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Original Article

Effect of culture medium on polymer production and temperature on recovery of polymer produced from newly identified *Rhyzopus oryzae* ST29

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Abstract

Thermotolerant fungal isolate ST29 was identified by observing on cell morphology and molecular technique based on internal transcribed spacer (ITS) gene to be *Rhizopus oryzae.* Among four culture media tested, the strain exhibited the highest growth in yeast malt extract (YM) medium (4.87 g/l), followed by Sabouraud dextrose broth (SDB) (4.25 g/l), potato dextrose broth (PDB) (4.10 g/l) and palm oil mill effluent (POME) (3.29 g/l), respectively, after 4 days cultivation at 45°C. However, the strain was found to produce polymer only in POME medium at 45°C, but not in the three synthetic media tested. Effect of temperature on separation of the biopolymer produced by this fungal strain was studied by incubating the culture broth in water bath with temperatures in the range of room temperature to 70°C. The biopolymer was recovered by filtration, centrifugation, and precipitation by adding 4 volumes of 95% ethanol, then freeze-drying. These temperatures therefore had no influence on the biopolymer yields (5.58-5.78 g/l) or on biomass yields (2.90-3.29 g/l).

Keywords: identification, separation, biopolymer, thermotolerant fungi, palm oil mill effluent

1. Introduction

Palm oil mill effluent (POME) is the mixed effluent generated from two major sources : sterilizer condensate and decanter effluent during the extraction of palm oil. Characteristics of POME include having high organic matter (BOD and COD values of 58,475 mg/l and 110,400 mg/l, respectively), high total solids and suspended solids (71,939 mg/l and 43,280 mg/l respectively), as well as oil & grease (25,600 mg/l) but low nitrogen content (900 mg/l) with acidic pH (4.5) and high temperature (70-90°C) (Pechsuth *et al.,* 2001). The POME is therefore a high strength wastewater and contains nutrients that could be utilized by many microorganisms.

Preliminary studies indicated that the thermotolerant fungal isolate ST29 produced biopolymer during cultivation in palm oil mill effluent (POME). To produce this biopolymer in defined medium, screening on different synthetic media was conducted in this study. In addition, it was anticipated that part of the polymer may be attached to the fungal mycelium, therefore, the effect of temperature on separation of this biopolymer from the fungal mycelium was also studied. Prior to these studies, the biopolymer-producing fungal strain was identified.

2. Materials and Methods

2.1 Fungus

Thermotolerant fungal strain ST29 was isolated from a palm oil mill and kept in the Culture Collection of Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Hatyai, Thailand. The strain was maintained on potato dextrose agar (PDA) at 4°C and subcultured every month.

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2.2 Identification of the thermotolerant fungal isolate ST29

The thermotolerant fungal isolate ST29 was identified based on cell morphology and molecular technique. For cell morphology, the strain cultured on Sabouraud dextrose agar (SDA) at 45°C for 4 days was used to observe the cell morphology under microscope (Sutton *et al.,* 1998). For molecular technique, the isolate ST29 was identified based on internal transcribed spacer (ITS) region. The primers ITS1 and ITS5 were used in polymerase chain reaction (PCR) to amplify the internal transcribed spacer (ITS) regions of the isolate and the basic local alignment search tools (BLAST) on the NCBI website (http://www.ncbi.nlm.nih.gov) used in identification.

2.3 Effect of culture medium on biopolymer production

Spore suspension was prepared by adding 10 ml of 0.1% Tween 80 onto PDA agar slant of 5 d fungal isolate ST29 and its concentration was adjusted to 2.4×10^6 spores/ml. The prepared starter culture (10%) was inoculated into palm oil mill effluent (POME), yeast malt extract (YM) medium, Sabouraud dextrose broth (SDB) and potato dextrose broth (PDB) in a 250 ml flask with the initial pH of 4.5. The cultivation was done in triplicate and incubated on a shaker (200 rpm) at 45°C for 4 days. The biopolymer production in these media was observed visually and its concentration as well as mycelium dry weight were determined at the end of cultivation. The culture broth was filtered to separate the mycelium, then centrifuged (8,000 x *g* for 20 min at 4°C), and the supernatant mixed with four volumes of 95% ethanol, stirred and kept at 4°C overnight. The precipitated biopolymer was obtained after centrifugation, then freeze dried. For determination of dry cell weight (DCW), the mycelium pellets were washed twice with distilled water, dried at 60°C to constant weight, then cooled and weighed (Selbmann *et al.,* 2002).

