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Extracting flavonoid from *Ginkgo biloba* using lignocellulolytic bacteria *Paenarthrobacter sp.* and optimized via response surface methodology

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Abstract: *Ginkgo biloba* flavonoids are important natural bioactive compounds with strong physiological effects. To develop an eco-friendly and effective method for extracting flavonoids from plants, a microbial method involving lignocellulose-degrading bacterial enzymes was developed to improve flavonoid extraction from *Ginkgo biloba* leaves. In this study, a newly isolated bacterial strain, *Paenarthrobacter* sp. S1.3, was employed for the fermentation processing of *Ginkgo biloba* leaf powder. The enzymatic characteristics of xylanase, CMCase, polygalacturonase, and β-glucosidase from *Paenarthrobacter* sp. S1.3 were also investigated. The major fermentation parameters for the microbial extraction of *Ginkgo biloba* flavonoids were optimized using response surface methodology. Under optimal conditions (30.6 °C, initial pH 7.6, and fermentation time 25 h), a total flavonoid yield of $34.15 \pm 0.52\,mg\,g^{-1}$ dry weight was obtained, which was 2.11 fold and 1.42 fold higher than the yields obtained from the unfermented and

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ethanol extraction methods, respectively. The optimized results also suggest that the polygalacturonase and β-glucosidase might play an important role in flavonoid extraction. Fermentation utilizing lignocellulose-degrading bacteria was a feasible green approach to improve the extraction of natural compounds from plants. © 2021 Society of Chemical Industry and John Wiley & Sons, Ltd

Key words: *Ginkgo biloba*; flavonoids; *Paenarthrobacter* sp.; lignocellulose degrading bacteria/enzyme; response surface methodology

Introduction

Ginkgo biloba is a gymnosperm deciduous tree belonging to the genus Ginkgo, which is known as the 'living fossil' as it is the only remaining species in the division Ginkgophyta.[1,2] The *Ginkgo biloba* leaves are rich in all kinds of flavonoids including flavonol glycosides, biflavones, proanthocyanidins, and isoflavonoids.[3] As the main ingredients of *Ginkgo biloba* leaf extract, *Ginkgo biloba* flavonoids have many physiological effects such as anti-oxidation,[4–6] radical scavenging,[7,8] protecting human melanocytes from H_2O_2-induced oxidative stress,[9] anti-inflammatory,[10,11] anticancer,[12–14] vascular protection,[15,16] and neuroprotective effects.[17,18] The *Ginkgo biloba* extract (EGb) has been used widely in medicines, health-care products, food additives, and cosmetics.

There are several modern methods for the extraction of flavonoids from *Ginkgo biloba*, including supercritical fluid,[19,20] multi-stage countercurrents,[21] deep eutectic solvent[22,23] and microwave-assisted extraction.[24,25] In addition to the physicochemical methods mentioned above, biological methods such as enzyme-assisted extraction[26–29] and microbial extraction[30,31] have also made important progress.

Lignocellulose is one of the major components in the plant-cell wall and is also considered the most abundant polymer in nature. Flavonoids and polyphenols are also linked to the complicated and rigid lignocellulose structure via the covalent bonds.[32] The recalcitrance of the lignocellulose therefore also enhances the difficulty in extracting the plant bioactive compounds.[33] The utilization of the lignocellulolytic enzyme and lignocellulose-degrading microbe has been studied for a long time. Several studies have shown their potential in extracting various types of value-added and bioactive compounds such as phenolic compounds,[34] carbohydrates,[35] fruit juice,[36] and pigments.[37] So far, many fungi and bacteria have been found capable of producing lignocellulolytic enzymes such as cellulases,[38–41] hemicellulases,[42–44] peroxidases, and laccases.[45–49]

The utilization of lignocellulolytic enzymes could contribute to improving the extraction of flavonoids from *Ginkgo biloba*,[26,27,50,51] and fermentation using lignocellulose-degrading microorganisms could be another innovative approach to improving the extraction of *Ginkgo biloba* flavonoids. The flavonoids are usually linked with pectin and hemicellulose. Thus, the lignocellulolytic enzymes can be used for pretreatment in flavonoid extraction.[52] It was also shown that the pre-fermentation of *Ginkgo biloba* leaves with *Aspergillus niger* increased the total flavonoid yield,[30] and the solid-state fermentation of Ginkgo seeds with *Bacillus natto* obtained a higher total flavonoid yield.[31]

