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Evaluating Bioplastic Production of Glucose Metabolic Perturbations *Pseudomonas Putida* Strains from Waste Frying Oil

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Waste frying oil (WFO) can take a heavy toll on people' health and the environment; however, they can still be useful in reality if they are appropriately collected and recycled. Specifically, we can take their advantages to create a nutritious carbon source for bacteria growth. In this research, due to a strong desire to enhance the value of such products, *Pseudomonas putida* KT2440 is employed to produce polyhydroxyalkanoates (PHA) which is a group of natural biodegradable polyesters synthesized by microorganisms. Two deficient glucose assimilation chassis strains, which are the *Δglk* and *Δgcd* created through knock-out genes encoding glucose dehydrogenase (*gcd*) and glucoskinase (*glk*) respectively, are compared with *P.putida* KT2440 WildType (WT) for optimization of PHA production. Batch cultivation coupled with an approach of standard submerged fermentation is used in order to monitor the biosynthesis of PHA and the growth profile of producer strains. The results indicate a significant improvement of PHA yields when using WFO at a concentration of 20 g/L and applying gene knock-outs, especially*Δglk*. Although there are still drawbacks, our results basically proclaim that the amount of gained PHA by using WFO is higher than glycerol which is the material of choice for PHA production. Additionally, the successful incorporation of PHA with Tylosin, an antibiotic belonging to the macrolide group, could be the fresh direction in fights against antibiotics resistance. To sum up, *P. putida* can convert greasy wastes into biopolymers boasting wide applications in diverse fields, which paves the way to develop the straightforward procedure to recycle and value this waste on a real life.

1. Introduction

In today's world, hardly would there be anything that won't affect the environment, and the only difference is the measure of the impact they have on the environment. Environmental pollution is multiplying due to various anthropogenic activities, and poisonous impacts of waste frying oil (WFO) on the environment have been raising concerns worldwide. Basically, WFO is regarded as spent oil which has been employed for deep frying and is no more viable for further consumption. Waste cooking oil can be harmful to the microorganisms and other organisms. The presence of toxic, organic and volatile compounds such as acrylamide, aldehyde, 4-hydroxymethylfurfural in WFO has been known to have mutagenic and carcinogenic activities (Hageman et al., 1988). Additionally, toxic compounds in the oil can be readily dissolved into the water and absorbed into living cells, eventually killing plants and animals. Furthermore, the consumption of heated cooking oil or the produced toxic compounds can cause inflammation, endothelial dysfunction, high blood pressure and neurodegenerative diseases (Chun-Yi et al., 2014). If it is not properly processed, these materials would be a significant cause of some physical and environmental unfavorable conditions. To exemplify, when it is disposed directly into the nature without any plan for processing, it can coat plants and animals and deplete their oxygen, which results in suffocation and millions of deaths of these species. Another significant example is air pollution of its offensive odor, which will reduce the quality of the air we breathe in and eventually lead to respiratory diseases. They can

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also hamper the reproduction rate of plants and animals, which would undoubtedly affect the sustainability of these. Before long, humans would start feeling this scarcity on the food supply chain. To be more severe, these detrimental impacts are more prominently visible because of the proliferation of population as well as commercial establishments engaging in the food and beverages sector including hotels, restaurants, road-side eateries and even street vendors. This is due to the fact that they could dispose of hundreds of tons of WFO without planned or scientific methods of processing on a daily basis. There is also the large proportion of people which have a tendency to throw away oil-rich products without hesitation into drains or sewers. There is even the case where the WFO of households or manufactures is directly released into rivers, lakes or ocean, which is ubiquitously aired on the media. In Malaysia, an estimated 540,000 tons of WFO from vegetable and animal fats are discarded each year without being treated as wastes (Kheang et al., 2006). It is estimated that around 1–2.5 million metric tons per year of WFO are generated in the European Union (EU) countries. However, the current level of collection is very low, as it varies between 0.7 and 1 million metric tons per year (Kulkarni et al., 2006) (Mannu et al., 2020). They are a testament to the majority of spent cooking oil from houses disposed of in the drainage and soil. Although it is impossible to tackle the aforementioned issues in the near future, WFO is not an utterly useless thing, so throwing it away without any consideration will definitely be imprudent. If having a suitable method for processing, WFO will be treasures in lieu of worthless things. It has a host of commercial value owing mainly to its ability to be recycled into useful commercial products (Orjuela and Clark, 2020). Another feasible solution of recycling WFO will be presented in this study, where WFO is made use of producing PHA which is basically a family of intracellular polyesters accumulating in some bacteria for energy and carbon storage.

