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Brewer's rice - A potential substrate for cosmeceutical bio-ingredient production by solid state fermentation using *Aspergillus oryzae*

Dang Lelamurni Abd. Razak*, Nur Yuhasliza Abd. Rashid, Anisah Jamaluddin, Amsal Abd. Ghani, Azlina Mansor and Musaalbakri Abdul Manan

Enzyme and Fermentation Technology Programme, Biotechnology and Nanotechnology Research Centre, Malaysian Agricultural Research and Development Institute (MARDI), MARDI Headquarters, Persiaran MARDI-UPM, 43400 Serdang, Selangor, Malaysia.
Email: danglela@mardi.gov.my

ABSTRACT

Aims: Brewer's rice is one of the by-products from rice processing industry that is rich in bioactive compounds but currently underutilized. Exploitation of agro-industrial by-products as substrates in solid-state fermentation processes provides value-addition to these underutilized by-products. The purpose of this research is to evaluate the potentiality of brewer's rice as a source of cosmeceutical or cosmetic bio-ingredient by utilizing solid-state fermentation process.
Methodology and results: Brewer's rice was submitted to solid-state fermentation with *Aspergillus oryzae* from MARDI's Collection of Functional Food Culture (CFFC). Extracts of unfermented and fermented brewer's rice were later subjected to determination of biological content and biological activities, as well as measurement of their phenolic and organic acids content. The extract of fermented brewer's rice exhibited an increase in total phenolic and total flavonoid content and showed enhanced 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging and ferric-reducing activities. Additionally, it was also found that the tyrosinase and elastase inhibition activities of fermented brewer's rice extract is significantly higher with nearly 7- and 57-fold, respectively, than the unfermented extract. Ferulic and kojic acid – two of the most important compounds in cosmeceutical formulations, were also detected in fermented brewer's rice extract.
Conclusion, significance and impact of study: Antioxidant, anti-pigmentation and anti-wrinkle properties of brewer's rice were successfully enhanced by fermentation with *A. oryzae*. Fermented brewer's rice extract has high potential to be developed as functional bio-ingredient for cosmeceutical as well as nutraceutical products.

Keywords: Aspergillus oryzae, brewer's rice, fermentation, cosmeceuticals

INTRODUCTION

Cosmeceuticals – a fusion term of cosmetic and pharmaceutical – is defined as a cosmetic product that wields a pharmaceutical therapeutic benefit. Cosmeceuticals represent the fastest growth segment in global and highly competitive skin-care market (Tsai and Hantash, 2008) and non-invasive effective products of natural origins are currently preferred by global consumers, compared to synthetic ingredients. There are a lot of cosmeceuticals or cosmetic ingredients developed through biotechnological system such as plant cell culture (Zappelli *et al.*, 2016) and fermentation (Chiba, 2007).

Solid-state fermentation (SSF) is a biotechnological process that has been long used to improve product properties. Basically, SSF can be defined as a process conducted using non-soluble materials that acts as physical support and source of nutrients to the microbes used in the process, in absence or near absence of free water (Pandey, 2003; Couto and Sanroman, 2006). Solid-state fermentation can be adapted for production of value-added products due to its eco-friendly approach, low cost as well as low polluting effluents.

As reported by Ellaiah *et al.* (2002), agro-industrial residues are generally considered as one of the best substrates to be used in SSF processes. For example, SSF of cocoa pod husk, cassava peel and palm kernel cake with *Rhizopus stolonifera* LAU 07 enhanced the nutritional qualities and antioxidant activities of all the solid substrates (Lateef *et al.*, 2008). According to Arasaratnam *et al.* (2001), rice processing by-products or wastes have gained importance as supports for fungal growth during the production of glucoamylase under SSF. Filamentous fungi are one of the most important groups of microorganisms that can be utilized in SSF. The genus *Aspergillus* is among the extensively studied and utilized fungi, such as being used to produce many types of enzymes such as esterase, tannase and lipase (Hölker *et al*., 2004).

