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Research paper

Biodiesel production from oleaginous yeast, *Cryptococcus* sp. by using banana peel as carbon source

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HIGHLIGHTS

- Monosaccharides were obtained from banana peel, pretreated with sulfuric acid.
- Cultivated with banana peel, *Cryptococcus* sp. obtained 34% lipid contents.
- Lipid contains high mono-saturates fatty acids, high biodiesel quality.
- Persulfate was used in lipid extraction and showed high extraction efficiency.

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ABSTRACT

An oleaginous yeast, *Cryptococcus* sp., was isolated from a traditional Korean fermented fish and used for bio-oil production with banana peel as a feedstock. Pretreatment of banana peel with 1% sulfuric acid resulted in up to 4.5 g L^{-1} of glucose (11.2% yield), and 18.1 g L^{-1} of fructose (45.2% yield). *Cryptococcus* sp., when grown on the pretreated banana peel, accumulated lipid up to 34.0%; and the lipid had a high degree of mono-saturation, which gives the resulting biodiesel better quality. Lipids were extracted using the pseudo-Fenton reaction based on persulfate as an oxidant, with the extraction efficiency of 91.1%. The data suggest that the new and potent oleaginous yeast, *Cryptococcus* sp., together with such cheap feedstock as the banana peel, can offer a competitive route for the production of bio-oil

1. Introduction

Global warming and energy crisis, two of the most serious problems that the world is facing, have a lot to do with fossil fuels: their intensive exploitation and consequent depletion. These issues can be resolved, though not entirely, by introducing renewable means, and biofuels like biodiesel can be a solution, at least in the transportation sector. Biodiesel is produced from biomass-derived triglyceride; some of the current sources include soybean oils, rapeseed oils, and corn oils (Felizardo et al., 2006). This renewable fuel, however, relies almost completely on food-based sources, which is ethically and even economically unacceptable at least in an ultimate sense. Thus, non-food sources such as microbial oils have been intensively studied as alternatives. The production of oils via microbial cultivation has advantages: oils can be continuously produced all year round and production does not require large areas of arable land (Angerbauer et al., 2008; Liu and Zhao, 2007; Papanikolaou and Aggelis, 2002; Zhao et al., 2008).

Microbes that can accumulate oils in the form of lipid bodies are called oleaginous microbes (Ma, 2006). The most common and, perhaps, the most promising of all are photoautotrophic microalgae, as they require only sunlight and carbon dioxide for their growth. Their practical application, however, has been limited due to their overly slow growth rate and low final cell concentration.

An alternative is heterotrophic microbes, especially oleaginous yeasts; they have fast growth rates and high lipid productivities. A critical issue of this heterotrophic approach, which is the need of expensive growth substrate, can be overcome by using cheap feedstocks such as food wastes, like Jerusalem artichoke (Sung et al., 2014) and cheese whey (Seo et al., 2014). One promising yet unexplored candidate is banana peel, as the banana market is the biggest fruit market throughout the world. In 2013, over 106 million metric tons of banana were produced (Statista, 2016), and the production is annually increasing. In fact, because it is a waste and the amount produced is tremendous, the disposal of banana peel is highly costly, so the beneficial use of the peel would contribute to the economy associated with banana in a general sense. The peel contains various minerals and nutrients and, most

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of all, about 60% of carbohydrates by weight (Anhwange et al., 2009); thus, it can be used in numerous applications including wine ethanol production (Tewari et al., 1985). Considering all, we attempted to examine its potential as a low-cost growth substrate for the cultivation of oleaginous yeasts, specifically a newly isolated *Cryptococcus* sp.

2. Materials and methods

2.1. Isolation of oleaginous yeasts

A traditional Korean fermented fish, Jeotgal, contains a significant amount of fish lipid, and *Cryptococcus* sp. (KCTC 27583) was isolated from a Jeotgal purchased in Suncheon Korea. Sabouraud Dextrose medium, which contained 40 g L^{-1} of dextrose, 10 g L^{-1} of peptone, 0.4 g L^{-1} of chloramphenicol, and 0.04 g L^{-1} of gentamicin, was used for the first enrichment of yeasts. 0.5 mL/L of gentamicin and chloramphenicol were included to eliminate undesirable bacteria. Diesel was used as a sole substrate when the yeasts were grown on agar plates as the second enrichment so that only oil-degraders could grow. After series of sub-culturing with 72-h incubations at 30 °C, single colonies of the yeasts were obtained. Through 18S rDNA sequencing (SolGent Co.), strains of oleaginous yeasts, including *Cryptococcus* sp., were identified (MK 758064). Their lipid bodies were then observed with fluorescent microscope after staining with Nile red.