As a natural soil resident, *P. putida* is capable of utilizing divergent carbon substrates and thriving in versatile nutritional environments. Multiple carbon sources such as carbohydrates, organics acids and aromatic compounds can be broken down and transformed into building blocks and energy equivalents for cell growth in *P. putida* through cyclic central metabolism pathways including the Embden–Meyerhof–Parnas , Entner–Doudoroff , pentose phosphate pathways as well as the tricarboxylic acid cycle (Sudarsan et al.,2014) (Nikel et al., 2016), which could yield different amounts of NADH/NADPH to meet energy demands, support the biosynthesis of cellular components for the overall cell biomass and produce bio-products such as amino acids and lipids (Spaans et al., 2015). Additionally, *P. putida* attracts a lot of attention of researchers due to its promising capability to produce medium-chain-length PHA. Basically, PHA functions as a reducing equivalent pool to maintain cellular redox balance and defend against oxidative stress (e.g. from nutrients such as N and P being limited or xenobiotic compounds oxidative degradation process such as toluene) through the fatty acid synthesis and oxidation process. Because of its plastic-like properties and biodegradability, bioplastics made from PHA could potentially replace traditional petroleum plastics. In light of a strong desire for improvement of PHA biosynthesis, previous studies have mainly focused on nutrient limitation (e.g. N, P, S or Fe) (Chen and Jiang, 2018), overexpression of PHA synthesis pathways, shutting down competitiveness pathways (Zhuang et al., 2014), enhancing the NADH or NADPH supply (Ling et al., 2018), changing cell growth patterns for rapid proliferation, morphological engineering to increase the cell size (Jiang et al.,2012), the optimization of promoter or the ribosome binding site (Li et al., 2016) or engineering alternative extremophilic bacteria chassis (Tan et al., 2014). In general, the economic competitiveness of PHA production depends on the cost of feedstock, the PHA yield and the cost of downstream processing. Among these factors, commercial production of PHA is mainly hindered by the cost of the carbon source, which can account for up to 50% of the total cost (Favaro et al., 2019). Fortunately, the large amount of glycerol, which is easily attained from biodiesel, has become an ideal low-cost feedstock for the production of PHA (Poblete-Castro et al., 2014). Previous studies have extensively investigated the conversion of singular carbon sources into PHA in *P. putida*, including glycerol (Poblete-Castro et al., 2014), which is an effective way not only to potentially reduce carbon source costs, but also to increase carbon conversion efficiency. Aside from the large availability of glycerol, the huge amount of WFO, which is not appropriately processed, should be considerably noticed; therefore, in this research, we would give a new way to deal with the residual oil, which would demonstrate that using WFO to create valuable PHA is palpably possible.

PHA consists mostly of short-chain-length hydroxyalkanoic acids with monomers of three to five carbon atoms and medium-chain-length hydroxyalkanoic acids with monomers of six to fourteen carbon atoms. A less common class of PHA is long-chain-length hydroxyalkanoic acids, which has monomers of more than fourteen carbon atoms. PHA is ubiquitous in the field of nanomedicine, given their high loading capacity, biocompatibility, lack of toxicity and biodegradability. It also outweighs other bioplastics in the medical field because its monomers 3-hydroxybutyric acid and 4-hydroxybutyric acid are recognized by the human body as degradation products which are swiftly and naturally removal from the body (Griffin, 1993). Moreover, PHA-based drug delivery systems have proven to be highly effective due to the fact that the drug can easily reach a targeted goal with the aid of targeting ligands and also provide a controlled release of the incorporated drugs. It is no surprise that PHA has been utilized as a drug delivery system for cancer therapy, infections and other diverse applications;

however, US Food Drug Administration (FDA)-approved PHA-based medicines for treatment are unavailable. Until now, PHA-based therapies are in the experimental stages, and clinical trials are significantly lagging.