Rice-derived bioactive compounds are now widely used as active ingredients in health food, pharmaceutical and cosmeceutical products and as food additives. In

***Corresponding author**

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Malaysia, brewer's rice or *temukut,* is one of the by-products of the rice processing industry that can be targeted for production of value-added products. Brewer's rice is a mixture of broken rice, rice germ and rice bran and considered as having a lower quality by-product than rice bran (Tan *et al.*, 2013). This underutilized by-product possesses functional compounds and antioxidants that can be applied in cosmeceuticals and health markets. Since the exploitation of brewer's rice as a source of cosmeceutical ingredient has not been investigated, this present study is undertaken to evaluate its potentiality by utilizing fungal solid-state fermentation process. In this preliminary study, *Aspergillus oryzae* was used to enhance the cosmeceutical properties of brewer's rice. The findings of this study shall be used for further experiments in an effort to increase the value of brewer's rice by converting it into an active ingredient for high-value cosmeceutical products.

MATERIALS AND METHODS

Fermentation process and extraction procedure

Fungal cultures of *A. oryzae* from MARDI's Collection of Functional Food Cultures (CFFC) were used in this study. Solid-state fermentation was conducted according to a method by Shankar and Mulimani (2007) with some modifications. Thirty grams of brewer's rice (BRR) was added to Erlenmeyer flasks and autoclaved at 121 °C for 15 min. Thirty-five mL of sterilized distilled water and 1% fungal spores (10^6 spores/mL) were added into each flask and the content were mixed thoroughly. All samples were incubated at 32 °C for 16 days. The fermented samples were then harvested, and oven dried at 50 °C for 24 h.

All samples were subjected to a modified procedure of hot water extraction by Lee *et al.* (2008). One gram of sample was mixed with 5 mL distilled water and boiled for 15 min in a water bath. The samples were then centrifuged for 15 min at 10,000 rpm and the supernatant was collected and filtered using Whatman filter paper. The filtrates were then kept at –20°C for further analysis. All experiments were performed in triplicate.

Determination of biological component and biological activities of unfermented and fermented BRR extracts

Total phenolic content, total flavonoid content and antioxidant activities

i. Total phenolic content (TPC)

The total phenolic content assay was carried out according to a method by Okmen *et al.* (2009) with some modifications. Five mL of Folin-Ciocalteu reagent (Merck) and 7.5% Na_2CO_3 (4 mL) was mixed to 1 mL aliquot of each sample. The mixture was allowed to react at room temperature for 2 h in the dark. The absorbance was read at 765 nm using a UV-Vis spectrophotometer (VARIAN, Cary 50). Calibration curve was plotted by using 0 to 200 ppm gallic acid as a standard. The standard curve equation was $y = 0.0097x + 0.1152$ with $R^2 = 0.9987$. Results are expressed as mg/g gallic acid equivalent (GAE).

ii. Total flavonoid content (TFC)

The total flavonoid content of samples was determined according to a method as described by Chang *et al.* (2002), with some modifications. One mL of each sample was added to 0.3 mL of 5% $NaNO_2$ solution. After 5 min incubation, 0.3 mL of 10% $AlCl_3$ solution was added to the mixture, followed by incubation for 6 min. Then, 1 M NaOH solution (2 mL) was added. The final volume of the mixture was brought to 10 mL by using distilled water and the final mixture was allowed to react for 15 mins. Absorbance was measured at 430 nm using a UV-Vis spectrophotometer (VARIAN, Cary 50). Quercetin, in the concentration of 0 - 100 ppm was used as a standard. The total flavonoid content was calculated from a quercetin calibration curve ($y = 0.0087x + 0.0328$, with $R^2 + 0.9947$), and the result was expressed as mg/g quercetin equivalent (QE).

iii. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging antioxidant assay

The assay was carried out with reference to a method by Thaipong *et al.* (2006). Freshly prepared DPPH working solution (2850 µL) was mixed with 150 µL of sample. The concentration of unfermented and fermented extract used in this study was 200 mg/mL. The mixture was allowed to react in the dark for 30 min. Absorbance was measured at 515 nm using a spectrophotometer (VARIAN, Cary 50). Ascorbic acid was used as the positive control. The percentage of scavenging activity of each sample was determined by using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = [(A_{blank} - A_{sample}) / A_{blank}] \times 100$$

iv. Ferric reducing antioxidant power (FRAP)