2.2. Cultivation condition

The medium for *Cryptococcus* sp. growth contained 0.5 g of NH_4Cl, 2.7 g of KH_2PO_4, 0.95 g of Na_2HPO_4, 0.097 g of $MgSO_4$, and 10 mL of trace element in 1 L of distilled water, and the pH was adjusted to 6.5. The trace element contained 10 mL of 25% HCl, 1.5 g of $FeCl_2{\cdot}4H_2O$, 70 mg of $ZnCl_2$, 100 mg of $MnCl_2{\cdot}4H_2O$, 6 mg of H_3BO_3, 190 mg of $CoCl_2{\cdot}6H_2O$, 2 mg of $CuCl_2{\cdot}2H_2O$, 24 mg of $NiCl_2{\cdot}6H_2O$, and 36 mg of $Na_2MoO_4{\cdot}2H_2O$ in 1 L of distilled water. 30 g L^{-1} of glucose, fructose, and maltose were used as carbon sources for the characterization, and all cultivations were performed in a shaking incubator at 175 rpm and 28 °C.

Growth curves were obtained (Fig. 2) by measuring optical density at 600 nm (OD_{600}) using an UV–Vis spectrophotometer (DR 5000, HACH).

2.3. Banana peel preparation and pretreatment

Bananas, imported from the Philippines, were purchased from a market in Daejeon, South Korea. Peels were thoroughly washed with distilled water, dried at 50 °C for 4 days until the weight remained unchanged, and ground to form powder with a grinder. This banana peel powder (BPP) was then stored until needed.

For pretreatment, 40 g L^{-1} of BPP was suspended in a 1.0% (v/v) sulfuric acid solution in a stainless steel reactor (⌀ 26 x 132 mm with 70 mL of volume) coated with Teflon (Sung et al., 2014), and it was treated in an oil bath (WiseBath), at 80 °C, 100 °C, and 120 °C for 20, 40, and 60 min. As a control, 40 g L^{-1} of BPP was autoclaved at 121 °C for 20 min without the acid. After the pretreatment, the solid residue was filtered through a 0.22 μm filter, and then pH was adjusted to between 6 and 7 with NaOH. Sugar contents were analyzed by a high performance liquid chromatography (HPLC) equipped with an Aminex HPX-87 column (Bio-Rad Laboratories Inc., USA).

Glucose yield and fructose yield (% w/w) were then calculated by the following equation (Eq. (1)).

$$Yield = \frac{Final\ sugar\ content}{Total\ carbohydrate\ content} \times 100 \quad (1)$$

Final glucose and fructose contents (g L^{-1}) were analyzed by HPLC, and total carbohydrate (g L^{-1}) of banana peel was measured using phenol-sulfuric acid assay (Taylor, 1995). The pretreated BPPs were then used as carbon sources for *Cryptococcus* sp. cultivation, and all the experiments were done in triplicate.

2.4. Lipid extraction

As a reference for total lipid extraction, the Folch et al. method (1957) was used, but with slight modifications. After cells were cultivated for 72 h, they were centrifuged at 2000 rpm for 10 min and washed with deionized water. Harvested cells were then frozen in liquid nitrogen and lyophilized at −75 °C for 4 days using freeze dry system (Labconco, USA). 10 mg of dry biomass was suspended in 2 mL of chloroform: methanol solution with 2:1 (v/v) ratio and vortexed for 10 min, and then 1 mL of chloroform solution, containing 0.5 mg of heptadecanoic acid (internal standard), 1 mL of methanol, and 300 μL of H_2SO_4 were added to the solution. After vortex-mixed for 5 min, it was heated at 100 °C for 20 min. It was then cooled down at room temperature and 1 mL of deionized water was added, and the organic layer was separated from inorganic layer by centrifugation at 4000 rpm for 10 min. The final lipid contents in organic layer were analyzed through gas chromatography (GC) equipped with a HP-INNOWAX column (Agilent, USA, 300 mm × 0.32 mm ×0.5 μm, Standard: RM-3(C14–C24)).