2. Methods

2.1 Strains and medium composition

The strains used in this study are *P. putida* KT2440 wildtype (WT), Δ*glk* and Δ*gcd*, which are kindly provided by Prof. Pablo I. Nikel, Denmark Technical University, Lyngby, Denmark. Two deficient glucose assimilation chassis strains, e.g. *Δglk* and *Δgcd* are created through the knock-out gene encoding glucose dehydrogenase and glucoskinase of WT strain. Due to a lack of the gene relating to glucose assimilation, these strains potentially own the capacity to use alternate carbon sources. All strains are preserved at −80°C in working seed vials (2 mL) with solution of LB medium and 30% of glycerol (w/v). Precultures and cultures have been performed on a defined mineral salt medium containing (in g/L): K_2HPO_4 14.6, $NaH_2PO_4.2H_2O$ 3.6; Na_2SO_4 2; $(NH_4)_2SO_4$ 2.47, NH_4Cl 0.5, $(NH_4)_2$-H-citrate 1, glucose 5, thiamine 0.01 and antibiotic 0.1. Thiamine is sterilized by filtration (0.2 µm). The medium is supplemented with 3 mL of trace element solution, 3 mL of a $FeCl_3.6H_2O$ solution (16.7 g/L), 3 ml of an EDTA solution (20.1 g/L) and 2 ml of $MgSO_4$ solution (120 g/L). The trace element solution contains (in g/L): $CoCl_2.H_2O$ 0.74, $ZnSO_4.7H_2O$ 0.18, $MnSO_4.H_2O$ 0.1, $CuSO_4.5H_2O$ and $CoSO_4.7H_2O$.

2.2 Bacterial cultivation

To begin with, *P. putida* was streaked on TSA medium and incubated for 24 h at 30 °C. Next, single colony was picked from the plate and transferred into 50 mL shake flasks containing 10 mL of Tryptic soy broth (TSB) before being incubated overnight under aerobic conditions at 30 °C and shaking speed at 180 rpm. To start the PHA-producing process from WFO, 1 L baffled shake flasks containing 200 mL of defined culture medium were modified to an initial OD_{595nm} at 0.1. Subsequently, 2.5 mL of this fluid was carefully transformed into another flask which contained 2.5 mL of a defined mineral salt medium, 62.5 µg/L of a trace element solution, 5 mL of WFO at concentration of 20 g/L and 17.5 mL of distilled water. The next step is when incubating it at 30°C by precisely 168 h before it was used for biomaterial-extraction step. Each culture was carried out in triplicate. Another test was similarly prepared; however, there was small alterations, which were utilizing glycerol at concentration of 10g/L for the replacement of WFO and the period of 48 h for incubation.

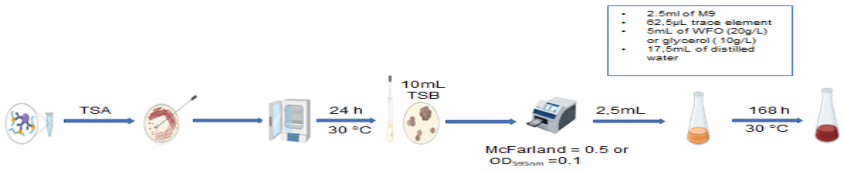

*Figure 1: The proced*ure for cultivating bacteria.

2.3 PHA extraction

To obtain pure PHA, biomass was harvested by centrifugation at 10,600 ×g, which was then washed twice with NaCl solution containing Tween 80 (0.01 %, w/v) and freeze dried. After freeze-dried cells were washed by using methanol to remove residual fatty acids, PHA was extracted from the cells in a Soxhlet apparatus using javel water as solvent. This PHA solution was concentrated in a rotary evaporator before it was precipitated by using 10 volumes of ethanol. The final step was when the polymer was dried at room temperatures. The PHA content (%) was defined as the percentage of PHA in the cell dry weight (CDW). All steps of experiments from section 2.2 and 2.3 were performed for all 3 strains including WT, *Δglk* and *Δgcd* to obtain PHA. Outcomes were then analyzed by the two-way ANOVA test. PHA acquisition was used to experiment the ability combining with antibiotics, particularly Tylosin.

