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Expression of Furfural Reductase Improved Furfural Tolerance in Antarctic Bacterium *Pseudomonas extremaustralis*

(Pengekspresan Furfural Reduktase Meningkatkan Ketoleranan Furfural dalam Bakteria Antartika *Pseudomonas extremaustralis*)

Ahmad Bazli Ramzi[1,*], Matthlessa Matthew Minggu[1], Ummul Syafiqah Ruslan[1], Mohamad Hazwan Fikri Khairi[1] & Peer Mohamed Abdul[2,3]

[1]*Institute of Systems Biology (INBIOSIS), Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor Darul Ehsan, Malaysia*
[2]*Research Centre for Sustainable Process Technology, Faculty of Engineering and Built Environment, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor Darul Ehsan, Malaysia*
[3]*Program of Chemical Engineering, Faculty of Engineering and Built Environment, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor Darul Ehsan, Malaysia*

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ABSTRACT

Whole-cell biocatalysis using Antarctic bacteria is presently hampered by a lack of genetic information, limited gene tools and critically, a poor range of cultivation conditions. In this work, biological engineering strategy was employed for developing *Pseudomonas extremaustralis*, a metabolically-versatile and biopolymer-producing Antarctic bacterium, as a new whole-cell biocatalytic host. For this purpose, gene cloning and plasmid construction were carried out for overexpression of furfural reductase (FucO), an industrially-important enzyme for degradation of toxic furfural compound commonly found in lignocellulosic biorefinery. *FucO* gene from *Escherichia coli* BL21 was cloned in pJM105 plasmid and transformed into competent cells of *P. extremaustralis* to generate a biologically-engineered pFucO strain. For functional characterization of the enzyme, furfural reductase activity was assayed, where the *P. extremaustralis* pFucO strain exhibited increased furfural reductase activity of about 15.6 U/mg, an 18.8-fold higher than empty plasmid-carrying control pJM105 strain (0.83 U/mg). Furfural detoxification activity using whole cells was also determined by which the overexpression of FucO led to increased tolerance and cell growth with an OD_{600} value of 5.3 as compared to the control pJM105 strain that was inhibited with 10 mM furfural during 48-hour cultivation. Therefore, the findings obtained in this study successfully demonstrated the development of *P. extremaustralis* as biocatalytic host for the production of recombinant furfural reductase. The bioengineering would serve as a modular biotechnological platform for polar strain and bioproduct development tailored towards industrial biotechnology applications.

Keywords: Antarctic bacteria; biological engineering; furfural reductase; *Pseudomonas extremaustralis*; whole-cell biocatalysis

ABSTRAK

Penggunaan bakteria Antartika sebagai biopemangkin keseluruhan sel buat masa ini adalah sangat terbatas akibat kurangnya maklumat genetik, alatan genetik yang terhad dan ketidakserasian keadaan pengkulturan. Dalam kajian ini, strategi kejuruteraan biologi digunakan untuk membangunkan *Pseudomonas extremaustralis*, sejenis bakteria yang berasal dari Antartika yang mempunyai metabolisme versatil, berupaya menghasilkan biopolimer dan sesuai digunakan sebagai perumah baru biopemangkin keseluruhan sel. Untuk mencapai tujuan ini, pengklonan gen dan pembinaan plasmid telah dilakukan bagi penghasilan furfural reduktase (FucO), sejenis enzim yang penting dalam penguraian sebatian toksik furfural yang kerap ditemui dalam proses penapisan sisa lignoselulosa. Gen *FucO* daripada *Escherichia coli* BL21 diklonkan ke dalam plasmid pJM105 dan dimasukkan ke dalam sel kompeten *P. extremaustralis* untuk menghasilkan strain terjurutera biologi pFucO. Untuk pencirian enzim dan kefungsian bakteria terubah suai

biologi ini, aktiviti furfural reduktase telah diasai dengan strain *P. extremaustralis* pFucO menghasilkan sebanyak 15.6 U/mg, iaitu 18.8 kali ganda lebih banyak daripada sel yang mengandungi plasmid kawalan pJM105 (0.83 U/mg). Aktiviti penguraian furfural menggunakan seluruh sel juga dilakukan yang mana pengaruhan enzim furfural reduktase berjaya meningkatkan pertumbuhan sel oleh strain pFucO yang menunjukkan OD_{600} sebanyak 5.3 selepas 48 jam dikultur berbanding strain kawalan pJM105 yang direncatkan apabila diuji dengan 10 mM furfural. Penemuan kajian ini membuktikan *P. extremaustralis* terjurutera biologi ini berjaya dibangunkan sebagai perumah biopemangkin untuk penghasilan furfural reduktase rekombinan. Strategi kejuruteraan biologi ini akan menjadi pelantar berasaskan bioteknologi untuk pembangunan strain dan penghasilan bioproduk kutub untuk aplikasi bioteknologi industri.

