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BIOPROSPECTING OF CELLULOLYTIC AND BIOSURFACTANT-PRODUCING BACTERIA FOR ORGANIC WASTE TREATMENT

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ABSTRACT

Increasing amounts of organic wastes such as cellulosic materials and waste oils which are difficult to manage using conventional physico-chemical processes necessiate the need for the development of more effective waste treatment approaches. One of the most attractive efforts is using cellulolytic enzymes and biosurfactants from microorganisms for the treatment of recalcitrant cellulosic materials and waste oils. Those biomolecules are not only effective for specifically removing targeted contaminants, but also easy to produce using cheap raw materials and non-toxic. To obtain native cellulolytic enzymes- and biosurfactants-producing bacteria which are expected to work optimally for organic waste treatments in Indonesia, we conducted bioprospecting in pine forest and hot spring ecosystems of Raden Soerjo Forest Park, Batu, East Java. As the results, two bacterial strains which exhibit cellulolytic activity and biosurfactant producing ability were isolated and characterized. HS1 strains isolated from hot spring was identified as *Bacillus subtilis* sub sp. *Inaquosorum* and is of interest due to its ability to produce cellulolytic enzyme and biosurfactant at high temperature which is a valuable feature for large-scale waste treatments.

KEY WORDS : Waste treatment, Cellulolytic Enzymes, Biosurfactants, Bioprospecting, Thermophilic Bacteria

INTRODUCTION

Recently, the rate of organic wastes released into the environment is increasing at an alarming rate (Makarichi *et al.*, 2018). Most of the organic wastes are disposed directly into landfills which increase the emission of greenhouse gas and unpleasant odor, as well as the contamination of ground water (Ma *et al.*, 2018). Moreover, current efforts on the treatment of those organic contaminants are rendered difficult due to the presence of recalcitrant components such as cellulosic materials and waste oils.

Cellulosic materials from paper and pulp, agricultural, and forestry industries, and foods, among others, are the single most voluminous byproduct of our society (Khan *et al.*, 2016). Those wastes are commonly treated using heat and chemical compounds which are uneconomical, energy-consuming, and detrimental to the environment. Similar problems also hindered the effective treatment of waste oils. Synthetic surfactant used in the treatment of petroleum hydrocarbons and its derivatives, waste frying oils, and used engine oils, among others, are expensive and often toxic (De *et al.*, 2015). Thus, the development of alternative treatment methods for cellulosic and oil wastes that are cheaper, more reliable, and more environmentally friendly than current processes are needed.

One of the most attractive treatment methods are the use of cellulolytic enzymes and biosurfactants

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from microorganisms. Those compounds act specifically towards certain substrates, are easy to produce from cheap raw materials, and generally non-toxic (Vijayakumar and Saravanan, 2015). Cellulosic enzymes included cellobiohydrolase (EC 3.2.1.91), cellulase (EC 3.2.1.4) and β-glucosidase (EC 3.2.1.21) work specifically towards degradation of cellulosic materials to fermentable sugars that can be converted to high value products such as bioethanol and organic acids (Chia-wen *et al.*, 2015). While, biosurfactants are surface active biological compounds which are able to solubilize hydrophobic substrates such as oils and hydrocarbon and ease their removal from contaminated environments (Santos *et al.*, 2016).

To obtain native cellulolytic enzymes- and biosurfactants-producing bacteria which are expected to work optimally for organic waste treatments in Indonesia, we conducted bioprospecting in pine forest and hot spring ecosystems of Raden Soerjo Forest Park, Batu, East Java. One thermotolerant strain and one mesophilic strain were isolated and their potency to produce cellulolytic enzymes and biosurfactants were also evaluated.

MATERIALS AND METHODS

Sampling site and method: Sampling was conducted in Pine forest and hot spring at Raden Soerjo Forest Park, Batu, East Java, Indonesia. Soil sample were collected from different sites of the pine forest at 112°32'00,6" E Longitude 07°44'21,7" S Latitude, stored in sterile container, and immediately brought into the laboratory. Sediment sample were collected from different sites of the hot spring at 112° 32' 0'' E Longitude and 7° 44' 31'' S Latitude in sterile insulated water bottles and immediately brought into the laboratory.

