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Effect of sequential twin screw extrusion and fungal pretreatment to release soluble nutrients from soybean residue for carotenoids production

Running Title: Effect of twin screw extrusion and fungal pretreatment to hydrolyze soybean residue

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Abstract

Background: Soybean residue (okara) is an agricultural by-product, which is rich in protein and fiber. This study evaluated a novel sequential process which combined fungal pretreatment (F) and twin screw extruder (E), to hydrolyze okara. The sequence of the pretreatment steps, and extruder at screw speeds 200 rpm (200) or 600 rpm (600), were tested. Next, soluble nutrients were extracted to create Fokara, EFokara200, EFokara600, FEokara200 and FEokara600 okara media.

Results: All the prepared okara media could support the growth and carotenoids production by the yeast *Rhodospiridium toruloides* (*R. toruloides*). This suggested that okara proteins and polysaccharides were successfully hydrolyzed by extrusion and fungal pretreatment, into soluble nutrients. *R. toruloides* accumulated the highest biomass of 23.7 mg mL⁻¹ DCW, when grown on FEokara600 media. This was higher as compared to commercial YPG media (18.7 mg mL⁻¹ DCW). On the other hand, *R. toruloides* accumulated the highest carotenoids production of 13.2 µg mL⁻¹ when grown on EFokara200 media as the nutrient source. This was comparable to the carotenoids production of 13.1 µg mL⁻¹ when *R. toruloides* was grown on YPG media.

Conclusion: Extrusion in combination with fungal pretreatment, is a low cost process, to hydrolyze and re-use okara, for carotenoids production.

Keywords: Pretreatment, Soybean residue, Okara, Twin screw extrusion, Fungal pretreatment, Carotenoids

INTRODUCTION

Soybean residue (okara) is the yellowish white "pulp" residue after aqueous extraction from the soybean, has taken place. It is a by-product generated from the soybean industry, during the manufacture of soymilk and tofu.¹ Worldwide, 14 million tons of okara are being produced annually.² Although okara is considered a by-product, it is nutrient rich. Okara contains 25 - 33%, protein, 10 - 20% lipids, and 40 - 60% soluble and insoluble fibers.^{3, 4} The dietary fiber in okara consists of 46 - 51% cellulose, 16 - 18% hemicellulose, and 1.4 - 2% lignin.⁵ These fibers could be hydrolyzed into fermentable simple sugars such as mannose, galactose and glucose.² Okara also contains a good amino acid profile, minerals, isoflavonoids, and phytosterols.^{6, 7} However, it is highly underutilized at present, as a large proportion is dumped into landfills or incinerated.^{8, 9} This is partly due to its high moisture content of 70 - 80%, which makes it easily perishable.¹⁰ It would be desirable to find other ways to valorize okara, which is still nutritionally rich.

Studies have found that it is essential for okara to be pretreated, to hydrolyze its lignocellulosic structure, which hinders nutrient accessibility. Various pretreatment methods have been employed, such as high hydrostatic pressure treatment, microfluidization, high-energy wet media milling, and enzymatic pretreatment.¹¹⁻¹³ Enzymatic hydrolysis is the most effective, but it is expensive as large quantities of enzymes are required.^{14, 15}

A largely unexplored technique used for pretreatment of okara, is twin screw extrusion. During extrusion, the effect of temperature and mechanical shear, causes a change in the physical structure and protein structure of the sample. It has been applied to a wide range of lignocellulosic biomass, to increase the nutrient and protein digestibility, mineral bioavailability, and especially soluble fiber content.¹⁶⁻¹⁹ During extrusion, the covalent bonds between cellulose, hemicellulose and lignin, are broken. This enables cellulose to be more accessible to enzymes, to be broken down into monosaccharides. Recently, a study combined a continuous process of twin screw extrusion with external addition of enzymes, to increase hydrolysis efficiency, which resulted in an increase in the yield of soluble sugars from sweet corn.^{20, 21} However, external addition of enzymes is expensive. A simple, low cost and eco-friendly alternative, is biological pretreatment. This is the use of a suitable fungi strain, which can secrete the appropriate enzymes to hydrolyze okara.