Compared with physicochemical methods, biological methods are more environmentally friendly and more efficient.[53–56] Microbe- and enzyme-assisted extraction normally use mild reaction conditions for extraction, which can reduce energy consumption and costs in the facility. Avoiding high temperatures can also be a strategy for recovering temperature-sensitive and value-added by-products.[57,58] However, the applications of microbe or enzyme assistance in large-scale extraction have been limited due to the high production costs of enzymes and the drying process after treatment for recovering the enzymes and products.[58]

The present study aimed to explore a microbial method involving bacteria fermentation that can improve the extraction of flavonoids from plants. The lignocellulose-degrading bacterial strain *Paenarthrobacter* sp. S1.3, which was newly isolated from forest soil, was employed for the fermentation processing of *Ginkgo biloba* leaf powder. The characteristics of the lignocellulase from *Paenarthrobacter* sp. S1.3 were investigated. The fermentation conditions were optimized using response surface methodology. The results suggested that the lignocellulose-degrading bacterial *Paenarthrobacter* sp. S1.3 has shown potential in increasing the extraction efficiency of the flavonoids from *Ginkgo biloba*, while maintaining a low production cost.

Methodology

Ginkgo biloba leaves

The *Ginkgo biloba* leaves used in this study were bought from the Pizhou Ginkgo Institute (Pizhou, China). The *Ginkgo biloba* dry leaves were ground into powder, the particle size of which was controlled by sieving through a sifter with 425 μm mesh.

Bacterial strain and medium

The lignocellulose-degrading bacterial strain *Paenarthrobacter* sp. S1.3 used in the present study was newly isolated from the soil in the forest near Kingfisher Lake (Thunder Bay, Canada) by our laboratory. The isolated strain S1.3 was identified by 16S rDNA gene sequencing. The liquid and agarose solid medium of Luria–Bertani (LB) broth was used to culture the seed strain. The minimal salt medium formulation contained ammonium nitrate (NH_4NO_3) 1.0 g L^{-1}, potassium phosphate monobasic (KH_2PO_4) 0.5 g L^{-1}, sodium phosphate dibasic anhydrous (Na_2HPO_4) 1.5 g L^{-1}, sodium chloride (NaCl) 1.0 g L^{-1}, magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$) 0.2 g L^{-1} and yeast extract 8 g L^{-1}. The minimal salt medium was supplemented with different gradient concentrations of *Ginkgo biloba* leaf powder (10, 20, 30, 40, g L^{-1}).

Chemicals

The beechwood xylan and carboxymethyl cellulose (CMC) were obtained from Megazyme (Irishtown, Ireland) and Acros organics (Morris Plains, USA), respectively. The polygalacturonic acid and 4-nitrophenyl *β*-D-glucopyranoside (pNpG) were purchased from Sigma (St Louis, USA). The other chemicals were analytical grades and commercially available in Sigma.

Analysis of enzymatic activities

The enzymatic activities of xylanase, CMCase, and polygalacturonase (PG) were measured with the spectrophotometric method as described in the literature[59,60] with minor modifications. Ten microliters of diluted crude enzyme was mixed with 20 μL of 1.0% substance solution (pH 5.0). The mixture was reacting in a 50 °C water bath for 10 min, then it was instantly cooled down and 60 μL of DNS reagent was added. Then the mixture was heating in the boiling water for 5 min. Water (200 μL) was added to each well and 200 μL mixed liquid was taken for measuring the absorbance at 540 nm. Polygalacturonase (PG) activity was analyzed, referring to the above protocols using polygalacturonic acid as the substrate. For the assay of the *β*-glucosidase activity, the cell-free supernatant 10 μL and 20 μL of 1 mM 4-nitrophenyl *β*-D-glucopyranoside (pNPG), prepared in 50 mM sodium acetate buffer at pH 5.0, were mixed and incubated at 50 °C for 10 min. The reaction supernatant was added to 60 μL of 100 mM sodium bicarbonate and diluted with 200 μL deionized water. Then, 200 μL of the diluted reaction solution was transferred to a 96-well microplate to measure the absorbance at 400 nm. The controls were carried out without the enzyme (cell-free supernatant) or the substrate.