Isolation of bacteria: Bacteria were isolated in CMC (carboxymethyl cellulose) Agar media (Naresh *et al.*, 2019) using serial dilution technique. Ten grams of soil or sediment sample was diluted in 90 mL of sterile NaCl 0.85% in 250 mL conical flask and kept it in orbital shaker at 100 rpm to get a homogenized soil suspension. Serial dilution was made and dilution of 10^{-2} and 10^{-3} for hot spring sediment and 10^{-9} and 10^{-10} for pine forest soil sample were spread onto CMC Agar plates and incubated at 30 °C for 48h.

Screening of Cellulolytic Activity: The cellulolytic activity of one isolate from pine forest soil sample (S1) and one isolate from hot spring sediment sample (HS1) which showed growth on CMC agar plate were evaluated using CMC plate assay (Mohammad *et al.*, 2017). Single colony of S1 was streaked onto CMC agar plate and incubated at 30 °C for 48 h, while single colony of HS1 was streaked onto CMC agar plates and incubated at 40 °C and 50 °C for 48h. After incubation, the agar medium was overflown with Congo Red solution (1% w/v) for 30 minutes. The Congo Red solution was then discarded, and the plates were further treated by flooding with 1 M NaCl for 30 minutes. The formation of clear zone of hydrolysis indicated cellulose degradation.

Screening of Biosurfactants Production: The ability of S1 and HS1 isolates to produce biosurfactants was screened using hemolytic activity assay (Ibrahim, 2018). In this assay Nutrient Agar (NA) supplemented with 5% sheep blood was used. Single colony of S1 was streaked onto blood agar plate and incubated at 30 °C for 48h, while single colony of HS1 was streaked onto blood agar plates and incubated at 40 °C for 48h. Hemolytic type was confirmed according to hemolysis result and clear zone color.

Identification and characterization of the bacterial isolate: S1 and HS1 were streaked onto Nutrient Agar plates incubated at 30 °C for 24h. Shape, color, elevation and margin of the colonies were characterized. Gram staining of the isolates were also conducted using Grams Stain-Kit (HiMedia, India).

16S rRNA Gene Amplification and Sequencing. Genomic DNA of HS-1 was extracted and purified using Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, USA). Amplification of the target region of the 16S rRNA 27F gene were conducted using 27F (52- AGA GTT TGA TCM TGG CTC AG-32) and 1492R (52- TAC GGY TAC CTT GTT ACG ACT T-32) primers (Satyapal *et al.*, 2018). The PCR product purification was conducted using DNA Clean & Concentrator™-5 (Zymo Research, USA). The purified PCR products were sequenced by First Base (Malaysia). The deduced sequences were compared for homology against the NCBI database of 16S ribosomal RNA sequences using Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and 16S-based ID from EZBioCloud (https://www.ezbiocloud.net/identify) (Yoon *et al.*, 2017).

RESULTS AND DISCUSSION

Sampling was conducted in February (rainy season), where the temperature of the sampling site at the pine forest was 22.5 °C and the pH was 6.2. While the sampling site at the hot spring was 50.1°C and the pH was 6.3. The sampling site was fed with plant litter, which might indicate the presence of microorganisms that can utilize the cellulosic materials from those dead plant materials.

One colony of bacteria was isolated from each sampling site, the one isolated from the soil of pine forest was termed as S1 and the other isolated from hot spring was termed as HS1. S1 isolate was gram-negative cocciwhich formedwhite round colonies withflat elevation and entire margin. Meanwhile, HS colony was endospore-forming, Rod-shaped Gram-positive which formed white round colonies with raised elevation and lobate margin. Molecular identification was also conducted for HS1 isolate based on partial 16S rRNA gene sequencing. Alignment with reference sequences from NCBI database of 16S ribosomal RNA sequences using Basic Local Alignment Search Tool (BLAST) and 16S-based ID from EZBioCloud revealed that HS-1 was 99.93% and 99.86% identical with *Bacillus subtilis* sub sp. *inaquosorum*, respectively. As for S1 isolate, molecular identification will be conducted in the future study.

As expected, both isolates showed cellulolytic activity indicated by the presence of clear zone around the colonies was observed in CMC agar after 48 hours incubation at 40 °C (Fig. 1a) and 50 °C for HS1 and at 30 °C for S1 (Fig. 1b). Both isolates also showed hemolytic activity which were related to the ability to produce biosurfactant. Green discoloration around HS1 and S1 colonies on blood agar indicated the alpha hemolysis activity of both isolates (Eldin *et al.*, 2019).

