

Full Length Research Paper

Enhancing riboflavin production through seawater-derived *Candida tropicalis*

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Screening of *Candida* spp. capable of riboflavin production from seawater samples in Chonburi province was conducted. Total of 47 isolates were identified as *Candida tropicalis* and *Candida krusei* with similar percentages of distribution. Among those identified isolates, only two of *C. tropicalis*, MICBUU002 and MICBUU019, were found to be able to produce riboflavin with concentrations of 254.22 ± 5.51 and 199.53 ± 0.74 ug/ml, respectively, on day-5 of incubation at 30°C in a medium broth containing 2% glucose as a sole carbon source. In contrary, none of *C. krusei* isolates showed the capability to produce riboflavin. The results implied that the capability of *C. tropicalis* to produce riboflavin was not an exclusive property of this specie since not every isolates of this *Candida* had this capability, at least at the detectable level that could be measured.

Key words: *Candida*, *C. tropicalis*, riboflavin, vitamin B2.

INTRODUCTION

Morphological shape of many species of *Candida* spp. yeast cell is usually round to oval and divides itself by budding. It may also form septate hyphae or pseudohyphae in favourable conditions. Different species may utilize different types of carbohydrates for growth (Kurtzman and Fell, 1998). *Candida* spp. could be found in several natural habitats, either terrestrial or aquatic environments. Some species could live with other living organisms. *C. albicans* is a normal flora on skin and vagina of healthy humans. However, it could also be an opportunistic pathogen when host's immune system is weak such as that found in immune-compromised patients (Molero et al., 1998).

There are over 163 species of *Candida* spp. identified by biochemical characteristics (Kurtzman and Fell, 1998) and only a few of those were isolated from seawater and sea sediments; for example *C. neustonensis* (Chang et al., 2010), *C. parapsilopsis*, *C. tropicalis*, *C. guilliermondii*, *C. intermedia*, *C. quercitrusa*, *C. rugosa*, *C. zeylanoides*, *C. membranifaciens* (Roth et al., 1962; Wang et al., 2008b). Some species of *Candida* spp. may synthesize enzymes or bio-molecules such as riboflavin that could be biotechnological importance. For example, *C.*

membranifaciens (Wang et al., 2008a, b) *C. guilliermondii*, *C. flareri* (Levine et al., 1949) and *C. famata* (Heefner et al., 1992) were reported that they could produce considerable amount of riboflavin as a primary metabolite. Among those species, only *C. membranifaciens* was reported to be an isolate from marine environment (Wang et al., 2008a).

Riboflavin, also known as vitamin B2, is a water-soluble vitamin. It is a precursor of flavin mononucleotide (FMN) and flavin adenine nucleotide (FAD), which are the components of flavoproteins (Bacher et al., 2000; Fischer and Bacher, 2005). These coenzymes are required for redox reactions of many cellular metabolic processes such as amino acid pathways, anabolic pathways, folate pathway (Massey, 2000).

Riboflavin has important roles in medical applications. Therapeutics that relies on natural substances such as vitamins may be the best method for curative purposes. Riboflavin could alleviate and prevent migraine symptom (Bigal et al., 2002; Breen et al., 2003; Woolhouse, 2005), night blindness (Graham et al., 2007) or anemia (Faber et al., 2005; Makola et al., 2003). Furthermore, enzymes involved in riboflavin production pathway may be the drug

targets for antibacterial activity against some pathogenic bacteria (Fischer and Bacher, 2008). For the applications in food industry, riboflavin could be added in foods such as yoghurt and soft drinks in order to give the yellow color or to enhance food's nutritional value (Stahmann et al., 2000).

As long as the problems of global pollution are concerned, the manufacture of any valuable natural products should use the procedures that support environmental-friendly orientations. The method that use microorganism such as *Ashbya gossypii*, *Candida famata* or *Bacillus subtilis* to produce riboflavin was preferred than chemical methods because the previous method offered lower cost of production and also produces less toxic wastes including CO₂ released to environments (Stahmann et al., 2000). Therefore, this study investigated the potential use of a microorganism, *Candida* spp., in riboflavin production to support the eco-friendly concept.