Kata kunci: Bakteria Antartika; biopemangkin seluruh sel; furfural reduktase; kejuruteraan biologi; *Pseudomonas extremaustralis*

Introduction

Antarctica is home to various extremophilic microbes living in extreme environments, including low subzero temperatures and nutrient-limited conditions. The unique properties of Antarctic microbes have led to increased bioprospecting efforts in the polar regions that aimed at discovering industrially-important enzymes and metabolites derived from new microbial isolates. The advent of recombinant DNA technology has facilitated the cloning of anti-freeze proteins and cold-active enzymes such as proteases, lipases and cellulases possessing higher specific activities at cold temperatures useful in detergent, food product additives and complex substrate degradation (Ali et al. 2013; Hashim et al. 2013; Sarmiento et al. 2015).

Despite the tremendous potential of bio-product development from extremophiles, particularly from the polar regions, existing biotechnological applications were primarily based on molecular cloning and recombinant protein productions in traditional hosts such as *Escherichia coli* and *Pichia pastoris*. Compared to the mesophilic hosts, the polar microbes exhibit distinct advantages of having an innate capacity to metabolize recalcitrant compounds, including diesel, phenols and plastics hence providing the beneficial attributes important for whole-cell biocatalysis and waste biotransformation (Martínez-Rosales et al. 2012). For unlocking the full biocatalytic potentials of the polar microbes, there is an urgent need to address the critical issues in the biological engineering of non-traditional hosts, including genetic tractability, genome sequence information and genetic toolkit availability. To date, only a limited number of Antarctic bacteria such as *Pseudoalteromonas haloplanktis* have been genetically-modified for recombinant protein expression but the use of the strict psychrophilic *Pseudoalteromonas* spp. as whole-cell biocatalytic hosts have been associated with poor expression and reduced growth at moderate (higher than 22 °C) temperature (Fendrihan & Negoiţã 2017; Piette et al. 2011).

With the versatile metabolic growth and availability of sequenced genome, Antarctic-derived *Pseudomonas extremaustralis* represents a promising biocatalyst host alternative to the traditional microbes. *P. extremaustralis* has been previously demonstrated to grow at a broader temperature profile ranging from 4 °C to 37 °C, is capable of degrading diesel, ethanol and lignin derivatives, and is highly resistant to oxidative stress (Tribelli et al. 2015, 2012). Importantly, this Antarctic bacterium can also produce biosurfactant and industrially-important biopolymers such as polyhydroxyalkanoate (PHA) and alginate (Raiger Iustman et al. 2015; Tribelli et al. 2015). As compared to strict aerobe *P. putida* KT2440 strain, *P. extremaustralis* has been shown to exhibit upregulated gene regulation under anaerobic conditions hence offering a major advantage for driving gene expression and production of oxygen-sensitive enzymes essential in the production of valuable compounds such as 1,3-propanediol and adipic acid (Jiang et al. 2016; Raj et al. 2018; Tribelli et al. 2019).

Considering the versatile growth profile and bio-product synthesizing abilities of *P. extremaustralis*, the main objective of this study was to demonstrate the enhancement of the biocatalytic activity of this Antarctic bacterium using a specially-devised biological engineering strategy. In this study, *P. extremaustralis* bacteria was successfully engineered to overexpress furfural reductase enzyme for biodegradation of furfural, a major fermentation inhibitor in lignocellulosic microbial biorefinery. The biological engineering strategy employed and results obtained from this work represent the first

study examining the development of biologically-engineered *P. extremaustralis* as biotechnological platform for furfural biocatalytic reduction and future polar strain and bio-product development.

Materials and Methods

STRAINS AND CULTURE CONDITIONS

Pseudomonas extremaustralis (DSM 25547) was employed in this study for cultivation, genetic modification and fermentation experiments. *E. coli* TOP10 was used for molecular cloning and plasmid maintenance experiments. Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract and 10 g/L sodium chloride) with kanamycin (50 µg/mL) was used for bacterial growth and plasmid propagation experiments. Cultivation for *E. coli* and *P. extremaustralis* was carried out at 37 °C and 25 °C, respectively.