2.4 Effect of temperature on recovery of the biopolymer

The fungal isolate ST29 was cultivated in POME as described above. The effect of temperature on separation of the biopolymer from the mycelium was studied by incubating the culture broth in water bath at room temperature, 40°C, 50°C, 60°C and 70°C for 15 min, then centrifugation and precipitation the polymers from the supernatant as described above.

3. Results and Discussion

3.1 Identification of the thermotolerant fungal isolate ST29

On Sabouraud dextrose agar, colonies grew very fast and appeared as white cotton-like colonies then became brownish-grey to blackish-grey depending on the age of sporulation. Rhizoids were found at the junctions of the stolons and sporangiophores, broad hyphae and chlamydospore (Figure 1). The strain was identified as *Rhizopus* sp. and distinguished from other members by the presence of well-developed rhizoids situated opposite sporangiophores (Diaz *et al.,* 2000). Further identification by using molecular technique was carried out based on ITS region of this fungal isolate which was amplified by polymerase chain reaction (PCR) using primer ITS1 and ITS5 and homology search was performed using BLAST search of NCBI (Park *et al.,* 2001). The sequence of the isolate ST29 showed homology with *Rhizopus oryzae* with 98% identity (Figure 2).

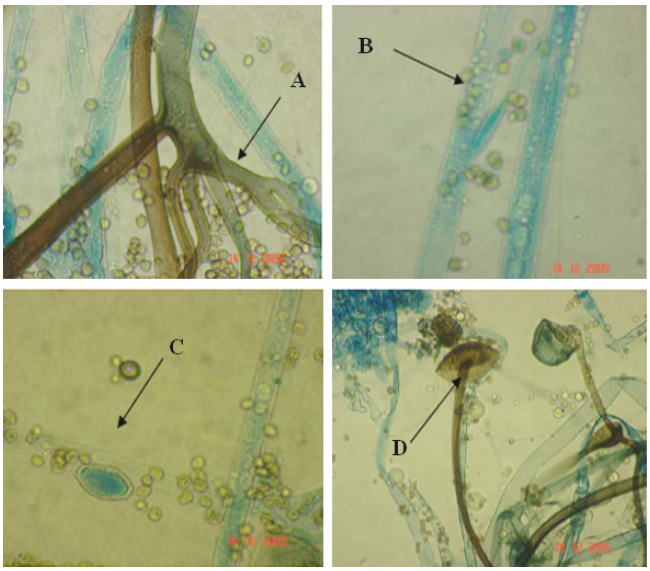

Figure 1. Microscopic morphology of *Rhizopus* sp. ST29 on Sabouraud dextrose agar
(A) brownish rhizoids,
(B) broad hyphae,
(C) chamydospores,
(D) sporangiophores with columella

3.2 Effect of culture medium on biopolymer production

Among four different media tested, *Rhizopus oryzae* ST29 grew better in the media of YM, SDB and PDB than in POME, giving biomass yields of 4.87, 4.25, 4.16 and 3.29 g/l, respectively, after 4 days cultivation at 45°C. However, the strain was observed to produce biopolymer only in POME cultivation but not in these three media. This may be due to the fact that POME contained high C/N ratio which is known to enhance the formation of biopolymer (Sutherland, 1996). Analysis of the C/N ratio of POME in this study gave the value of 40:1 which was much higher than those of YM (8:1), SDB (6:1) and PDB (10:1). In addition, POME also contains high organic matter (36,000 g/l COD, 10 g/l oil) but was low in nitrogen content (1.04 g/l). Composition of dried POME was reported to be 34-44% N-free extract, 23-32% ether

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Query  1    CGGAAGGATCATTAACTAATGTATTGGCACTTTACTGGGATTTACTTCTCAGTATT
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  19   CGGAAGGATCATTAACTAATGTATTGGCACTTTACTGGGATTTACTTCTCAGTATT

Query  61   GCTTCTATACTGTGAACCTCTGGCGATGAAGGTCGTAACTGACCTTCGGGAGAGAC
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  79   GCTTCTATACTGTGAACCTCTGGCGATGAAGGTCGTAACTGACCTTCGGGAGAGAC

Query  121  GACATATAGGCTATAATGGGTAGGCCTGTTCTGGGGTTTGATCGATGCCAATCAGG
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  139  GACATATAGGCTATAATGGGTAGGCCTGTTCTGGGGTTTGATCGATGCCAATCAGG