Since the thorough study of *Candida* spp. in Thailand in terms of its distributions in natural habitats and its potential applications in biotechnology such as riboflavin production has not been reported elsewhere, the investigation is needed to gain such knowledge. Therefore, this study aimed to isolate and identify *Candida* spp. from the local natural resource which was seawater samples in Muang Chonburi and further investigated their capabilities to produce riboflavin in shaking flasks condition in which glucose was selected as a sole carbon source in riboflavin production medium. This monosaccharide was used, firstly, because it is generally the most easily utilized carbon source for microorganisms. Secondly, this simple sugar is also the primary substrate that could convert to guanosine-5'-triphosphate (GTP), the first precursor of riboflavin synthetic pathway in *C. famata* (Lim et al, 2001). Thirdly, the maximum riboflavin yields produced by *Ermothecium ashbyii* and *Ashbya gossypii* were illustrated when glucose was used as a carbon source at an incubation temperature of 30°C (Özbas and Kutsal, 1986), the same temperature employed in this study.

MATERIALS AND METHODS

Seawater sampling

Seawater samples were randomly collected from the coastal area of Chonburi province (Approximate Latitude (°E) /Longitude (°N)) (Google maps, 2011): 13.3641/100.9749, 13.3620/100.9748, 13.3043/100.9023, 13.3045/100.9016, 13.3022/100.8975 and 13.2747/100.9208. Samples were obtained at subsurface level according to the method described by Pollution Control Department (2001, 2004) with some modifications. In brief, each seawater sample was filled in a 50 ml centrifuge tube (Axygen), closed the cap tightly, put the tube in a plastic bag and stored the sample on ice immediately. Isolation of yeast was handled within 24 h after

sample collection.

Isolation of yeasts

Each seawater sample was pipetted into a 50 ml flask containing sterile YPD broth (1% yeast extract (Biomark), 2% peptone and 2% dextrose (Himedia) supplemented with 100 ug/ml chloramphenicol (Sigma)) for the proportion of seawater:YPD = 1:10. Samples were incubated at 30°C 150 rpm (innova4340, New Brunswick Scientific) and collected them on day-5 for serial dilutions and aseptically spreading on YPD agar. Samples were incubated at 30°C overnight and single colonies that showed typical colony morphology of yeast were picked and re-streaked on sterile YPD agar (Same components as YPD broth but with an addition of 1.5% agar). Prepare wet mount of each isolated colony and visualize cells under a microscope (Olympus CH30) in order to confirm yeast cell morphology.

Identification of *Candida* species

Inoculum preparations and species identification of *Candida* sp. were conducted using RapID™ Yeast Plus System kit (remel) according to manufacturer's instruction.

Screening for isolates capable of riboflavin production

A single colony from stock cultures was transferred to a 50 ml flask containing 20 ml YPD broth at 30°C 150 rpm (innova4340, New Brunswick Scientific) for 24 h. The culture was taken to measure the optical density at 600 nm (Thermo He λIOS γ) and adjusted to OD₆₀₀ = 1.0. This culture (0.2 ml) was transferred to a 50 ml flask containing 20 ml riboflavin production medium (2% Glucose, 0.5% (NH₄)₂SO₄, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O, 0.01% CaCl₂·2H₂O, 0.01% NaCl (Ajax Finechem) and 0.2% yeast extract and grew at 30°C 150 rpm (innova4340, New Brunswick Scientific) for 5 days. The culture was collected and centrifuged at 14000 rpm at 4°C for 10 min (Sartorius, Sigma 1-14). The culture supernatant was further used for riboflavin assay.