A new method for cell breakage was adopted based on the oxidative power of persulfate. For that, 10 mL of *Cryptococcus* sp. cells (DCW: 2.3 g L^{-1}) that were cultivated for 72 h was first treated with different concentrations of sodium persulfate: 1, 2, and 3 mM, at 90 °C (Seo et al., 2016) for 1 h. A hydrogen peroxide-based treatment (0.5% H_2O_2) (Seo et al., 2015) was also used, for comparison. After the cells were disrupted, the lipids were retrieved with 5 mL of chloroform with shaking at 150 rpm for 1 h. The chloroform layer was separated by centrifugation at 1500 rpm for 5 min and removed, and the total lipid was recovered using the evaporator (Park et al., 2014). Lipid extraction efficiency was calculated according to the following equation (Eq. (2)).

$$Lipid\ extraction\ efficiency = \frac{Weighed\ lipid\ after\ evaporation}{Lipid\ content\ in\ 10\ mL\ cultivated\ cell} \times 100 \quad (2)$$

2.5. Statistical analysis

One-way analysis of variance (ANOVA) test and *t*-test were carried out for all the results to verify the statistical significance.

3. Results and discussion

3.1. Characterization of cryptococcus sp.

With a hypothesis that good oil-degraders may also accumulate lipid well, fermented fish, which typically contains a significant amount of fish oil, was selected as a targeted sample for the isolation, and several yeasts were isolated and identified through 18S rDNA sequencing. Among them, *Cryptococcus* sp. was selected and used in the subsequent experiments, because of the following features: fast growth rate, high lipid content, and ability to grow well with a number of different carbon sources (Fig. 2). The cell had size of 5–10 μm, and lipid bodies were clearly observed in red after Nile red staining, (Fig. 1).

For cultivation, 30 g L^{-1} of glucose, fructose, and maltose, which are major sugar components in the banana peel, were used and resulted in doubling times of 2.11, 2.35, and 2.45 h, respectively. Even without optimization, lipid contents of the cells after cultivations resulted around 30.4 ± 1.7% (DCW: 3.4 g L^{-1}).

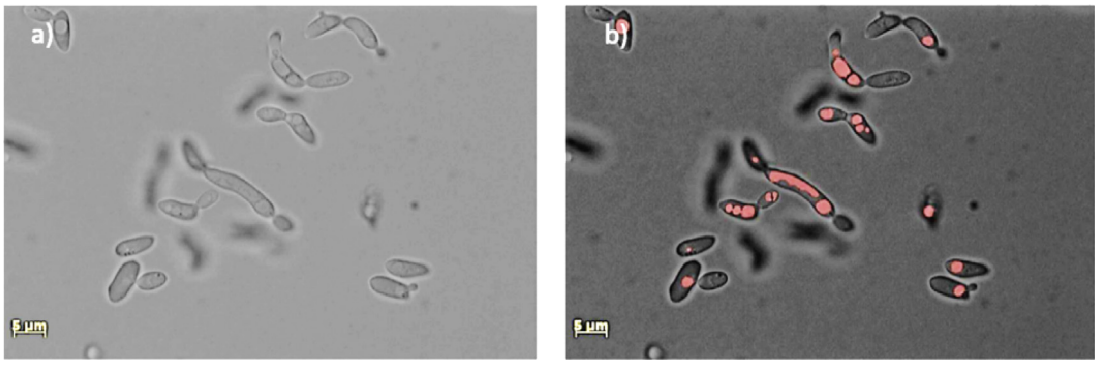

Fig. 1. Microscopic images of (a) *Cryptococcus* sp. and (b) lipid bodies stained with Nile red . (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