Query  181  CCTTTCTTCCTTTGGGAAGGAAGGTGCCTGGTACCCTTTACCATATACCATGAATT
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  241  ATTGAAAGTATAATATAATAACAACTTTTAACAATGGATCTCTTGGTTCTCGCATC
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Sbjct  259  ATTGAAAGTATAATATAATAACAACTTTTAACAATGGATCTCTTGGTTCTCGCATC

Query  301  AAGAACGTAGCAAAGTGCGATAACTAGTGTGAATTGCATATTCGTGAATCATCGAG
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  541  CATCGTGCTTTTTTGGTCCAACCAAAAAACATATAATCTGGGGGTTCTGCCAGCCA
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Query  601  ATAT----------TTTAACTATGATCTGAAGTCAAGTGGGACTACCCGCTGAACT
            ||||          ||||||||||||||||||||||||||||||||||||||||||
Sbjct  619  ATATTTTAATGATCTTTAACTATGATCTGAAGTCAAGTGGGACTACCCGCTGAACT
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Figure 2. The sequence of the isolate ST29 showed homology with *Rhizopus oryzae* with 98% identity (BLAST search of NCBI from web http://www.ncbi. nlm.nih.gov).

Table 1. Effect of temperature on recovery of biopolymer and biomass from the culture broth of *Rhizopus oryzae* ST29 after 4 days cultivation in palm oil mill effluent at 45°C

Temperature (°C)	Biopolymer(g/l)	Dry cell weight (g/l)	Final pH
Room temperature	5.58 ± 0.16 [a]	3.29 ± 0.34 [a]	6.21 ± 0.04 [a]
40	5.62 ± 0.20 [a]	2.90 ± 0.07 [a]	6.69 ± 0.03 [b]
50	5.64 ± 0.14 [a]	3.03 ± 0.05 [a]	6.18 ± 0.04 [a]
60	5.67 ± 0.11 [a]	2.98 ± 0.27 [a]	6.20 ± 0.04 [a]
70	5.78 ± 0.13 [a]	3.07 ± 0.04 [a]	6.06 ± 0.25 [c]

Values were mean ± SD of triple determinations and values followed by the same letters are not significantly different by Duncan's multiple range test (DMRT) (P = 0.05).

extract, 11-12% fiber, 8-11% protein, 2.5% total sugar and 11-14% ash (Agamuthu and Tan, 1985). *Rhizopus* sp. was previously reported to have the ability to produce an exo-polysaccharide (EPS) (Sankpal *et al.,* 2001) which is serologically active (Miyazaki *et al.,* 1979), possessing anti-tumour properties (Yang *et al*., 2000; Chihara *et al*., 1970) and as a heat-stable antigen (Notermans and Soentoro, 1986). In addition, several species of fungi are used as traditional medicines in treatment of different human diseases such as hepatitis, hypertension, hypercholesterolaemia, and gastric cancer (Park *et al*., 2001). It is therefore essential to characterize the biopolymer from *Rhizopus oryzae* ST29 for further exploitation and this has been under investigation.

3.3 Effect of temperature on recovery of the biopolymer

The effect of temperature on separation of the biopolymer from mycelium of *Rhizopus oryzae* ST29 was studied at room temperature (~30), 40, 50, 60 and 70°C for 15 minutes. These temperatures were chosen with the aim of decreasing the biopolymer. Results indicated that there was no difference in the biopolymer yields (1.85-1.92 g/l) and biomass yield (5.59-5.72 g/l) (Table 1). Therefore, the biopolymer can be extracted at room temperature at lower cost than treating at higher temperatures. Temperature at 70°C was previously reported to be the minimum temperature necessary to obtain optimum viscosity of the polysaccharide gum for its recovery (Gaddy and Patton, 2005). However, it was noted that 70°C was the minimum temperature tested in that study (70-120°C). In general, the effect of temperature depends on the type and viscosity of the polymer under study.

4. Conclusion

The thermotolerant fungal isolate ST29 was identified as *Rhizopus oryzae.* The strain produced biopolymer during cultivation in palm oil mill effluent (POME) at 45°C, but not in the media tested (YM, SDB and PDB). The tested temperature (room temperature to 70°C had no effect on recovery of the biopolymer from the culture broth of *R. oryzae* ST29.

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