PLASMID CONSTRUCTION

Broad host range vector pJM105: pTac RFP on pCM66T (Addgene plasmid # 74746; hereafter denoted pJM105) was used for transformation and genetic manipulation of *P. extremaustralis*. The pJM105 plasmid carried minimal IncP replicon and constitutive *tac* promoter (Marx & Lidstrom 2001). To confer furfural-degrading capability in *P. extremaustralis*, *FucO* gene encoding furfural reductase was cloned into the pJM105 plasmid yielding pFucO plasmid. The *FucO* gene originated from *E. coli* BL21 (DE3) was amplified using the bacterial genomic DNA and cloned by cutting the template pJM105 plasmid with *Eco*RI restriction enzyme and assembled using primers FucO Fwd-5'-CACACAGGAAACAGAATTCTATGGCTAACAGAAT GATTTTCAACGAA-3') and FucO Rev CTTGCCATG *TATCTCCTT*TTACCAGGCGGTATGGTAAAGCTC-3′). The *Eco*RI restriction enzyme site and ribosomal binding site (RBS) were underlined and italicized, respectively. Briefly, the fragments of the *FucO* gene and linearized pJM105 plasmid were isothermally-assembled in 1:5 vector:insert ratio by incubating with NEBuilder® HiFi DNA Assembly Master Mix for 1 hour (Ramzi et al. 2018). The assembled fragments were then chemically-transformed into *E. coli* Top10 competent cells (Ramzi et al. 2018). The resultant *E. coli* transformants carrying the assembled pFucO construct were evaluated via Colony PCR and further verified via DNA sequencing. All bacterial strains and plasmids employed in this study were listed in Table 1.

TRANSFORMATION AND FURFURAL DETOXIFICATION BY RECOMBINANT *P. extremaustralis*

Two plasmids, namely pJM105 control plasmid and pFucO expression plasmid, were transformed into *P. extremaustralis* using electroporation methods as described previously (Wang et al. 2010). The plasmids were prepared unmethylated (*Dam- Dcm-*) by inoculating and extracting the plasmids from *E. coli* K12 ER2925 (New England Biolabs, USA). LB plus kanamycin plates were used for the transformation and selection of *P. extremaustralis* harboring pJM105 and pFucO plasmids, respectively. Positive transformants carrying the recombinant *FucO* gene were confirmed via Colony PCR and Sanger sequencing. Functional expression of the recombinant *FucO* gene by the engineered pFucO *P. extremaustralis* strains were conducted via enzymatic assay and furfural tolerance. Tolerance against furfural was determined using 10 mM furfural according to previous reports on furfural toxicity to native microbes (Abdul et al. 2013; Wang et al. 2011). For whole-cell furfural detoxification experiments, *P. extremaustralis* strains carrying respective pJM105 and pFucO plasmids were grown in LB plus kanamycin with 10 mM furfural for 48 h at 25 °C. Bacterial growth was determined by measuring the OD_{600} value every 12-hour intervals.

ANALYTICAL METHODS AND ENZYMATIC ASSAY

UV-visible spectra, enzyme assays and furfural reduction were measured using a UV-Vis spectrophotometer (DU730, Beckman Coulter, USA). The protein concentration was determined using a Nanodrop UV-Vis spectrophotometer (Thermo Scientific, USA). For determination of furfural reductase activity, cells from furfural fermentation experiments were collected and subjected to cell lysis using CelLytic B reagent (Sigma Aldrich, USA). The resultant crude extracts were used for subsequent enzymatic assays. Furfural reductase activity was determined using methods previously described (Wang et al. 2011). Briefly, the reaction mixture consisting of 10 mM furfural, 0.2 mM NADH and the appropriate dilution of protein extract in 200 mM sodium phosphate buffer (pH 7.0) was assayed at 25 ºC. Determination of furfural reduction was performed by measuring the decrease of NADH absorbance value at 340 nm (ε= 6,220 M^{-1} cm^{-1}). One unit (U) of furfural reductase activity is defined as the amount of enzyme required to reduce 1 mole of NADH per minute.