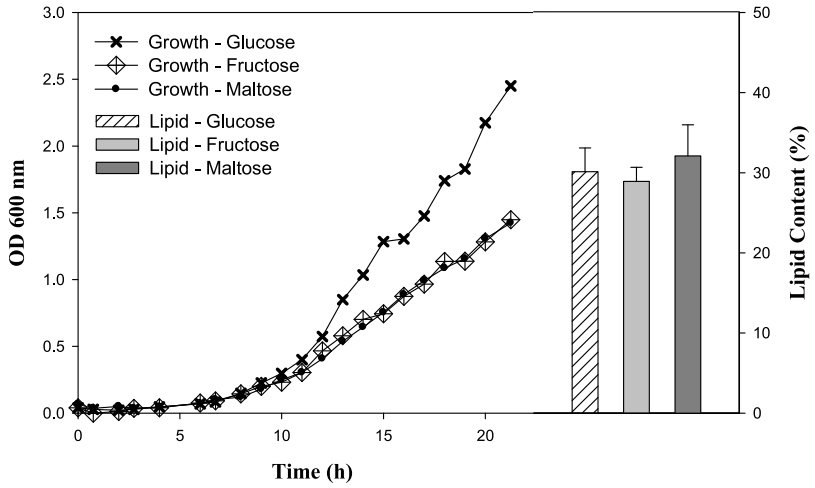

Fig. 2. Growth curve and lipid contents of *Cryptococcus* sp. cultivated with glucose, fructose, and maltose.

3.2. Sugar content of pretreated banana peel

After autoclave only, the BPP solution contained 2.4 g L^{-1} of glucose and 1.7 g L^{-1} of fructose, which corresponded to only about 6.0% and 4.3% yields, respectively. When the BPP was pretreated with heat only, the pretreatment efficiency was so poor that the amount of monosaccharides formed was low.

Regarding glucose, the acid-aided treatment performed best: e.g., 100 °C for 40 min led to the highest concentration of 4.5 g L^{-1} and 11.2% of yield among the results of various pretreatment conditions (Fig. 3). Fructose yield, on the other hand, was the highest at 120 °C for 40 min, resulting in 45.2% of yield and the maximum concentration of 18.1 g L^{-1} (Fig. 4). All this showed that the banana peel indeed contains sufficient carbohydrates, all of which can be easily retrievable as monosaccharides for the cell cultivation. High temperature (e.g. 120 °C) had a negative impact on the overall efficiency, however, because glucose and fructose were dehydrated to form furfural under an acidic condition. The Molisch's test (Agarwal, 2009) turned the BPP solution to slight violet, indicating that furfural was formed.

3.3. Lipid production and biodiesel quality

A maximum lipid content, 34.0% (DCW: 3.3 g L^{-1}), was obtained when cultivated with the BPP treated at 120 °C for 40 min, while a minimum lipid content, 26.3% (DCW: 2.8 g L^{-1}), was obtained with the BPP pretreated at 100 °C for 40 min (Fig. 5). When cultivated with the treated BPP, *Cryptococcus* sp. generally had lipid contents similar to when cultivated with other substrates: 30.7% versus 30.4%. The maximum lipid productivity was 1.12 g L^{-1} with the BPP, which was higher than 1.03 g L^{-1} of the one cultivated with other substrates. This implied that the BPP could serve as a cheap substitute for the expensive purified sugars with better productivity and performance.

To see if the resulting biodiesel satisfied criteria required for transportation fuels, quality assessment was carried out. Fatty acid compositions of the lipid extracted from *Cryptococcus* sp. were analyzed, and they work as variables to calculate cetane number (CN), oxidation stability (OS), cold filter plugging point (CFPP), and iodine value (IV), which are requirement standards deciding the quality of biodiesel. Although results were different depending on substrates, lipid of *Cryptococcus* sp. turned out to be better than vegetable oils in terms of biodiesel quality, as shown in Table 1.

CN is related with ignition delay time and combustion quality; high CN value represents short ignition delay, resulting in high-quality engine performance. BPP (80 °C/60 min) showed the highest value, passing the EN standard, while some vegetable oils do not meet the standard. OS is the relative susceptibility of the fuel to degradation by oxidation and determined by the correlation with the ratio of polyunsaturated methyl ester (Pullen and Saeed, 2012). Even though only BPP (80 °C/60 min) exceeded the EN standard, all other BPPs showed higher values than other vegetable oils. Regarding CFPP and IV, all BPPs satisfied the standard (except for BPP (80 °C/20 min)), indicating that they all contain high amount of saturated fatty acids and higher melting point with better oxidation stability.