Determination of total flavonoids

The spectrophotometric method was used to determine the total flavonoid content. The method principle is as follows: under the alkaline conditions, flavonoids dissolved in ethanol and combined with trivalent aluminum ions to form a red complex that has maximum absorption at 510 nm wavelength. In a certain concentration range, the concentration and absorbance are following Beer–Lambert's law. To determine the total flavonoids released during the fermentation process, 100 μL of the culture supernatant (centrifuged to remove bacterial cells and fermentation residues) mixed with 175 μL ethanol was used as the sample to determine the total flavonoid content. Rutin was used as the standard in this study. The sample or standard solution (or water for blank control) 275 μL was mixed with 100 μL sodium nitrite solution ($NaNO_2$, 50 g L^{-1}), 150 μL aluminum nitrate solution ($Al(NO_3)_3$ 100 g L^{-1}), and 350 μL sodium hydroxide solution (NaOH 200 g L^{-1}). After 15 min incubation, 200 μL of the mixture was transferred to a 96-well microplate. Absorbance at 510 nm was determined with an Epoch Microplate spectrophotometer (BioTek, USA). The flavonoid content was calculated according to the standard calibration curve. All experiments were performed in triplicate. The yields were expressed as milligrams of total flavonoids per gram of *Ginkgo biloba* leaf powder (dry weight).

Microbial extraction

The fermentations were carried out in 125 mL triangular flasks containing 20 mL medium (initial pH 8.0) with 100 μL of 12 h culture as the inoculum, incubated at 30 °C under aerobic conditions at 200 rpm in the shaker (VWR® Incubating Orbital Shaker Model 3500, VWR, Mississauga, Canada). Then 0.20 g of ginkgo leaf powder was added to 20 mL of minimal salt medium in a 125 mL triangular flask covered with aluminum foil and sterilized at 115 °C for 15 min. The supernatant was centrifuged to determine the total flavonoids.

The control I contained the Ginkgo biloba leaf powder and minimal salt medium but it is without any bacteria inoculation. Control II was ethanol extraction, which uses an equal volume of 60% ethanol solution as a substitute for the inoculated minimal salt medium.

Single factors optimization

To investigate the factors affecting the flavonoid yield, fermentations were performed with different initial pH levels (5, 6, 7, 8, and 9), temperatures (25, 30, 35, 37, 40 °C), leaf powder concentrations (1–5 g L^{-1}), and fermentation times (12, 24, 36, 48, 60, 72, and 84 h). All the experiments, under different conditions, were performed in triplicated.

Box–Behnken design

The response surface methodology was used to optimize the fermentation conditions for the total flavonoid yield. The experiment was designed using the Box–Behnken method with SYSTAT 12 software (Systat Software Inc., San Jose, USA). Based on the results of the preliminary single-factor experiments, the temperature (X_1), initial pH (X_2), and fermentation time (X_3) were determined as independent variables, and the total flavonoid extraction yield (Y) was used as response value. The Box–Behnken design with three factors and three levels including three replicates at the center point was performed to generate the second-order response surface. The F-test at the 0.05 significance level, coefficient of determination (R^2), and the lack of fit were used to measure the goodness of fit of the second-order polynomial model. The fitted contour plots were obtained with the response surface methods-contour / surface program in SYSTAT 12 software.

Results and discussions

Characterization of the bacterial strain

The newly isolated bacterial strain *Paenarthrobacter* sp. S1.3 was closest to the reported strain *Paenarthrobacter nitroguajacolicus* P1-D12 (accession number: MK318583.1) with a similarity of 99% according to the Basic Local Alignment Search Tool (BLAST) results of the 16S rRNA gene sequence against the National Center for Biotechnology Information (NCBI) 16S rDNA database. The morphological and physiological characters of strain *Paenarthrobacter* sp S1.3 were shown in the supplmental material Table S1. The strain shows positive results in gram staining, motility, urease, and the production of various lignocellulases. The strain also shows negative in methyl red. Furthermore, it doesn't have the ability in reducing nitrate and utilizing ornithine and citrate. Moreover, it does not produce H_2S, indole, and hydrogen peroxidase. The strain *Paenarthrobacter* sp. S1.3 exhibited xylanase, CMCase, polygalacturonase, and *β*-glucosidase effects, which offer enormous application potential for the microbial extraction of flavonoids from plants. In this study, therefore, strain *Paenarthrobacter* sp. S1.3 was chosen for extracting flavonoids from *Ginkgo biloba* leaves.

Lignocellulase activity

The lignocellulase activity produced by *Paenarthrobacter* sp. S1.3 in different fermentation conditions was determined and the enzymatic characteristics of lignocellulase are shown in Fig. 1. The maximum activity of PG, *β*-glucosidase, xylanase, and CMCase was produced at the