Therefore the objective of this study was to use twin screw extrusion, in combination with low cost fungal pretreatment, to hydrolyze and improve the nutrient accessibility of okara. We evaluated the amount of soluble nutrients released, by simple water extraction. Then it was used as an added nutrient source to ferment the carotenoids producing yeast, *Rhodospiridium toruloides* (*R. toruloides*). To the best of our knowledge, this is the first biorefinery technology to transform okara into the biobased product, carotenoids, through microbial fermentation. The ability of the yeast to metabolize the soluble nutrients from okara for growth and

carotenoids production, was used as an indicator to reflect the extent of hydrolysis and release of soluble nutrients from okara.

MATERIALS AND METHODS

Okara sample

Fresh okara was supplied by Unicurd Food Company, Singapore. The okara was carefully placed into aliquots, and sealed in air tight polythene bags. It was stored at -20°C in the dark.

Pretreatment of okara using fungal pretreatment and twin screw extrusion

Fungal pretreatment (F) of okara were carried out as previously reported.²² Twin screw extrusion (E) processing of okara was carried out using two screw speeds, 200 rpm and 600 rpm. Depending on the sequence of the pretreatment, two sample series were created in this study. In the 'EF' series, okara was first extruded (E) at 200 rpm or 600 rpm screw speed, followed by biological fungal pretreatment (F). This was termed as treatment group EFokara200 and EFokara600, respectively. In the 'FE' sample series, okara first underwent fungal pretreatment (F), followed by extrusion (E) at 200 rpm or 600 rpm screw speed. This was termed as treatment group FEokara200 and FEokara600, respectively. The treatment group with fungal pretreatment of okara alone, was termed as Fokara. Extrusion of all okara samples, were carried out at a temperature of 160°C, using the twin screw extruder from Thermo Scientific (Process 11 Parallel Twin-Screw Extruder).

Moisture content analysis

The moisture content (%) was determined using the Sartorius moisture analyzer MA160. A sample of approximately 2 g was placed on a disposable aluminum pan, and dried at 105°C, until constant dry weight.

Extraction of soluble nutrients from pretreated okara as added nutrient source for fermentation of *R. toruloides*

After extrusion and biological pretreatment of okara, soluble nutrients were extracted from the treatment groups to form Fokara, FEokara200, FEokara600, EFokara200, and EFokara600.²² These okara media served as an added nutrient source, for the fermentation of *R. toruloides*. The addition of 20 % (w/v) glycerol into each okara media, was also included as the carbon source. Commercial YPG (Yeast extract-peptone-glycerol) media which contained 20 % (w/v) glycerol, and 10 g L⁻¹ yeast extract and 20 g L⁻¹ peptone (YP) was included for comparison. All fermentation were carried out in 250 mL Erlenmeyer flasks, in a total volume of 50 mL, shaking at 250 rpm for 72 h, at 30°C.

Total soluble solids content in prepared okara media

The total soluble solids in all of the prepared okara media, were measured using a hand held refractometer (Kruss DR 101-60), and the results were expressed as °Brix.²³

Growth of *R. toruloides*

To measure the growth of *R. toruloides* during fermentation, samples were appropriately diluted and measured using a Nano Drop 2000 UV spectrophotometer. Growth was measured based on optical density (OD), at the wavelength of 600 nm.²⁴ OD values were converted into dry cell weight (DCW) using a standard curve.

Extraction and quantitation of carotenoids produced by *R. toruloides*

At the end of fermentation, the total amount of intracellular carotenoids produced by *R. toruloides* grown on YPG, Fokara, EFokara200, EFokara600, FEokara200 and FEokara600 media were quantitated. To do this, 1 mL culture media was centrifuged at 10,000 g for 10 mins, and washed twice with ultra pure water. Next, the cell pellet were disrupted with glass beads, using a Fast Prep bead grinder (MP Biomedicals, Solon, OH, USA). Carotenoids were extracted into acetone, until the cell pellet was colorless. Then the acetone fraction, which contained the carotenoids, were analyzed by high performance liquid chromatography (HPLC) equipped with a photodiode array detector (Agilent 1100 Series instrument, Agilent Technologies, CA, USA). The column used was Li-Chrospher 100 RP-18 (Merck), and a flow rate of 0.5 mL min⁻¹ was applied.²⁵ Quantitation of the carotenoids torularhodin and β -carotene, were based on standards obtained from CaroteNature (Ostermundigen, Switzerland).