BPP (80 °C/60 min) showed the highest quality, satisfying the EN standards in all the categories. Interestingly, even the oil

Table 1
Biodiesel quality assessment for vegetable oils and *Cryptococcus* sp. cultivated by various substrates and pretreated banana peel (WRI, 2014).

	Cetane number	Oxidation stability	Cold Filter Plugging Point (CFPP)	Iodine value
EN standard	>51	>6	<0 in summer <−10 in winter	<120
Soybean oil	49	1.3	−5	128
Corn oil	53	1.2	−12	101
Sunflower	44	4.4	−9	132
Cryptococcus sp.				
Glucose	49.3	5.01	−16.5	87.8
Fructose	49.8	5.12	−16.5	84.7
BPP (80 °C/20 min)	41.6	4.06	−16.5	138.5
BPP (80 °C/40 min)	48.8	4.92	−16.5	90.9
BPP (80 °C/60 min)	55.7	7.88	−16.5	45.7
BPP (100 °C/20 min)	49.4	5.04	−16.5	87.0
BPP (100 °C/40 min)	49.7	5.12	−16.5	84.8
BPP (100 °C/60 min)	49.6	5.11	−16.5	85.5
BPP (120 °C/20 min)	49.2	4.99	−16.5	88.6
BPP (120 °C/40 min)	47.1	4.64	−16.5	102.0
BPP (120 °C/60 min)	48.1	4.79	−16.5	95.6

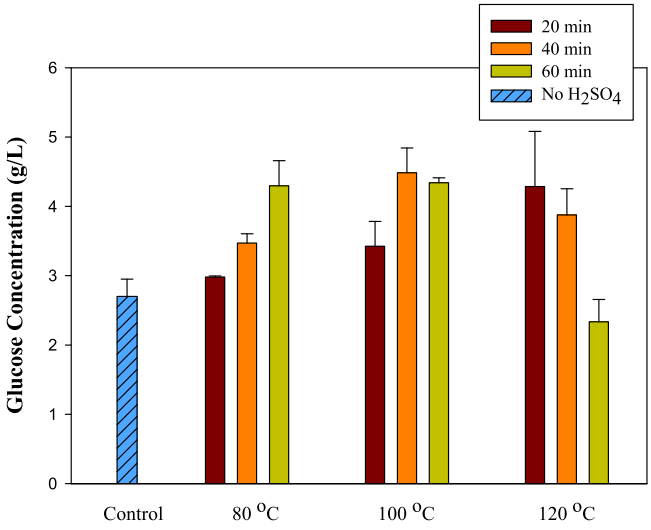

Fig. 3. Glucose concentrations in pretreated banana peel with various pretreatment conditions.

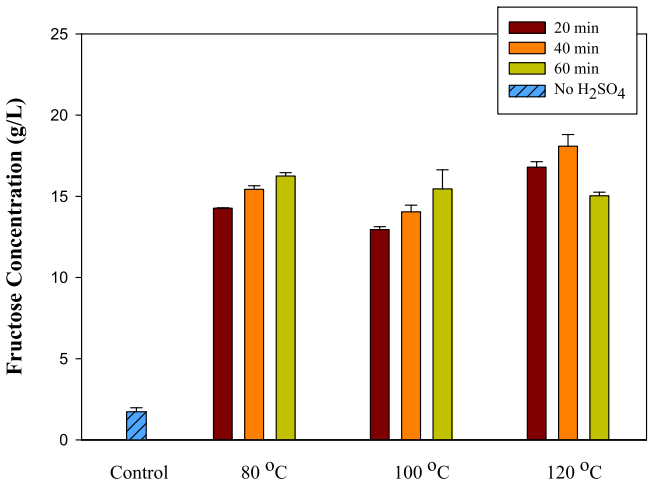

Fig. 4. Fructose concentrations in pretreated banana peel with various pretreatment conditions.