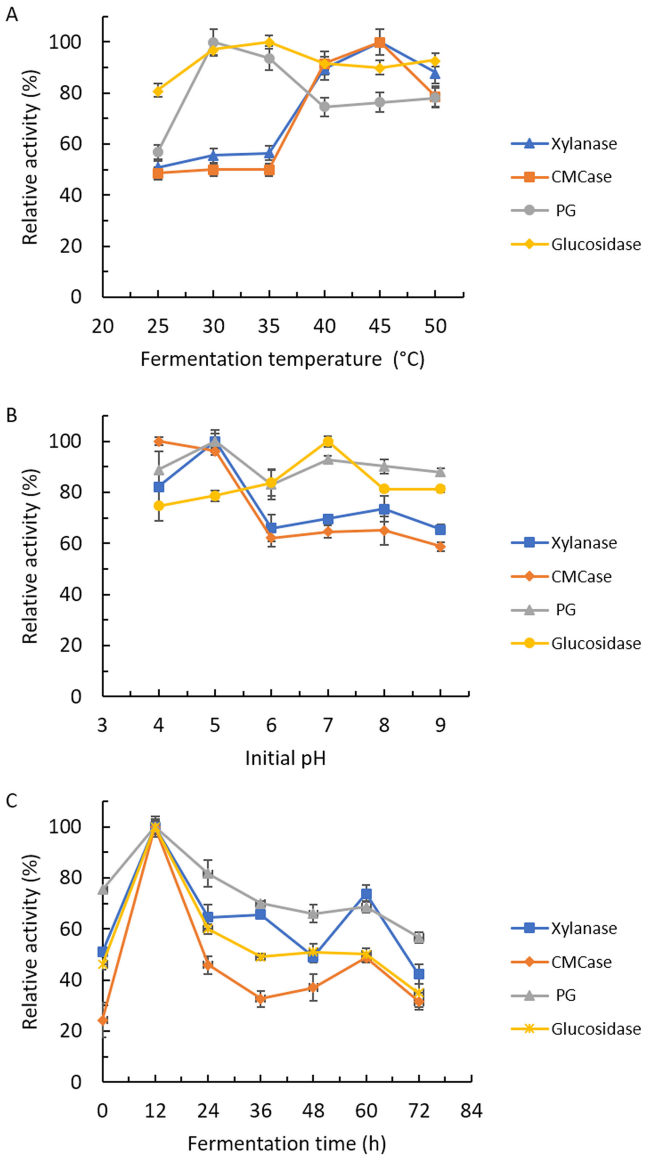

Figure 1. Enzymatic characteristics of lignocellulase produced by *Paenarthrobacter* sp. S1.3. (a) Various temperatures at pH 5.0 and 12 h; (b) various initial pH values at 30 °C and 12 h; (c) various fermentation time at 30 °C and pH 8.

fermentation (with initial pH 8), with temperatures of 30, 35, 45, and 45 °C, respectively (Fig.1(a)). The optimum initial pH was pH 5 for the PG, xylanase, and CMCase activity, and pH 7 for *β*-glucosidase (Fig.1(b)). With the initial pH 5, the maximum PG, xylanase, CMCase, and *β*-glucosidase activity all appeared at 12 h (Fig.1(c)).

Effects of fermentation conditions

The *Ginkgo biloba* leaf powder was fermented with *Paenarthrobacter* sp. S1.3 in minimal salts liquid medium,