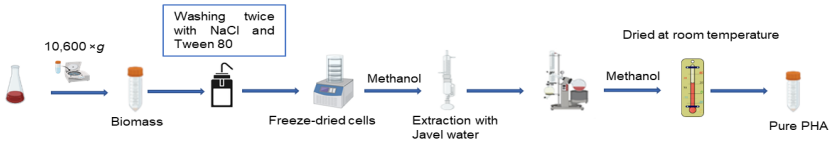

Figure 2: The procedure for PHA extraction

2.4 Antibiotic trapping

Pure PHA was dissolved in chloroform in the ratio of 1:1. PHA loaded with tylosin was prepared by the addition of 1 wt.% of the drug (related to the PHA mass) into the dissolving PHA and stirring for 15 min in a crockery. To determine the concentration of Tylosin combined with PHA, 100 mg of sample were dissolved in 5 mL of chloroform. 1 mL of this sample was then mixed with 10 mL of sodium phosphate buffer (0.1 mol/L, pH 7.4) for 30 min. The solution was filtered through a 0.45 µm cellulose acetate membrane before the determination of Tylosin was performed by using FTIR analysis. The experiments were run in triplicate.

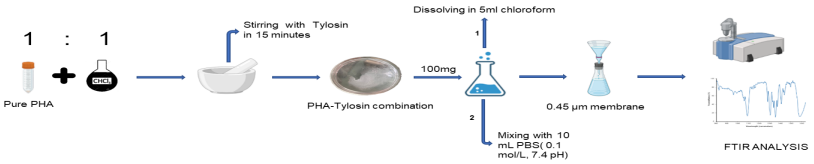

Figure 3: The procedure to determine the combination between Tylosin and PHA

3. Result

3.1 The figures for CDW and PHA gained from 3 various *P. putida* strains when cultivating in glycerol and WFO.

Table 1: The amount of CDW and PHA was obtained when using glycerol and WFO.

	CDW (g/L)		PHA (g/L)		The ratio of PHA to CDW (%)	
	Glycerol	*WFO*	*Glycerol*	*WFO*	*Glycerol*	*WFO*
Time for cultivating	48 h	168 h	48 h	168 h		
WT	7.38	16.5	6.10	12.87	83 %	78 %
Δ*glk*	7.85	17.7	6.12	13.275	78 %	75 %
Δ*gcd*	7.4	15.3	5.92	10.71	80 %	70 %

The two-way ANOVA test was performed to analyze the effects of cultivation in the medium of glycerol and WFO and three various *P.putida* strains on the yields of CDW and PHA. Simple main effects analysis showed that the growth medium did have a statistically significant effect on the production of CDW and PHA, p= 0.04 and 0.013 respectively (<0.05). With regard to the influence of various strains, while they had a statistically considerable impact on the creation of CDW and PHA with p=0.02 (<0.05) when being cultivated in WFO, the opposite was true for cultivation in glycerol with p= 0.234(>0.05). The detailed results were showed up in Figure 4 and 5.

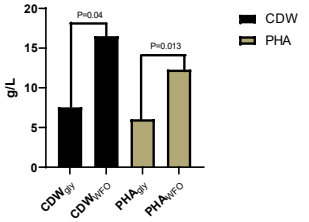

Figure 4: The difference between the amount of CDW and PHA when cultivating in the two various medium

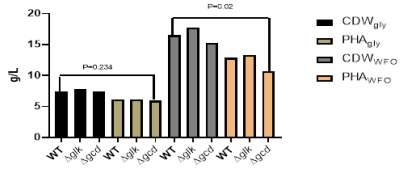

Figure 5: The difference between the amount of CDW and PHA when applying gene knock-outs in the two medium of cultivation.

3.2 Antibiotic combination

The FTIR result, which was the red line, illustrated the incorporation between Tylosin and PHA.