Statistical analysis

All experiments were repeated three times, and expressed as mean values $\pm$ standard deviations (SD). Data were analyzed using analyses of variance and Tukey test using KaleidaGraph™, Synergy Software. Significant levels were set at $p < 0.05$.

RESULTS AND DISCUSSION

Effect of pretreatments on the moisture content of okara

The parameters of the various okara pretreatments, and the effects of the pretreatments on the moisture content in okara is shown in Table 1. In this study, original fresh okara was determined to have a moisture content of 77.4 % (Table 1). This was similar to previous studies which reported okara to have a 80 - 85 % moisture content.^{10, 26}

After fungal pretreatment alone (Fokara), moisture content in okara increased to 79.7 %, as shown in Table 1. On the other hand, moisture content decreased in all other groups after pretreatments. In the 'EF' samples series, moisture content dropped to 23.0 % in EFokara200, and 54.7 % in EFokara600. For the 'FE' sample series, moisture was 38.6 % in FEokara200 and 57.1 % in FEokara600 (Table 1). Generally, a lower screw speed of 200 rpm reduced the moisture content in okara more, as compared to a higher 600 rpm screw speed. This is probably due to a longer resident time of the product in the extruder at a lower screw speed. This would have increased the duration of the sample exposed to heat, decreasing the moisture content in the sample.²⁷

Total soluble solid content in prepared okara media

The various pretreatments were performed, in order to increase the accessibility and solubility of okara nutrients. Hence, refractometry was used as an indicator of the total soluble solids in the okara media, expressed as °Brix.²⁸ As can be seen in Table 1, when soluble nutrients were extracted from fresh okara with no pretreatment (fresh okara), a very low °Brix of 0.15, was obtained. This indicated that a negligible amount of soluble nutrients, could be extracted from okara, without pretreatment. On the other hand, the total soluble solids which were extracted from okara with pretreatments, were found to be higher, ranging from a °Brix of 2.6 to 5.7. The highest °Brix of 5.7 were achieved with a sequential combination of extrusion at a screw speed of 600 rpm, followed by fungal treatment (EFokara600). A higher °Brix value suggested a higher amount of soluble nutrients, such as sugars.²⁹

Effect of okara media as an added nutrient source on the growth of *R. toruloides*

The prepared okara media were then tested as a potential added nutrient source for the fermentation of the red yeast *R. toruloides*. It was observed that all okara media were able to effectively support the growth of *R. toruloides* (Figure 1). This indicated that soluble and fermentable nutrients were indeed extracted into the okara media.

Overall, the growth of *R. toruloides* reached stationary phase by 72 h, in all treatment groups. When fungal pretreatment alone was used, the resultant Fokara media supported *R.*

toruloides to reach a final DCW of 17.2 mg mL⁻¹. In comparison, *R. toruloides* achieved a higher DCW of 23.4 mg mL⁻¹ and 23.7 mg mL⁻¹, when grown on FEokara200 and FEokara600 media, respectively. This strongly suggested that when extrusion was carried out after fungal treatment, the hydrolysis of okara increased. In addition, the screw speeds did not seem to have a significant effect in the 'FE' samples series. More interestingly, both the 'FE' treatment groups performed better as compared to conventional YPG media, which accumulated a lower DCW of 18.7 mg mL⁻¹ at the end of fermentation (Figure 1).