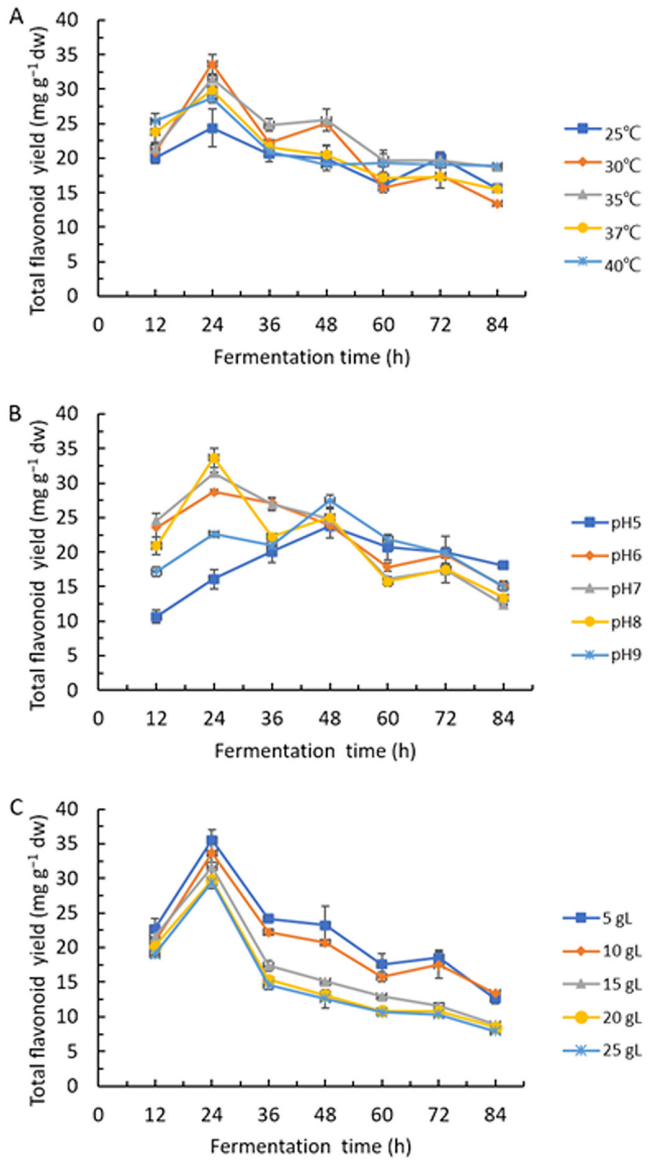

Figure 2. Effect of growth factors on microbial extraction yields of total flavonoids using *Paenarthrobacter* sp. S1.3. (a) Effect of temperature; (b) effect of initial (starting) pH; (c) fermentation time.

into which the flavonoids were released. As shown in Fig. 2, the incubation temperature, medium initial pH, fermentation time, and *Ginkgo biloba* leaf powder concentration had important effects on the total flavonoid yield. The highest flavonoid yield can be obtained at 30 °C (pH 8 and fermentation time 24 h), and gradually decreases with increasing temperature to 45 °C (Fig. 2(a)). The highest total flavonoid yield was obtained at pH 8 (Fig. 2(b)) because the flavonoids are more soluble in alkaline conditions. However, when the Ginkgo leaf powder content in the fermentation broth increases, the yield gradually decreases (Fig. 2(c)).

Table 1. Box–Behnken design matrix for optimization of the total flavonoid yield.

Run	X_1 (temperature)	X_2 (pH)	X_3 (time)	Y (yield, mg g-1)
1	−1 (25 °C)	−1 (7)	0 (24 h)	28.461 ± 2.85
2	1 (35 °C)	−1	0	29.029 ± 0.34
3	−1	1 (9)	0	18.818 ± 0.71
4	1	1	0	25.757 ± 0.24
5	−1	0 (8)	−1 (12 h)	20.052 ± 1.26
6	1	0	−1	21.405 ± 0.89
7	−1	0	1 (36 h)	20.641 ± 0.53
8	1	0	1	24.816 ± 0.18
9	0 (30 °C)	−1	−1	24.581 ± 0.88
10	0	1	−1	17.171 ± 0.76
11	0	−1	1	26.933 ± 2.00
12	0	1	1	20.935 ± 0.71
13	0	0	0	33.675 ± 0.30
14	0	0	0	33.205 ± 0.95
15	0	0	0	33.216 ± 2.12

Optimization of the fermentation

Response surface methodology was used to optimize the fermentation treatment of *Ginkgo biloba* leaf powder. Three factors (temperature, pH, and fermentation time) and three levels were selected for the Box-Behnken design. The ranges and the levels of the variables are shown in Table 1.

Multivariate regression fitting was used to obtain the quadratic polynomial regression equation of the total flavonoid yield (Y) versus the coded independent variables X_1 (temperature), X_2 (pH), and X_3 (fermentation time). The R^2 of the fitted model was 0.997, indicating that 99.7% of observed values could be explained by the fitted model and that there was a strong relationship between the factor variables and the response variables (experimental value of total flavonoids yield). The analysis of variance (Table 2) revealed that the regression, linear, quadratic, and interaction of the response surface quadratic model were all very significant ($P < 0.01$) and appropriate for the experiment's prediction.

The lack of fit (Table 3) was statistically insignificant ($P > 0.05$), showing that the regression equation was reliable for predicting the trends of the responses and that the total flavonoid yield could be explained by the temperature, pH, and fermentation time factors.

The regression coefficients and corresponding *P*-values are shown in Table 4. The linear terms X_1, X_2, and X_3 are all highly significant ($P < 0.01$), meaning that the total flavonoid yields were highly significantly affected by the temperature (positively), pH (inversely), and fermentation

Table 2. Analysis of variance for the response surface quadratic model.

Source	Df	Type I SS	Mean squares	F-ratio	*P*-value
Regression	9	413.119	45.902	187.582	0.000
Linear	3	120.643	40.214	164.339	0.000
Quadratic	3	279.839	93.28	381.194	0.000
Interaction	3	12.637	4.212	17.214	0.005
Residual error	5	1.224	0.245		
Total error	14	414.343			

Table 3. Lack of fit test for the response surface quadratic model.