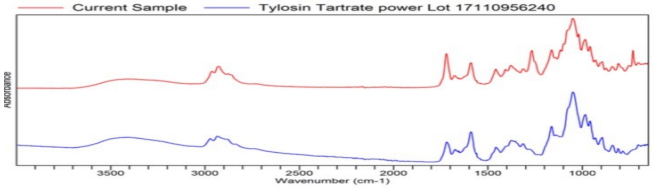

Figure 6: FTIR results of Tylosin-PHA combination compared to normal Tylosin

4. Discussions

It is obvious that *P. putida* KT2440 could utilize WFO and glycerol to create high biomass and the great amount of PHA with only using simple solvents and techniques. It is also notable that using WFO can result in the larger yield of CDW and PHA, whose figures are statistically significant with p= 0.04 and 0.13 respectively. Nevertheless, there is always a trade-off, which is a requirement of longer time for *P. putida* dissolving and consuming all the WFO with 168 h in comparison with 20 h by using glycerol. Among wild type and the 2 mutant *P. putida*, when they are cultivated in glycerol, albeit it does not have a statistically significant influence (p= 0.234> 0.05), Δ*glk* still acquires the best biomass and the largest content of PHA, which is 7.85 g/L and 6.12 g/L respectively. However, when making use of WFO, the yields of CDW and PHA are strongly created by Δ*glk* as against counterparts. The analysis results with p=0.02 is a testament to the fact that the WFO and eradication of *glk* could be a boon to the PHA production. From aforementioned outcomes, we can conclude that *P. putida* primary metabolism of glucose is by using glucokinase which is an enzyme encoded by *glk* gene; therefore, when there is the elimination of *glk*, *P. putida* is inclined to use other materials as carbon sources including glycerol and WFO instead of glucose. When it comes to Δ*gcd*, the yield of CDW and PHA is the least in WFO cultivation, which can confirm that cutting off glucose dehydrogenase, to some extent, does have a contrary impact. Regarding WT, it has almost the lowest figures compared with other 2 strains; therefore, glucose would be still the preferable resources if the glucose-transforming genes are not destructed. Although producing a lot of PHA, Δ*glk* also has its own drawbacks. Specifically, if it is not cultivated within approximately 18-24 h in a rich medium such as TSB, it would not grow or produce any PHA, which is not the case for the other strains.

Apart from the PHA extraction method mentioned above, PHA can be produced without centrifuge by using $0.45\mu m$ filter to eliminate carcasses. Subsequently, continuously pouring water and ethanol through the filter in order to purify the PHA before adding boiling chloroform into 3 marked petri dishes in such a way that it could immerse entirely the filter in the dishes. After exactly 15 s for waiting, taking the filter out and drying the petri dishes for about from 2 to 3 days. By using this special method, it can be applied for industrial scale. It would be great if fed-batch is used to maximize the PHA production simply because using WFO for culture may appear biofilm later on, which decrease PHA collected. It also noticeable that antifoam substances should be implemented due to the fact that appearances of foam may mitigate CDW, OD_{595nm} as well as bacteria growth and PHA content.

Our results also demonstrate that PHA have a potential to incorporate with antibiotics, which was successfully explored in other studies with various antibiotics like gentamicin sulfate, kanamycin sulfate and levofloxacin (Amini et al., 2018)(Kundrat et al., 2019)(Naveen et al., 2010). This outcome could provide a superior alternative to mitigate the antibiotics resistance or augment treatments using other polymeric carriers because they are otherwise defined as pharmaceutical apparatus or formulations that aid in the sustained, targeted release of a therapeutic Our results indicate the economical way, to some extent, to create PHA, and our application intensifies the ability to use PHA in fights against resistance of antibiotics which have been exacerbating.

5. Conclusion

We could reap a benefit from WFO if there is an appropriate method. The outcomes show that using WFO to extract PHA is extremely auspicious. It is also notable that eradication of *glk* gene could attain the higher amount of PHA; thus, this research could open the door for later studies to obtain the maximum content of PHA by using WFO. On the other hand, albeit antibacterial impacts of the Tylosin-PHA fusion were not carried out, the incorporation of Tylosin into PHA consolidated the application of PHA in drug-delivery systems, and last but not

least, our results could be positively fundamental to in-depth studies about officially using PHA as a method to treat infection in the future.

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