Riboflavin assay

Riboflavin assay of culture supernatants were determined by the measurement of the optical density at 440 nm in a spectrophotometer (Thermo He λIOS γ). Riboflavin standard (Sigma) was used for standard curve construction. Each sample was done in triplicate. Triplicate data were calculated for means and standard deviation (S.D).

RESULTS AND DISCUSSION

Candida spp. isolated from seawater samples were identified as *C. tropicalis* and *C. krusei* in a proportion of 53.19 and 46.81%, respectively. Microscopic morphology of *C. tropicalis* was round to oval shaped cell while *C. krusei* appeared to be elongated cells (Figure 1). Screening for riboflavin production from *C. tropicalis* isolates (MICBUU001 to MICBUU025) and *C. krusei* isolates (MICBUU026 to MICBUU047) showed that *C.*

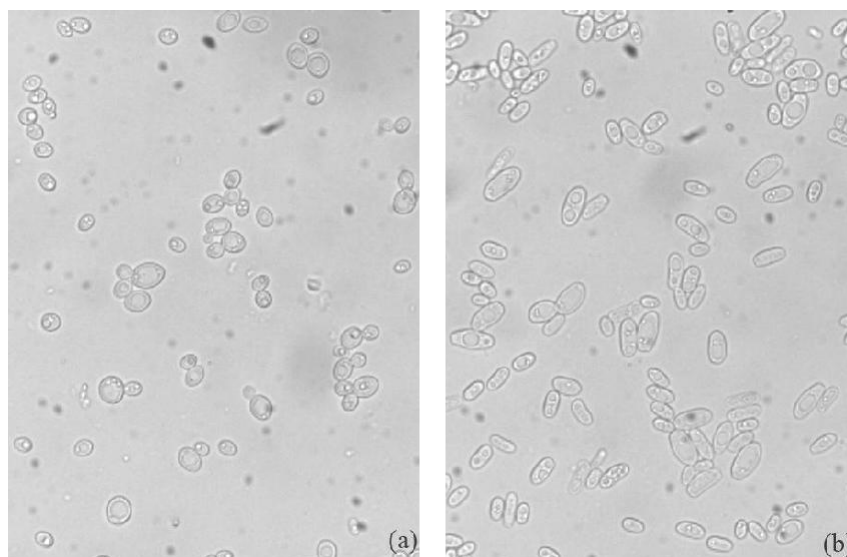


Figure 1. Cell morphologies of (a) *C. tropicalis* and (b) *C. krusei* grown in YPD broth at 30°C for 24 h using total magnification of 1000x.

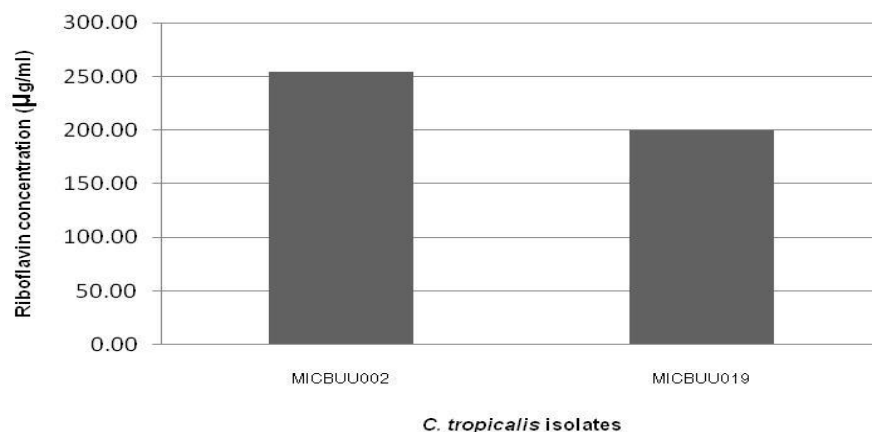


Figure 2. The production of riboflavin from *C. tropicalis* isolates.

tropicalis MICBUU002 and MICBUU019 could produce riboflavin with concentration over 254.22 ± 5.51 and 199.53 ± 0.74 µg/ml, respectively as shown in Figure 2.