The FRAP assay was performed as previously described (Benzie and Strain, 1999). Freshly prepared FRAP working solution (2850 µL) was mixed with 150 µL aliquot of each sample (200 mg/mL) and was allowed to react at room temperature in the dark. Absorbance was measured at 593 nm. The standard curve was constructed using 0 to 2000 µM $FeSO_4$ solution. The ferric reducing antioxidant activity was calculated from a ferrous calibration curve ($y = 1.0986x + 0.1042$, with $R^2 + 0.998$), and the result was expressed as mM/g ferrous equivalent (FE).

Anti-pigmentation and anti-wrinkle activity

i. Tyrosinase inhibition activity

To determine the anti-pigmentation or skin lightening potential of sample, tyrosinase inhibition activity was

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carried out according to the method by Alam *et al.* (2011). In a 96-well plate, a mixture of 40 µL of each sample (200 mg/mL), 80 µL of 0.1 M phosphate buffer (pH 6.8) and 40 µL of mushroom tyrosinase (31 U/mL) was prepared. Blank solutions, with and without enzyme as well as sample solutions without enzyme were also prepared. Forty µL of the reaction substrate (10 mM L-DOPA) was added to every sample and blank solutions, respectively. The final mixtures were incubated in the dark at 25 °C for 5 min. The dopachrome produced in the reaction mixture was determined by using a microplate reader (Molecular Devices, VERSA max). Measurement was taken at 475 nm and 100 µg/mL kojic acid was used as the positive control or reference inhibitor.

ii. Elastase inhibition activity

Anti-wrinkle potential of samples was evaluated by measuring their elastase inhibition activity using EnzChek™ Elastase Assay Kit (Molecular Probes, E-12056). The assay was conducted according to the manufacturer's protocols. Fifty µL of sample aliquot (200 mg/mL) was added to 100 µL of 0.5 U/mL porcine pancreatic elastase and incubated in the dark at room temperature for 15 min. Then, 50 µL of DQ™ elastin working solution (25 µg/mL) was added into the mixture followed by incubation in the dark for 30 min. Absorbance at 505/515 nm (Ex/Em) was measured using a fluorescent microplate reader (VARIAN, Cary Eclipse). N-methoxy (N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone) at a concentration of 0.01 mM was used as a reference inhibitor.

Tyrosinase and elastase inhibition activities was calculated using the following equation:

$$\% \text{ inhibition} = \{[(A - B) - (C - D)] / (A - B)\} \times 100$$

Where,
A = absorbance of blank solution with enzyme
B = absorbance of blank solution without enzyme
C = absorbance of sample solution with enzyme
D = absorbance of sample solution without enzyme

Determination of bioactive compounds composition of fermented BRR extract

Phenolic acids

An HPLC Alliance Separation Module (Waters 2695) equipped with a photo-diode array detector (Waters 2996) with a reversed-phase analytical column (150 mm × 4.6 mm × Bridge C18, 3.5 µm, Waters) was used in the determination of phenolic acid composition. The detector was set at ʎ= 280 nm, ʎ= 330 nm and ʎ= 360 nm. To separate the compounds, the mobile phase used was 0.1% formic acid and methanol in gradient condition at 40 °C with the flow rate set at 0.7 mL/min. Quantification of phenolic acids was performed using the calibration curves obtained by injecting known amounts of standard compounds – caffeic, coumaric, ferulic and sinapic. This experiment was conducted according to the method by Aleksandra *et al.* (2011).

Organic acids

The quantification of organic acids was performed using a Waters HPLC (2695) according to the method by Violeta *et al.* (2010). The organic acids in the sample were separated on a 250 mm × 4.6 mm, Extrasil ODS 5µm column. For simultaneous detection, the detector was set at ʎ= 210 nm and ʎ= 245 nm. The determination of organic acids was conducted in isocratic conditions at 30 °C, using 50 mM phosphate solution (pH 2.8) as the mobile phase with the flow rate of 0.7 mL/min. Quantification of organic acids was performed using the calibration curves obtained by injecting known amounts of individual standard compounds (oxalic, citric and kojic).