Source	Df	SS	Mean squares	F-ratio	*P*-value
Lack of fit	3	1.08	0.36	5.002	0.171
Pure error	2	0.144	0.072		
Residual error	5	1.224	0.245		

Table 4. Estimates of the regression coefficients.

Effect	Coefficient	Standard error	t	*P*-value
Constant	33.365	0.286	116.825	0.000
X_1	1.629	0.175	9.316	0.000
X_2	–3.29	0.175	–18.814	0.000
X_3	1.264	0.175	7.23	0.001
X_1*X_1	–4.263	0.257	–16.559	0.000
X_2*X_2	–3.586	0.257	–13.931	0.000
X_3*X_3	–7.374	0.257	–28.644	0.000
X_1*X_2	1.593	0.247	6.44	0.001
X_2*X_3	0.353	0.247	1.427	0.213
X_1*X_3	0.705	0.247	2.852	0.036

time (positively). The order of the effects of the three factors was: pH > temperature > fermentation time. The effects of quadratic and interaction terms on the total flavonoid yield are also highly significant ($P < 0.01$). The quadratic terms X2 1, X2 2 and X2 3 had highly significant inverse effects on the total flavonoid yield ($P < 0.01$). The interaction terms X_1*X_2, X_2*X_3, and X_1*X_3 had highly significant ($P < 0.01$), insignificant ($P > 0.05$), and significant ($P < 0.05$) positive effects, respectively.

To estimate the shape of the response surface, the contour plots were produced based on the fitted model. Figure 3(a)–(c), respectively, presented the effects of variable combination X_1–X_2 (temperature-pH), X_1–X_3 (temperature-fermentation time), and X_2-X_3 (pH-fermentation time) on the total flavonoids yield (Y) when the third variable was at the optimum level. All the contour plots in Fig. 3 were clear ellipses indicating that all the variable interactions are having effects on the respond value (flavonoids yield). Furthermore, all variables had an arched curvilinear effect on the total flavonoid yield; thus, the optimum conditions for the maximum total flavonoid yield were in the design range. Figure 3 shows that when the temperature and fermentation time increased from 25 °C and 12 h to 30.59 °C and 24.97 h, respectively, the yield of flavonoids also increased. However, once the condition passed that point, the yield gradually decreased. Similarly, the yield of the flavonoids would be gradually increasing when the pH is decreasing from 9 to 7.57 but the yield would be decreasing again after the pH is lower than 7.57.

The optimal response, 34.218 with a 95% confidence interval (33.520, 34.915), was obtained by canonical analysis. The coded factor values for the stationary point were 0.118 (X_1), −0.429 (X_2), and 0.081 (X_3), with corresponding experimental conditions: temperature 30.59 °C, pH 7.57, and fermentation time 24.97 h.

To check the fitness of the model, the quintuplicate verification experiments were performed with the predicted optimum levels of the variables: fermentation temperature 30.6 °C, pH 7.6, and fermentation time 25 h. The experimental value $34.15 \pm 0.52\ mg\ g^{-1}$ was identical to the expected value, demonstrating that the model had a reliable goodness of fit to predict the total flavonoid yield during the fermentation process with the *Paenarthrobacter* sp. S1.3 strain.

Effectiveness of microbe extraction compared to solvent extraction

To estimate the effectiveness of microbe extraction compared with traditional ethanol extraction, two control groups, I and II (without microbe inoculated and with 60% ethanol only, respectively), were used and the results were compared with microbe extraction (Fig. 4). The result shows that the yield of microbe extraction ($34.15 \pm 0.52\ mg\ g^{-1}$) is 2.11-fold and 1.42-fold higher than the control group ($16.19 \pm 0.93\ mg\ g^{-1}$) and 60% ethanol extraction ($23.99 \pm 0.85\ mg\ g^{-1}$).