TABLE 1. List of bacterial strains and plasmids used in this work

Strain or plasmid	Genotype or construct	Reference or source
Bacterial strains		
Escherichia coli TOP10	F⁻*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K λ⁻ *rps*L(Str^R) *end*A1 *nup*G	Invitrogen[a]
Pseudomonas extremaustralis (DSM 25547)	Wild type	DSMZ[b]
Plasmids		
pJM105	pJM105: pTac RFP on pCM66T plasmid harboring minimal IncP replicon and constitutive *Tac* promoter, Km^R	(Marx & Lidstrom 2001)[c]
pFucO	pJM105 containing *FucO* gene encoding furfural reductase, Km^R, *tac* promoter fragments	This study

Km, kanamycin; R, resistance. [a]Invitrogen Corporation, Carlsbad, CA, USA. [b]Leibniz-Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. [c]pJM105: pTac RFP on pCM66T was a gift from Mary Lidstrom (Addgene plasmid # 74746)

Results and Discussion

Plasmid Construction for Furfural Reductase Expression

In this work, biological engineering strategy was employed for developing *Pseudomonas extremaustralis* as a new and alternative Antarctic biocatalyst host. Compared to the other Antarctic microbes, this bacterial host was selected based on the expansive range of substrates and products that can be naturally metabolized, including environmentally- and industrially-important compounds including ferulic acid biosurfactant and biopolymers (Figure 1(a)). The bacterial capacity to produce biopolymers and utilize glucose and ferulic acid render *P. extremaustralis* as a potentially new platform strain for bio-product development from lignocellulosic feedstock. Despite these advantageous traits, *P. extremaustralis* lacks the ability to tolerate or metabolize furfural, a major inhibitory compound found in the pretreated-lignocellulosic hydrolysate. To this end, a biological engineering approach was specially-devised for expressing furfural-degrading activity in this Antarctic bacterium. For this purpose, plasmid construction was carried out for cloning and expression of the furfural reductase-encoding *FucO* gene from *E. coli* BL21. Expression of recombinant *FucO* gene was previously shown to increase furfural degrading capacity of engineered ethanologenic *E. coli* strains by converting furfural to the less toxic furfuryl alcohol as a final product (Wang et al. 2013, 2011). In this study, shuttle plasmid pJM105 was used for cloning the *FucO* gene under the control of constitutive *tac* promoter yielding pFucO expression plasmid (Figure 1(b)). The pFucO and pJM105 plasmids were then introduced to the bacterial cells via electroporation, resulting in engineered pJM105- and pFucO-harboring *P. extremaustralis* strains.

Functional Furfural Reductase Activity in *FucO*-Expressing *P. extremaustralis*

The biocatalytic capacity of bio-engineered microbial hosts requires a strong gene expression system by introducing functional genetic regulatory elements, particularly promoter and terminator. In this study, the functional expression of recombinant furfural reductase in *P. extremaustralis* was determined and compared against the control strain carrying empty plasmid pJM105. As depicted in Figure 2, specific furfural reductase activity of FucO-expressing *P. extremaustralis* was about 15.6 U/mg, an 18.8-fold higher than control

pJM105 strains (0.83 U/mg) hence demonstrating the high expression of furfural reductase under the control of *tac* promoter.

These results indicate the successful use of the *tac* promoter, one of the most widely-used bacterial promoters for directing protein production and establishment of genetic tools in industrially-important prokaryotic hosts (Markley et al. 2015; Ramzi et al. 2015). This finding highlights the important role of the broader growth profiles of *P. extremaustralis*, which may facilitate functional expression of the *E. coli*-derived promoter in this Antarctic bacterium. The robust cellular machinery, metabolic reconstruction models, and well-characterized transcriptome and physiological profiles will provide good genetic background information essential in developing the biocatalytic potentials of *P. extremaustralis* as new and alternative Antarctic expression hosts (Raiger Iustman et al. 2015; Tribelli et al. 2019, 2018).