In the 'EF' sample series, when *R. toruloides* was grown on EFokara200 media, it reached a final DCW of 14.3 mg mL⁻¹. This was the lowest growth, out of all the treatment groups. On the other hand, *R. toruloides* reached a DCW of 19.9 mg mL⁻¹ (Figure 1), when grown on EFokara600 media, which was comparable to YPG. It seemed that in EFokara200 treatment, a lower screw speed of 200 rpm, might be insufficient to shear okara, into smaller soluble molecules.³⁰ In the EFokara600 group, a higher screw speed of 600 rpm, seemed more effective to fibrillate the okara cell wall structure, which lead to an increased surface area of okara for subsequent fungal enzymatic action. This was consistent with a previous study, which showed that a higher screw speed increased sugar yields from soybean extrudates.⁵

Overall, the growth curve results demonstrated that the 'FE' treatment groups, were more effective in increasing the amount of soluble nutrients from okara, and supporting the growth of *R. toruloides*, as compared to the 'EF' treatment groups (Figure 1). This indicated that

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the sequence of pretreatment step, was an important factor. Fungal enzyme activity is well known to be affected by the moisture content of the substrate. Hence the 'FE' treatment groups might have performed better, due to the high initial moisture content of okara at 77.4 % (Table 1), which could be more conducive for fungal hydrolysis. A schematic diagram of the physical and biochemical changes occurring in okara during the 'FE' pretreatment process, is shown in Figure 2. During the first step of fungal pretreatment, carbohydrases such as cellulase would be released, to enzymatically degrade okara cellulose into soluble fiber and monosaccharides, such as glucose.³¹ The fungi can also secrete large amounts of proteases. This would have hydrolyzed proteins into soluble peptides and free amino nitrogen (FAN).^{22, 32, 33} FAN can serve as a nitrogen source for fermentation of *R. toruloides*. During the subsequent extrusion step, further disruption of fibers and shearing of the covalent and non covalent bonding between polysaccharides and protein linkages takes place³⁴. Thus this increased the amount of smaller and soluble molecules, which would have been extracted to serve as an added nutrient source for *R. toruloides* fermentation.³⁰

Characterization and quantitation of carotenoids produced by *R. toruloides*

The effect of using Fokara, EFokara200, EFokara600, FEokara200 and FEokara600 media as an added nutrient source, to support carotenoids production by *R. toruloides* was then evaluated. The results were compared to commercial YPG media. A total of 8.9 $\mu\text{g mL}^{-1}$ torularhodin and 4.3 $\mu\text{g mL}^{-1}$ β -carotene were produced by *R. toruloides* when it was grown on

EFokara200 media (Table 2). This was the highest amount of carotenoids produced by *R.*

toruloides, out of all the other okara media. This was also higher than $8.7 \mu\text{g mL}^{-1}$ torularhodin and $4.4 \mu\text{g mL}^{-1}$ β -carotene produced by *R. toruloides* when grown on commercial YPG media.

It was unexpected that EFokara200 media supported the highest carotenoids production by *R. toruloides*, as it resulted in the lowest growth, as shown in Figure 1. C/N ratio is one of the most influential parameter affecting microbial carotenoids production. A high C/N ratio during the initial phase of fermentation, is favors carotenoids accumulation but not biomass.^{35, 36} Therefore it may be possible that as okara was hydrolyzed to a lower extent in EFokara200 treatment group, resulting in a lower amount of soluble fermentable nutrients. However, with the supplementation of glycerol in all the media at the start of fermentation, a high carbon to nitrogen (C/N) ratio, would have resulted.

Using EFokara600 media as the nutrient source, the only carotenoid produced by *R. toruloides* was $9.6 \mu\text{g mL}^{-1}$ torularhodin. Although not as commercially attractive as β -carotene, torularhodin can quench singlet oxygen better than β -carotene, due to its longer polyene chain.³⁷ Hence, EFokara600 might be used as a media to produce a pure carotenoids fraction, which specifically contains only torularhodin.