Statistical analysis

Mean values and standard deviations were calculated from the data obtained from triplicate experiments. In determining the significance of the data, one-way analysis of variance (ANOVA) was conducted using Minitab Statistical Software (Version 14). Differences between means with a *p*-value of <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Biological components and biological activities of unfermented and fermented brewer's rice extracts

In this study, antioxidant properties of fermented brewer's rice extracts were investigated due to its importance in cosmeceutical and cosmetic products. Ingredients with antioxidant properties are responsible in protecting and repairing skin from free radical damage. Studied extracts were subjected to radical scavenging (DPPH) and reducing antioxidant (FRAP) assays. According to the results shown in Table 1, higher biological components and antioxidant activities was observed in fermented samples, compared to unfermented counterpart. Fermented brewer's rice extract showed a significant 47-fold increment of its total phenolic content, compared to unfermented sample. Contrary to total phenolic content, fermented samples showed only a slight increase in total flavonoid content.

The enhancement of total phenolic and flavonoid contents of the fermented brewer's rice extracts is an indication of secretion of cellulose/hemicellulose degrading enzymes such as β-glucosidase. These enzymes hydrolyze β-glucosidic bonds of conjugated phenolic compounds, leading to increase concentration of free polyphenols (Georgetti *et al.*, 2009). Phenolics are known to be responsible for antioxidant activity (Abo-Elmagd, 2014). The results showed that fermented brewer's rice possessed high antioxidant activities, which are positively correlated with its total phenolic content.

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This is consistent with a report from Hansakul *et al.* (2011), which suggested that the phenolic content of extract from rice materials can be correlated to their antioxidant activity.

Cosmeceutical-related enzymatic inhibition activities of unfermented and fermented brewer's rice extracts

The potential of fermented brewer's rice extracts as anti-wrinkle and anti-pigmentation or skin whitening agents was determined by performing elastase and tyrosinase inhibition assays, respectively. Skin darkening or hyper-pigmentation is caused by over-production of melanin pigment. Tyrosinase is the enzyme responsible in the melanin biosynthesis by the melanocyte cells in our skin. Therefore, inhibitor of tyrosinase is a crucial ingredient in cosmeceutical formulations targeting on controlling hyper-pigmentation or skin darkening induced by UV rays. On the other hand, elastase inhibitors function as anti-wrinkle or anti-aging agent in cosmeceutical formulations, responsible to block elastin from being degraded by elastase enzyme. Elastin is a highly elastic protein that provides firmness and elasticity to human skin.

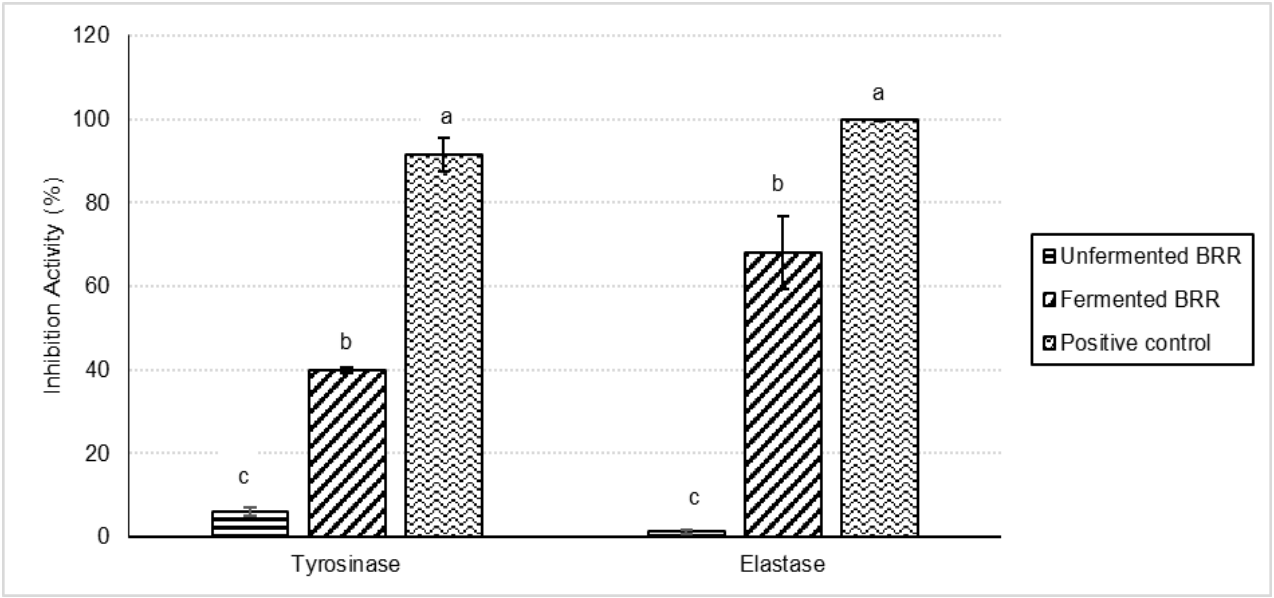
It was clarified from Figure 1 that fermented brewer's rice extract possesses anti-pigmentation and anti-wrinkle activities. The results showed moderate inhibition activity amounted to 39.9% and 68.1% on tyrosinase and elastase, respectively. Unfermented brewer's rice showed much lower inhibition activity on these two enzymes, amounted to less than 6.0%. Both unfermented and fermented brewer's rice extracts showed weaker inhibition than the reference inhibitors used in this study. As far as we know, there is no report on the evaluation of cosmeceutical-related activities of extracts obtained from fermented substrate used in this study.