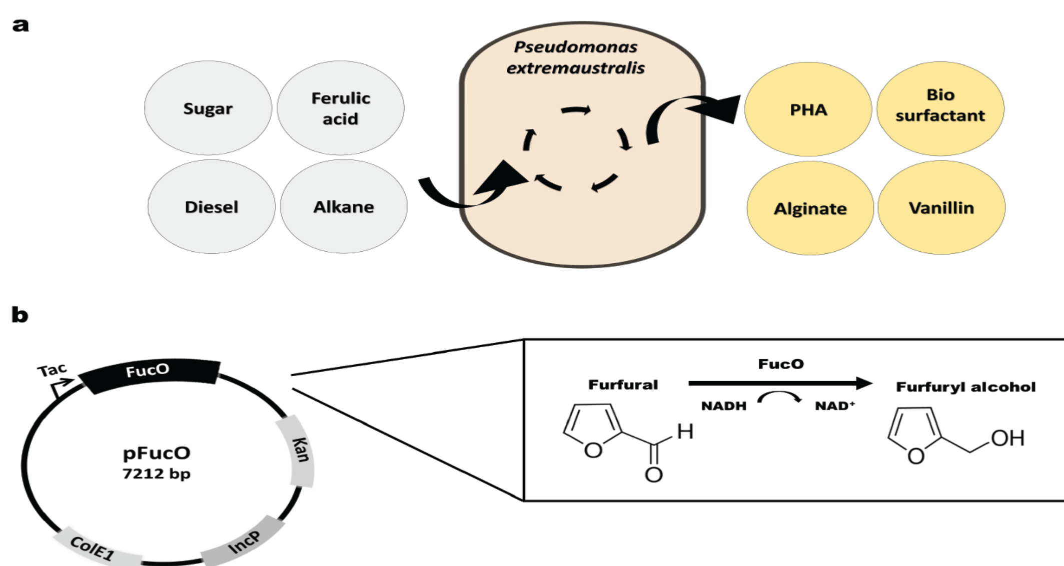

FIGURE 1. (a) Range of substrates and products that can be metabolized by *P. extremaustralis*. (b) Plasmid construct and biocatalytic reduction process of furfural to furfuryl alcohol by recombinant *FucO*-encoded furfural reductase

WHOLE-CELL DETOXIFICATION OF FURFURAL BY *P. extremaustralis* pFucO STRAINS

Furfural represents a major inhibitory compound formed in pre-treated lignocellulosic hydrolysate that contributed to the low-yielding bioprocessing performance and increased costs for additional furfural detoxification steps. Therefore, elevated tolerance of microbial cells is required to tolerate the presence of furfural and maintenance of cell growth during the cultivation process. In this study, furfural detoxification capacity in *FucO*-expressing *P. extremaustralis* was determined and compared against the control strains by cultivating both strains in the presence of 10 mM furfural. As demonstrated in Figure 3, the growth of control pJM105 strain without the *FucO* gene was inhibited by the inhibitory compound, while the *FucO*-overexpressing strain grew well and reached a 5.3 OD_{600} value over the 48-hour cultivation period. These results suggested that the presence of furfural caused a growth-inhibiting effect on *P. extremaustralis* without furfural reductase overexpression. Thus, functional expression of *FucO* succeeded in conferring furfural detoxification capacity as demonstrated in the bioengineered *P. extremaustralis* pFucO strain.

Similar growth-impeding effects of furfural were observed in native microbes, including *E. coli, Zymomonas mobilis,* and *Thermoanaerobacter pentosaceus*, of which a considerable amount of furfural was shown to cause significant growth retardation (Franden et al. 2009; Tomás et al. 2013; Wang et al.

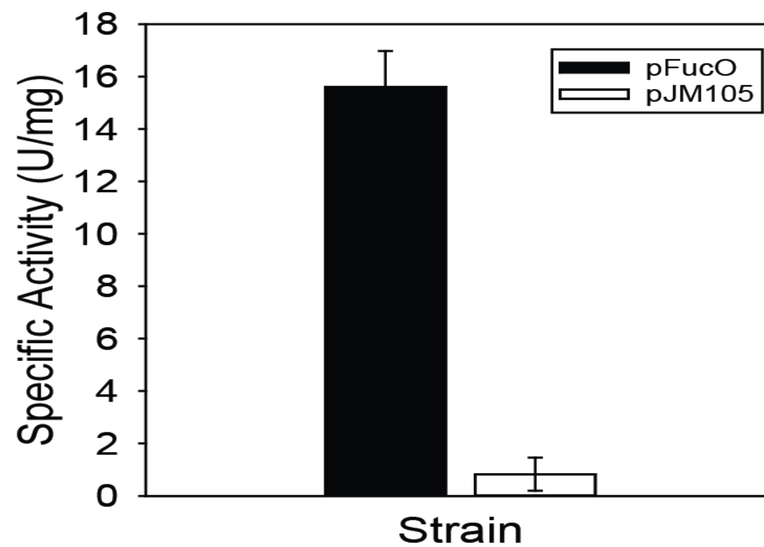

FIGURE 2. Increased furfural reductase activity of bioengineered *P. extremaustralis* pFucO strain as compared to control pJM105 strain. The specific activity of furfural reductase was determined based on the decrease of NADH absorbance value measured at 340 nm (ε= 6,220 M^{-1} cm^{-1}). The enzyme unit (U) is defined as the amount of enzyme required to reduce 1 mole of NADH per minute. Values and error bars represent the mean and standard deviation of at least triplicate measurements