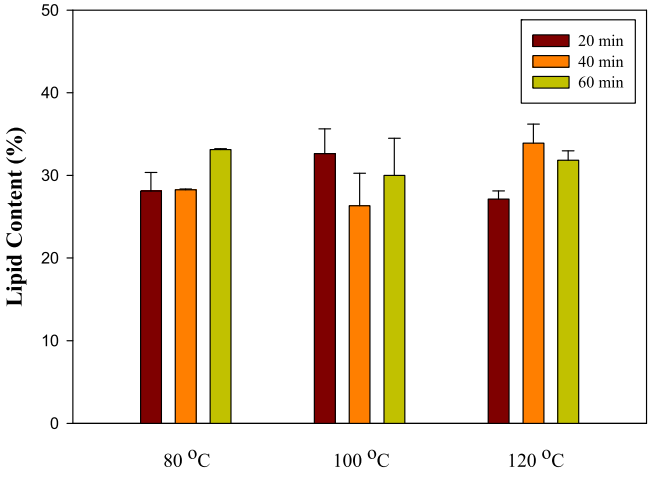

Fig. 5. Lipid contents of *Cryptococcus* sp. after cultivated with pretreated banana peel with various pretreatment conditions.

estimated to be the worst (BPP (120 °C/40 min)) showed better quality than the vegetable oils. If lipid contains more mono-unsaturated fatty acids, better biodiesel results from it (Fallen, 2009). In that sense, our isolated *Cryptococcus* sp. appeared to be well suited for this purpose. Also, the use of the banana waste as a feedstock can add economic competitiveness to it.

3.4. Lipid extraction using persulfate-based oxidation

Besides, oil extraction must be preceded by cell breakage to be sufficiently effective. To this end, we adopted persulfate, which is a cheap alternative to hydrogen peroxide for the Fenton reaction. It was found to be potent enough at all the tested concentrations, with the highest efficiency of 91.1% at 3 mM. This result, comparable to a hydrogen peroxide-based method (90.0%) in terms of value, was quite promising (Fig. 6); it is because the use of persulfate is approximately 13 times cheaper and yields lipid better suited for biodiesel production than that extracted with hydrogen peroxide (Seo et al., 2016).

3.5. Statistical analysis

After one-way ANOVA test and *t*-test, the statistical significances were analyzed for all the data. Regarding lipid contents, since all pretreated BPP contained enough sugar for the growth, the results did not show any significance when analyzed with the control. On the other hands, sugar concentrations of BPP after the pretreatments showed high significance levels, manifesting that pretreatment methods actually had major effect on the results.

4. Conclusions

This study showed that a food waste, banana peel, could be a cheap feedstock for the cultivation of oleaginous yeasts, such as our isolated *Cryptococcus* sp., on account of its property of containing rich sugars like glucose and fructose. Using the pretreated banana peel, *Cryptococcus* sp. accumulated 34.0% lipid with 1.12 g L^{-1} of lipid productivity, which was even higher than with pure forms of sugars. Besides, extracted oil was found to have exceptional quality so as to form high-grade biodiesel, and it appeared to be more so with the effective and economical extraction method based on persulfate.

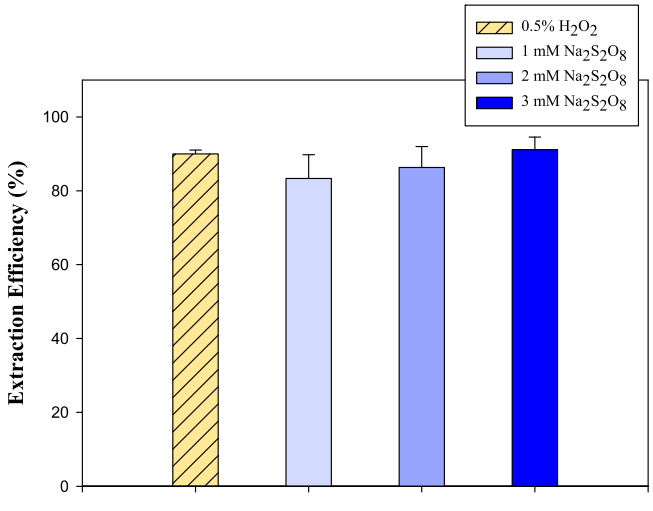

Fig. 6. Lipid extraction efficiency using hydrogen peroxide and different amount of persulfate.

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Appendix A. Supplementary data

Supplementary material related to this article can be found online at https://doi.org/10.1016/j.egyr.2019.07.012.

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