However, Chang *et al.* (2007) has reported that methanol extract of rice and rice fermented with *A. oryzae* did not show any anti-tyrosinase activity. Previous study on fermentation of rice bran using the same fungi showed improvement of tyrosinase and elastase inhibition activities, up to 35- and 7-fold respectively, compared to unfermented counterpart (Abd. Razak *et al.*, 2017).

Table 1: Total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activities of unfermented and fermented BRR extracts.

Sample	Biological Components		Antioxidant Activities	
	Total phenolic content (mg GAE/g sample)	Total flavonoid content (mg QE/g sample)	DPPH radical scavenging activity (%)	Ferric-reducing antioxidant power (mM FE/g sample)
Unfermented BRR	0.05 ± 0.00^{b}	1.13 ± 0.09^{b}	61.96 ± 0.03^{b}	11.40 ± 0.29^{b}
Fermented BRR	2.37 ± 0.14^{a}	1.78 ± 0.34^{a}	93.91 ± 0.40^{a}	58.16 ± 7.71^{a}

Footnote: Each value is expressed as mean ± SD. The values in the row with the same letter are not significantly different at the level of 0.05 (p>0.05). BRR = brewer's rice.



Footnote: The values with the same letter in each category are not significantly different at the level of 0.05 (p>0.05). BRR = brewer's rice.

Figure 1: Tyrosinase and elastase inhibition activity of unfermented and fermented BRR (brewer's rice).

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Bioactive compounds composition in unfermented and fermented brewer's rice extracts

The content of bioactive compounds, such as phenolic and organic acids, can be modified by the metabolic activity of microbes, enzyme hydrolysis and biochemical metabolism that occur during fermentation. As stated by Katina *et al.* (2007), synthesis of various bioactive compounds may happen as a result from structural breakdown of cell walls, induced by the fermentation process.

As displayed in Table 2, ferulic acid – a potent antioxidant and anti-aging agent (Kumar and Pruthi, 2014), was the major phenolic acid detected in fermented brewer's rice extract. Meanwhile, citric acid was the highest organic acid detected in fermented brewer's rice. This result was supported with a report by Tsao *et al.* (1999) which stated that *Aspergillus* sp. are known to produce high yield of citric acid during fermentation process. Citric acid is a type of β-hydroxy acid that is widely used as an antioxidant in cosmetic formulations (Kornhauser *et al.*, 2012), therefore the presence of citric acid in our fermented samples partly contributes to the fermented brewer's rice potential as cosmeceutical bio-ingredient. None of the phenolic and organic acids detected and quantified in fermented brewer's rice was present in the unfermented brewer's rice. Hydrolyzing enzymes such as α-amylase, xylanase, β-glucosidase and esterases are associated in the release of free and water-soluble phenolic compounds from their insoluble bound-form (Bhanja Dey and Kuhad, 2014). Fungi are widely acknowledged for their potential to overproduce a variety of organic acids, particularly *Aspergillus* sp. (Karaffa *et al.*, 2001).