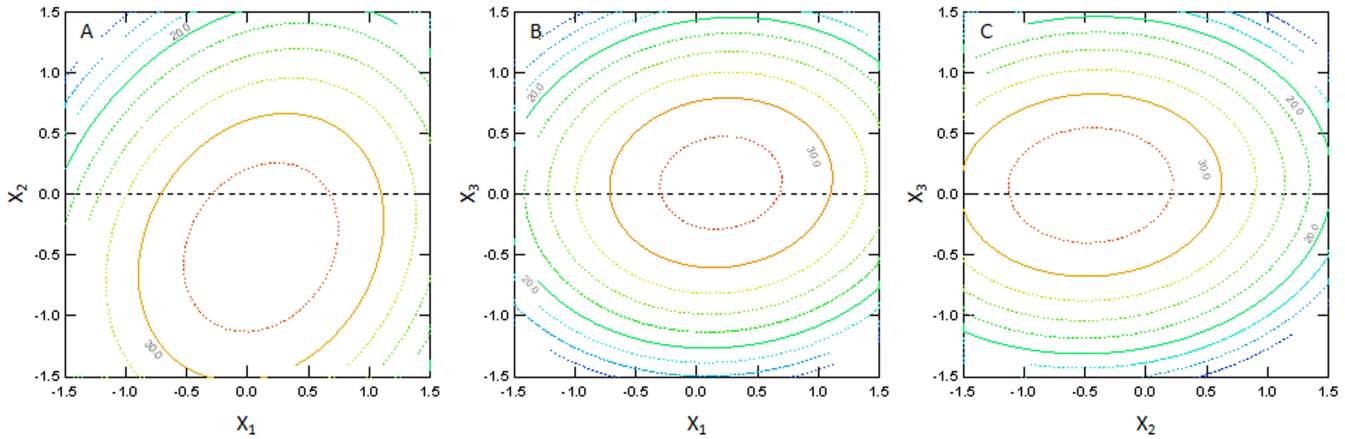

Figure 3. The contour plots between the temperature (X_1), pH (X_2) and fermentation time (X_3) showing the interactive effects on the total flavonoids yield (Y). (a) Y vs X_1, X_2; (b) Y vs X_2, X_3; (c) Y vs X_1, X_3.

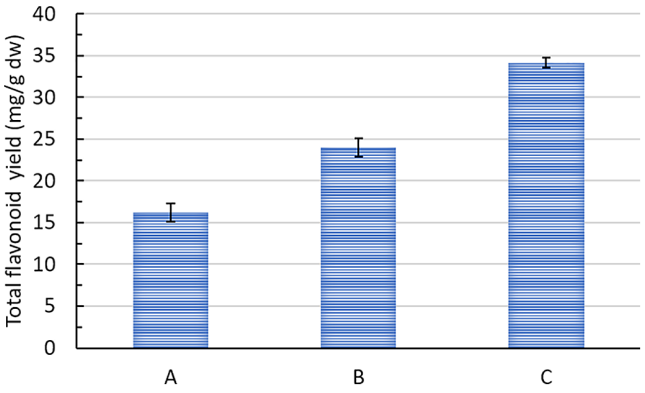

Figure 4. The extraction yield of control I (without inoculation, a), control II (60% ethanol extraction, b), and microbe extraction (c).

Discussion on the microbe extraction

Plants are an abundant bioresource of bioactive compounds such as flavonoids. The key to the extraction of plant bioactive compounds is the effective release and dissolution of these compounds from plant cell tissue into the extraction solution. The plant cell wall commonly consists of three sections: middle lamella (pectin), primary cell wall (cellulose, hemicellulose, and pectin), and secondary cell wall (cellulose, hemicellulose, and lignin) that formed in some plant cells.[61] The complex structure of the plant cell wall usually causes the extraction to be less efficient. Some physicochemical,[22,62–65] enzymatic,[26–29] and biological methods,[30,31] which could break down the intact cells and cell walls, have been used for the extraction of plant nature compounds. Interestingly, the lignocellulose-degrading fungi and bacteria have considerable potential not only for the conversion of biomass into biofuels and value-added products[66,67] but also for microbial extraction of bioactive compounds from plants.[30]

The *Paenarthrobacter* is a genus of gram-positive aerobic bacteria from biomass-containing natural environments. It previously belonged to the genus *Arthrobacter* and was reclassified as *Paenarthrobacter* genus based on phylogenetic groupings, 16S rDNA, peptidoglycan homogeneity, polar lipid profiles, and quinone systems.[68] Several species within the *Paenarthrobacter* genus could degrade nicotine,[69,70] atrazine,[71] l-proline,[72] iprodione,[73] sulfamethazine,[74] and lignin.[75]

In this study, the lignocellulose-degrading capacity of *Paenarthrobacter* sp. S1.3 was demonstrated. The microbial method involving *Paenarthrobacter* sp. S1.3 was developed to extract flavonoids from *Ginkgo biloba* leaves, and the microbial extraction conditions, were optimized using response surface methodology. The condition of microbe extraction is one of the important parameters in industrial application. The optimized condition and yield in this study and other studies are summarized in Table 5. Even though the use of the microbe requires more time in extracting the flavonoids, it can extract more flavonoids and does not require the use of organic solvent. Furthermore, our study suggested that the extraction can be conducted under mild conditions (low pressure and low temperature), which can avoid damaging the flavonoids and other value-added by-products.[81]

Under optimized fermentation conditions, polygalacturonase and β-glucosidase also show very high activity. Pectinases are also considered to be the most common enzyme for extracting bioactive polyphenols in the cell wall because these polyphenols have a higher affinity to pectin rather than cellulose and hemicellulose.[58,82] Thus, these results suggest that the polygalacturonase and β-glucosidase are playing an

Table 5. Summary of the yields and reaction conditions in this and other studies.