The use of FEokara200, resulted in a total carotenoids production of $5.0 \mu\text{g mL}^{-1}$ torularhodin and $4.3 \mu\text{g mL}^{-1}$ β -carotene by *R. toruloides*. On the other hand, a higher $5.8 \mu\text{g mL}^{-1}$

¹ torularhodin and 4.4 $\mu\text{g mL}^{-1}$ β -carotene, was produced using FEokara600 media. Overall, the 'FE' series seemed to support a higher biomass production (Figure 1), but lower carotenoids production by *R. toruloides* (Table 1). We postulate that more fermentable sugars and FAN were released from the 'FE' pretreatment, which would have resulted in a lower C/N ratio at the start of fermentation, in the 'EF' samples series. This would correlate with the fact that a lower C/N ratio favours biomass but not carotenoids production.³⁸

Conclusion

In conclusion, this study demonstrated the feasibility of twin screw extrusion and fungal pretreatment to release soluble nutrients from okara. All of the prepared okara media, could support growth and carotenoids production by *R. toruloides*, as well as commercial YPG. Extrusion in combination with fungal pretreatment was shown to be more effective to hydrolyze and release soluble nutrients from okara, than fungal pretreatment alone. In addition, the sequence of pretreatments step was determined to be an important factor. The highest growth accumulated was 23.7 mg mL^{-1} DCW by *R. toruloides* when grown on FEokara600, which was higher than commercial YPG medium (18.7 mg mL^{-1} DCW). The highest total carotenoids produced was 13.2 $\mu\text{g mL}^{-1}$, when *R. toruloides* was grown on EFokara200 medium, which was comparable to commercial YPG (13.1 $\mu\text{g mL}^{-1}$). In summary, our study showed a novel and cost efficient pretreatment process, to re-use okara to produce

carotenoids. Further optimization of the C/N ratio in okara media, could potentially increase the growth and carotenoids production of *R. toruloides*.

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Figure legends

Figure 1. Growth curves of *R. toruloides* using YPG, EFokara200, EFokara600, FEokara200 and FEokara600 nutrient media.

Figure 2. A schematic diagram of the hydrolysis of okara using fungal pretreatment followed by twin screw extrusion, in the 'FE' sample series.

Fig1.jpg

Fig2.jpg

Tables

Table 1. Process parameters using sequential extrusion and fungal pretreatment. Resulting moisture content (% w/w) of pretreated okara, and total soluble solids in prepared okara media.

Sample	Pretreatment process	Extruder rotation speed (rpm)	Moisture content (% w/w) $\pm$ SD	Total soluble solids (°Brix)
Fresh okara	-	-	77.4 $\pm$ 0.3	0.15 $\pm$ 0.01
Fokara	Fungal pretreatment only	-	79.7 $\pm$ 0.2	2.6 $\pm$ 0.03 ^a
EFokara200	Extrude okara $\rightarrow$ fungal pretreatment	200	23.0 $\pm$ 0.7	4.5 $\pm$ 0.1
EFokara600	Extrude okara $\rightarrow$ fungal pretreatment	600	54.7 $\pm$ 1.1	5.7 $\pm$ 0.1
FEokara200	Fungal pretreatment $\rightarrow$ extrude okara	200	38.6 $\pm$ 0.8	3.4 $\pm$ 0.1
FEokara600	Fungal pretreatment $\rightarrow$ extrude okara	600	57.1 $\pm$ 0.5	2.65 $\pm$ 0.08 ^a

Results represent the mean of three replications $\pm$ standard deviation. Values in the column followed by the same letter are not significantly different at $p = 0.05$.

Table 2. Production of carotenoids ($\mu\text{g mL}^{-1}$) by *R. toruloides* grown on media from okara hydrolyzed by extrusion and fungal pretreatment

	YPG	Fokara	EFokara200	EFokara600	FEokara200	FEokara600
Torularhodin ($\mu\text{g mL}^{-1}$)	8.7	6.8	8.9	9.6	5.0	5.8
β -carotene ($\mu\text{g mL}^{-1}$)	4.4	4.3	4.3	0	4.3	4.4
Total ($\mu\text{g mL}^{-1}$)	13.1^a	11.1	13.2^a	9.6	9.3	10.2

Results represent the mean of three replications $\pm$ standard deviation. Values in the column followed by the same letter are not significantly different at $p = 0.05$.