2011). In lignocellulosic bioprocessing, furfural was generated from pre-treatment methods, including acid and alkaline treatments that released up to 10 mM (~1 g/L) of furfural in many pentose-rich hydrolysates culminating in reduced bioethanol and biohydrogen productions (Abdul et al. 2013; Heer & Sauer 2008; Tomás et al. 2013). Interestingly, despite being known as an environmentally-robust microbial strain, wild-type *Pseudomonas* sp. was still affected by the inhibitory effects of furfural, hence underlining the importance of biological and metabolic

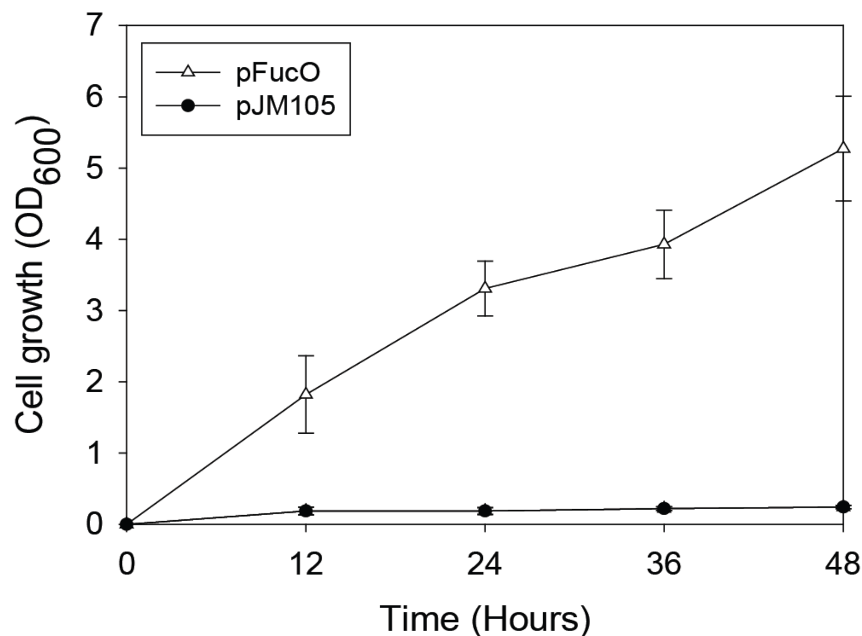

FIGURE 3. Whole-cell detoxification of engineered *P. extremaustralis* pFucO overexpressing recombinant furfural reductase against growth-inhibiting furfural compound. Cell growth (OD_{600}) of *P. extremaustralis* carrying control pJM105 and pFucO plasmid in LB media containing 50 µg/mL kanamycin and 10 mM furfural was measured over the 48-hour cultivation at 25 °C. Data points and error bars represent the mean and standard deviation of at least three independent fermentations

engineering approaches for alleviating the toxicity issues (Dietrich et al. 2013; Guarnieri et al. 2017). From these results, the employment of the biological engineering strategy is therefore essential in increasing the bacterial biocatalytic activity and, more importantly, providing furfural-detoxification activity in bioengineered *P. extremaustralis*. Considering that this bacterium has been previously shown to produce industrially-important compounds, specifically PHA and alginate (Raiger Iustman et al. 2015; Tribelli et al. 2015), the functional expression of recombinant FucO enzyme will facilitate future utilization of furfural-containing biomass hydrolysates as inexpensive and sustainable feedstocks for biotechnological production of the desired products. This work successfully demonstrated the feasibility of biological engineering of *P. extremaustralis* as a new Antarctic whole-cell biocatalyst host that can be further engineered to express heterologous enzymes or biosynthetic genes tailored toward addressing specific biotechnological applications.

Conclusions

In this study, biological engineering approach was employed for developing *P. extremaustralis* as a new biocatalytic host alternative to the commonly used Antarctic bacteria. Construction and transformation of plasmid pFucO expressing the *FucO* gene under the control of *tac* promoter have enabled elevated furfural reductase activity in bioengineered FucO-expressing *P. extremaustralis*. Furthermore, overexpression of the recombinant FucO conferred whole-cell furfural detoxification hence, demonstrating the enhanced biocatalytic performance of engineered *P. extremaustralis* pFucO strain. This study is the first to report the development of the Antarctic bacterium *P. extremaustralis* for furfural biocatalytic reduction using microbial engineering approaches.

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*Corresponding author; email: bazliramzi@ukm.edu.my