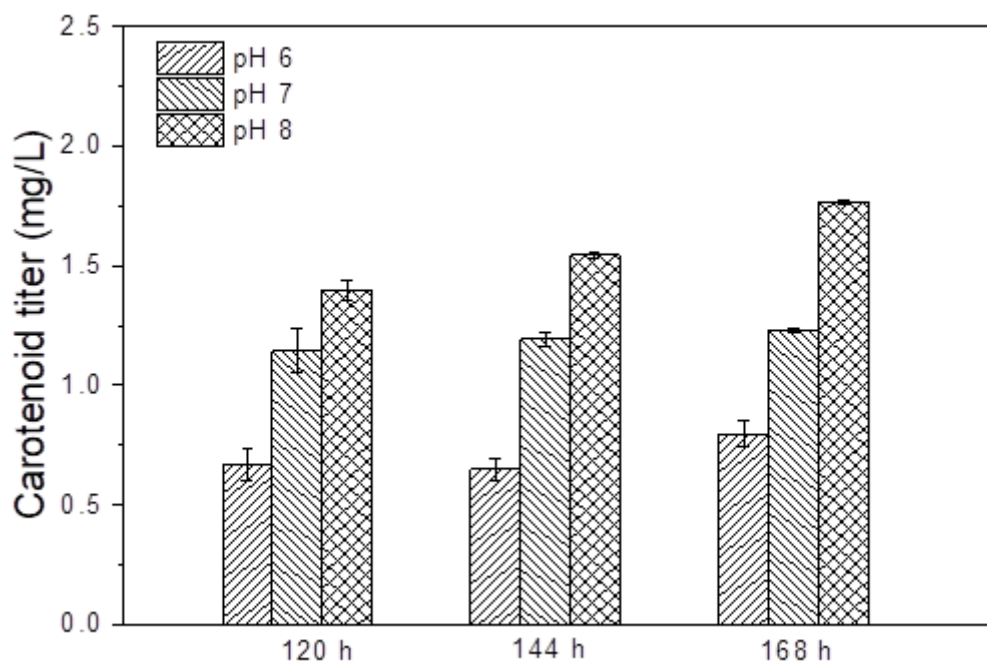
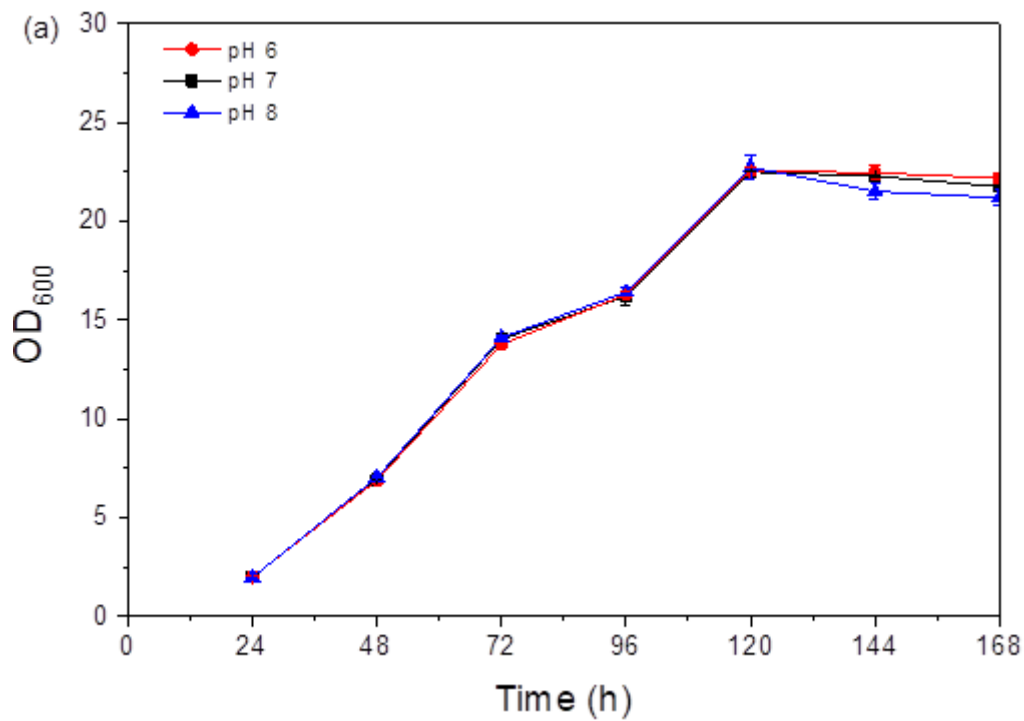


Figure 7

(a) Growth profile and (b) carotenoid production by *R. rhodochrous* during two-stage pH fermentation.