The aforementioned results showed the potent production of riboflavin by *C. tropicalis* which was consistent with a previous report that demonstrated the ability of this *Candida* specie to produce riboflavin (Buzzini and Rossi, 1998). Two isolates of *C. tropicalis* from this study were able to produce approximately 0.2 g/l riboflavin in the shaking-flask condition tested. The yield appeared to be less than the yield produced by *C. tropicalis* from the report by Buzzini and Rossi (1998). The reasons for this are likely to be from strain variation,

different growth conditions and different culture preparation. However, this yield of riboflavin was similar to the riboflavin concentration produced by *Candida guilliermondii* that utilized liquid brewery waste (Contasti and Bahar, 1988). Furthermore, the isolated strains produced ten-fold more riboflavin yield than that from *C. membranifaciens* subsp. *flavinogenie* W14-3 (Wang et al., 2008a). In comparison to other microorganisms, *C. tropicalis* from this studied produced two-fold more riboflavin yield than that from *B. subtilis* PRF93 (Sauer et al., 1996). However, in comparison to *Ashbya gossypii*, fungi that could overproduce riboflavin in the industrial-scale were not the same in every case. *C. tropicalis* could

produced more riboflavin than *A. gossypii* that grown in a medium containing up to 1% glucose for the maximum period of 9 days. The riboflavin yield could be approximately the same in comparison with *A. gossypii* that was cultured in a medium containing 3 or 5% glucose for 4 days (Tanner et al., 1949). However, there was also a condition that had less amount of riboflavin produced than that from *A. gossypii* that utilized cheese whey supplemented with 10 g/l bran as substrate (Ertrk et al., 1998).

Several factors could influence riboflavin yield produced by microorganism. Different components of growth medium or substrates may induce different riboflavin yields. In addition, the optimal growth conditions for riboflavin production in each microorganism are also different. Even though complex carbohydrates such as sucrose (Levine et al., 1949; Suzuki et al., 2009) or xylose (Chi et al., 2008) could be used to induce riboflavin production. Glucose was used in this study and the results demonstrated that it was adequate for *C. tropicalis* MICBUU002 and MICBUU019 to convert it to the detectable amount of riboflavin.

Apart from extrinsic factors, intrinsic factors could have significant role in riboflavin production. There were studies reported that the genetically engineered strains constructed in the laboratory could favour the riboflavin production. Jimenez et al. (2005) reported that *A. gossypii* that had a constitutive expression of *AgADE4*, a gene encoding phosphoribosyl pyrophosphate amidotransferase, could inactivate the inhibition of purine pathways and further enhanced riboflavin production. Phosphoribosyl pyrophosphate amidotransferase has a role in the synthesis of GTP, a precursor for riboflavin synthesis. Zhu et al. (2007) reported that a strain of *B. subtilis* that lacked the function of *pta*, a gene related to acetate pathway, showed the up-regulation of a gene encoding for acetolactate synthase. This resulted in an increased level of riboflavin synthesis. Though this study did not aim to use metabolic engineering to improve the yield of riboflavin from the yeast isolates, it could be conducted later after the potential limit for riboflavin production from the yeast isolates is investigated and characterization of riboflavin are elucidated by analytic tools such as high-performance liquid chromatography (HPLC).

This study showed the results that could contribute the basic information about natural isolates of *Candida* sp. regarding to its relevant in the production of riboflavin and perhaps other interesting metabolites waiting to be discovered.

Conclusions

This study demonstrated that *C. tropicalis*, MICBUU002

and MICBUU019, isolated from seawater samples in Muang Chonburi could produce riboflavin in a broth containing 2% glucose on day-5 of incubation at 30°C with concentrations of 254.22 ± 5.51 and 199.53 ± 0.74 ug/ml, respectively. *C. krusei* isolates were also investigated for riboflavin production. However, all of *C. krusei* isolates did not produce riboflavin.

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