Microbe pretreatment/extraction				
Species / enzyme	Samples	Treatment	Yield	References
Paenarthrobacter sp S1.3, lignocellulase	*Ginkgo biloba* leaf	24.97 h; 30.59 °C; pH 7.57	34.15 ± 0.52 mg g^{-1}	This study
Aspergillus niger Gyx086, lignocellulase	*Ginkgo biloba* leaf	61 h; 27.8 °C; pH 5.5	27.59 ± 0.52 mg g^{-1}	30
Penicillium decumbens, cellulase	*Ginkgo biloba* leaf	30 h; 60 °C; pH 6	28.3 ± 0.5 mg g^{-1}	26
Cellulase, protease	*Semen cuscutae*	45 min; 45 °C; pH 4.5	18.75 mg g^{-1}	76
Organic solvent				
Solvent	Samples	Treatment	Yield	References
Choline chloride / levulinic acid	*Ginkgo biloba* leaf	15 min; 50 °C; 40% water	10.32 ± 0.14 mg g^{-1}	77
Choline chloride / malonic acid	*Ginkgo biloba* leaf	53 min; 65 °C; 55% water	22.19 ± 0.71 mg g^{-1}	22
Other physicochemical methods				
Methods	Samples	Treatment	Yield	References
Ultrasonic	*Ginkgo biloba* leaf	250 W, 28.89 min; 40.74 °C; 60% ethanol	11.74 mg g^{-1}	78
Supercritical fluid	*Ginkgo biloba* leaf	CO_2/ethanol; 20 MPa, 90 min; 40 °C	0.36%	79
Microwave-assisted	*Malus domestica* root	1500 W, 20 min; 100 °C; 60% ethanol	17.1 mg g^{-1}	80

important role in the extraction of flavonoids from *Ginkgo biloba* leaves. Furthermore, some of these lignocellulolytic enzymes can transglycosylate the aglycone form of the flavonoids and convert them into the glycoside form of flavonoids, which would have a higher solubility in water.[26]

Even though microbe extraction has several advantages compared to traditional ethanol extraction, there are still several disadvantages and limitations in industrial applications.

One of the major obstacles is that biological extraction is sensitive to the reaction environment. Changes in the pH, temperature, and composition in the medium could significantly affect the performance of the microbes and enzymes. Genetic or protein engineering could be a potential strategy to overcome this difficulty. Lindahl *et al.* show that the mutated thermostable β-glucosidase can enhance the performance in extracting the flavonoids.[83] Another problem could be the high cost of enzyme production. One of the strategies is enzyme immobilization, which can increase the reusability of the enzyme and reduce the cost of enzyme production.[58,84]

As mentioned above, even though there are several limitations, microbe and enzymatic extraction are still considered to be promising methods for extracting a large quantity of water-soluble flavonoids under mild and environmentally friendly reaction conditions. To achieve more cost-effective extraction, several biotechnological approaches need to be developed further. Genetic engineering and direct evolution could be powerful techniques to improve the performance of microbes and enzymes. Based on our study, developing a cost-effective method for producing polygalacturonase and β-glucosidase would dramatically reduce the cost of flavonoid extraction.

Conclusion

A microbial method involving lignocellulose-degrading bacteria fermentation was developed to improve the extraction of flavonoids from *Ginkgo biloba* leaves in a greenway. The characteristics of the lignocellulase produced by *Paenarthrobacter* sp. S1.3 were demonstrated. The microbial extraction method applying *Paenarthrobacter* sp. S1.3 resulted in the increased yield of the total flavonoids compared to traditional ethanol extraction, indicating that fermentation employing lignocellulose-degrading bacteria was a feasible approach to improve the extraction of natural bioactive compounds from plants. Furthermore, polygalacturonase and β-glucosidase could play an important role during the fermentation. Microbial approaches using *Paenarthrobacter* sp. S1.3 seem to be encouraging, with appreciable benefits like high yield, eco-friendliness, and mild operating conditions.

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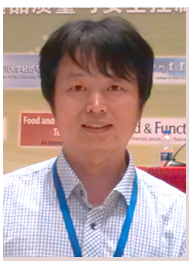

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