Table 2: Phenolic and organic acids content on fermented BRR extract.

Compound Group	Compound Type	Compound Content
Phenolic acids (µg/mL)	Caffeic	2.99 ± 1.27
	Coumaric	4.36 ± 0.99
	Ferulic	6.71 ± 0.36
	Sinapic	2.77 ± 0.19
Organic acids (mg/mL)	Oxalic	0.98 ± 0.30
	Citric	109.7 ± 1.39
	Kojic	0.05 ± 0.00

Footnote: Each value is expressed as mean ± SD

Based on the results of biological activities and bioactive compounds content in unfermented and fermented brewer's rice, due to the substantial amount of organic acids detected in fermented brewer's rice extract, it is possible to suggest that these acids play a major role in ferric-reducing and DPPH-radical scavenging activities. Known potent antioxidant compounds such as ferulic acid are also contributing to the antioxidant activity in the fermented brewer's rice extract. However, as explained by Leong and Shui (2002), the overall antioxidant activity in an extract is not necessarily indicated by the potent antioxidant compounds present. The complex mixture of many compounds in the fermented brewer's rice extract may have worked synergistically, thus can show different results by different method of antioxidant assays. As for unfermented brewer's rice, it is suggested that the predominant source of antioxidant activity is derived from other compounds such as vitamin E, oryzanol and ɣ-aminobutyric acid.

Tyrosinase and elastase are the two significant enzymes that involve in deterioration of the skin. The inhibition of tyrosinase activity of the fermented brewer's rice extract may be attributed to the presence of kojic acid in the extract. Kojic acid has been tested, reported and used extensively in cosmetic preparations as prevention of melanin overproduction in epidermal layers of the skin (Miyazawa *et al.*, 2003). On the other hand, elastase inhibition activity of fermented brewer's rice extract may be contributed by ferulic and caffeic acids detected in the extract. As reported by Saija *et al.* (1999) and Magnani *et al.* (2014), both compounds have been known as excellent protective agents against photo-aging of the skin, induced by UVA radiation which accelerates elastase synthesis leading to the degradation of skin elasticity and formation of wrinkle. Similar to antioxidant activity, the inhibition of these skin-degrading enzymes by the fermented brewer's rice extract can be attributed to the synergism that may exist between many types of compounds present in the extract.

The use of other extraction method and solvent may influence the bioactive compounds content and consequently, the biological activities of fermented brewer's rice extract. The presence of various bioactive compounds with different characteristics and polarities may or may not be soluble in particular solvent (Turkmen *et al*., 2006). Pengkumsri *et al.* (2015) has found that extraction with hexane enhanced the antioxidant activities of rice bran oil compared to extraction by cold-pressed, hot-pressed and supercritical fluid. The effect of other extraction methods or solvents on the biological activities of fermented brewer's rice is still unknown. Therefore, further investigation on the optimum extraction procedure is required to isolate the bioactive compounds that can further enhance the cosmeceutical-related activities of fermented brewer's rice.

CONCLUSION

The present preliminary investigation demonstrates that solid state fermentation with *A. oryzae* successfully improved cosmeceutical properties of brewer's rice. Compounds related to anti-pigmentation and anti-aging properties were also produced during fermentation of brewer's rice with *A. oryzae* – proving its potentiality as active bio-ingredient in cosmeceutical formulation. Synergistic effect among compounds may have played a vital role in improving the biological activities of fermented brewer's rice. In order to achieve the optimal production of bioactive compounds and cosmeceutical related bio-activities in brewer's rice, experiments on optimization of solid-state fermentation process is now on-going. To

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assess the efficacy of fermented brewer's rice extracts as cosmeceutical bio-ingredient, further *in vitro* study using skin cell lines will be commenced in the near future.

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