The production of cellulolytic enzyme and biosurfactantsby HS-1 isolate is amenable with previous reports. *Bacillus subtilis* sub sp. *inaquosorum* was reported to produce alkaline cellulase which is valuable enzyme for agricultural, food, and industrial wastes treatment and lipopeptide biosurfactant which can be utilized as agent for waste oil treatments (Gautam and Sharma, 2014; Regmi *et al.*, 2017; Varadavenkatesan and Murty, 2013). The ability of the HS-1 isolate to produce cellulolytic enzyme and biosurfactants at 50 °C is

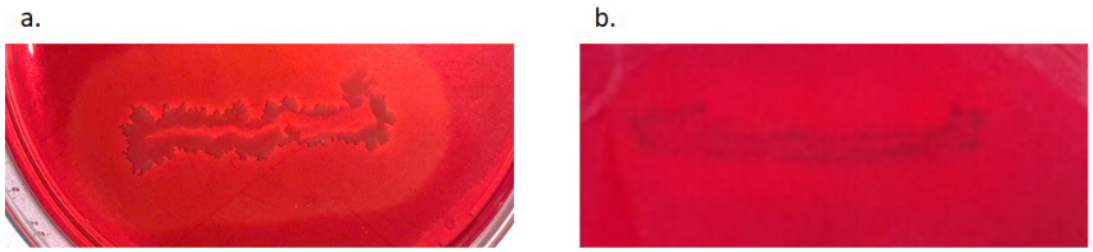

Fig. 1. Cellulolytic activity of (a.) HS1 isolate at 50 °C and (b.) S1 isolate at 30 °C indicated by clear zone surrounding CGR-1 colonies after treatment with Congo Red and NaCl.

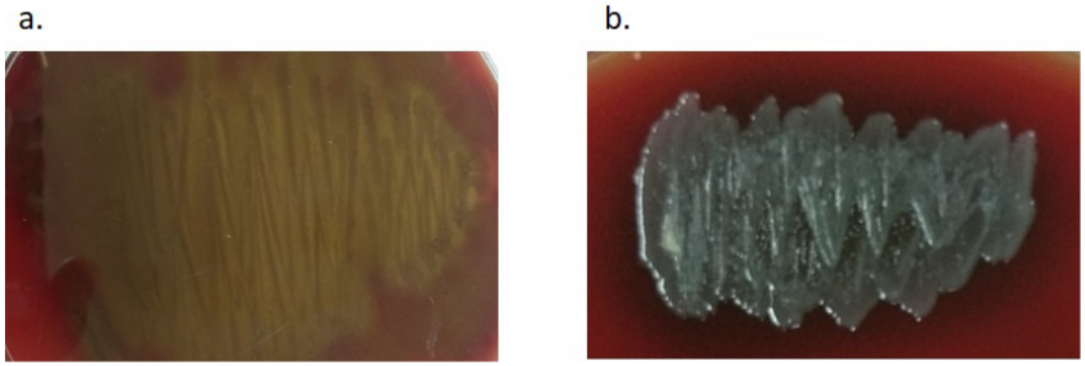

Fig. 2. Hemolytic activity of (a.) HS1 isolate at 40 °C and (b.) S1 isolate at 30 °C indicated by green discoloration surrounding the colonies.

industrially attractive, since waste treatments often require operation in harsh temperature condition (Cai *et al.*, 2017; Restaino *et al.*, 2018).

CONCLUSION

Bioprospecting of bacteria for waste treatment at Raden Soerjo Forest Park, Batu, East Java, Indonesia resulted in the isolation of two cellulolytic enzymes and biosurfactants-producing bacterial strains S1 and HS1. *Bacillus subtilis* subsp. *inaquosorum* HS1 isolated from hot spring, in particular, is of interest due to its ability to produce cellulolytic enzyme and biosurfactant at high temperature which is a valuable feature for large-scale waste treatments. In the future, research on the characterization and production of cellulolytic enzymes and biosurfactants using both isolates will be conducted. Furthermore, the application of both isolates for treating cellulosic materials from agricultural, food, and industrial wastes, as well as waste oils will also be performed.

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