

# Screening and Identification of Cellulase Producing Yeast from Rongkho Forest, Ubon Ratchathani University

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**Abstract** In this present study, a total of 82 yeast strains were obtained from 111 samples collected from various sources such as soil, tree barks and insect frass from RongKho forest, Ubon Ratchathani University. They were screened and identified for cellulase production. Based on their capabilities of degrading carboxymethyl cellulose as a sole carbon source in solid medium, 45 strains performed cellulose hydrolysis which was verified by the colorless halo in the solid medium. Among them, 3 strains namely UBU-SK6, UBU-JK8 and UBU-JK9/1 showed higher levels of enzyme activity. After the step of solid medium selection, yeast cells were grown in liquid medium containing 1% yeast extract and different concentration of carboxymethyl cellulose (0.5-1.5%) under shaking with 150 rpm at 30°C and for 168 h. The enzyme activity of these 3 strains exhibited 0.224, 0.238 and 0.26 UmL<sup>-1</sup>, respectively. Afterwards, these wild yeast strains were molecularly identified by sequencing the ITS1-5.8S-ITS2 and D1/D2 domains of the subunit (26S) ribosomal DNA. The identification of UBU-SK6, UBU-JK8 and UBU-JK9/1 showed 98-100% similarity as *Candida* sp. 05-7-186T, *Candida easanensis* and *Candida* sp. ST-390 respectively.

**Keywords** Cellulase Producing Yeast, *Candida* Sp., Cellulose Hydrolysis

## 1. Introduction

Cellulolytic microorganisms play an important role in the biosphere by reducing cellulose. During their growth on cellulosic material, cellulases are synthesized by microorganisms. Cellulases from microorganisms have attracted a great deal of attention because they are highly specific biocatalysts that act in synergy to release sugars, especially glucose which is of great industrial interest due to the potential for its conversion, for example, in the textile and paper industries and in animal feed [1-3] as well as the production of bioenergy as bioethanol and biofuels [4].

There is a wide variety of microorganisms in nature which

produce cellulases; only a few yeast strains have been seen as major producers of this enzyme. For this reason, explorations of biodiversity in the search for new biocatalysts by selecting microorganisms from nature represents a method for discovering new enzymes which may permit the development of bio-catalyst at an industrial scale. Currently, there is a great interest in finding microorganism species that are not yet known as interesting producers of inputs to industry. Moreover, biocatalysts also benefit in biotechnological processes which can replace many chemical processes, in terms of production of key products. As an example is the replacement of acid hydrolysis for enzymatic hydrolysis, as is well known in the industry [5].

Traditionally, yeasts have been identified and classified by cellular morphology and their distinctive reactions on a standardized set of fermentation and assimilation tests. These methods are laborious and time-consuming. Moreover, these characteristics are influenced by culture conditions and can provide uncertain results because of strain variability. Given these difficulties and the impracticality of identification most species from genetic crosses, molecular techniques, including the sequencing of rRNA genes and/or their flanking ITS regions are increasingly used for identification because the sequencing of rRNA genes and/or their flanking ITS regions have shown that many of these genotypic characteristics are strain specific for recognition of either species or genera. Moreover, these techniques are fast, easy and reasonably precise, and thus suitable for the rapid screening of isolates [6]. The main goals of the present study included the screening and identification of cellulase producing wild yeasts, isolated from samples collected from RongKho forest, Ubon Ratchathani University.

## 2. Materials and Methods

### 2.1. Yeast Isolation

Two grams of each sample from natural sources such as soil, mushroom, fruits (one wound per fruit) and insect frass was incubated in 5 ml of Yeast extract-Malt extract (YM)

broth (3 gL<sup>-1</sup> yeast extract, 3 gL<sup>-1</sup> malt extract, 5 gL<sup>-1</sup> peptone and 10 gL<sup>-1</sup> glucose) containing 0.1gL<sup>-1</sup> chloramphenicol and 2 gL<sup>-1</sup> sodium propionate to suppress bacterial and mold growth, respectively at 30°C for 2-3 days. An aliquot of 0.1 ml of each yeast sample suspension was spread on YM agar supplemented with 0.1 gL<sup>-1</sup>chloramphenicol and 2 gL<sup>-1</sup> sodium propionate. Plates were incubated at 30°C for 2-3 days. Colonies of yeasts were purified by repeated streaking three times on YM agar plates without the chloramphenicol and sodium propionate.

## 2.2. Solid Medium Selection

For qualitative analysis of cellulase production; all strains of yeast were incubated at 30°C for 2 days on carboxymethyl cellulose sodium salt (CMC-Na salt) agar (1%yeast extract, 0.5% CMC-Na salt and 1.8% agar). After incubation, cellulase-producing strains were identified based on the formation of a clear halo around the colonies, which were made visible by Congo red staining [6].

## 2.3. Index of Enzymatic Activity

The enzymatic activities were estimated according to the method reported by Anagnostakis and Hankin [7] who proposed an Enzymatic Activity Index (EAI), which is the ratio between the halo diameter and the colony diameter.

## 2.4. Determination of Carboxymethyl Cellulase Production

The carboxymethyl cellulase-positive isolates were evaluated for carboxymethyl cellulase activity by growing the culture in carboxymethyl cellulose-yeast extract medium containing 1% yeast extract containing and different concentrations of carboxymethyl cellulose (0.5-1.5%) under shaking with 150 rpm at 30°C for 7 days. Subsequently, the cells were pelleted by centrifugation, and the cell-free supernatant was used as the source of the enzyme.

Carboxymethyl cellulase activity was determined by measuring the release of reducing sugars from CMC-Na salt using the Somogyi–Nelson method [8]. Reaction mixtures contained 0.45 mL of 0.5% carboxymethyl-cellulose sodium salt in 50 mM potassium phosphate buffer, pH 7 and 0.05 ml of each enzyme fraction. Control lacked the enzyme fraction. After incubation at 50°C for 15 min, the reaction was terminated by adding 0.5 mL of Somogyi reagent. The mixture was vortexed, placed in a boiling-water bath for 10 min, and cooled to room temperature. A 0.5 mL of Nelson reagent was added. After being vortexed, the mixture was centrifuged to remove any precipitate, and the absorbance of the supernatant was measured at 660 nm. One international unit (IU) of enzyme activity was defined as the amount of enzyme required to release 1 micromol of glucose from CMC-Na salt in 1 minute under the assay condition.

## 2.5. Molecular identification

Pure isolated yeast cells from 3 mL of each 24-h culture were harvested by centrifugation and DNA extraction as described by Sambrook et al. (1989) [10]. Identification was carried out by sequencing the gene that encodes the 5.8S regions of ribosomal ribonucleic acid (RNA) and the spacer regions ITS-1 and ITS-2 after PCR amplification. To do so, the universal primers ITS4 (5' TCCTCCGCTTA-TTGATATGC 3') and ITS5 (5' GGAAGTAAAAGTCGTAACAAGG 3') were used. The D1/D2 domains of the 26S subunit were also sequenced by using the primers NL1 (5' GCATATCAA-TAAGCGGAGGAAAAG 3') and NL4 (5' GGTCCGTGTTTCAAGACGG 3'), according to the methodology described by Kurtzman and Robnett [9]; Esteve-Zarzoso et al. [11] and Leaw et al. [12]. The PCR product was purified by using QIAquick PCR Purification Kit and analyzed with 1.5% agarose gel electrophoresis. The PCR products were sequenced using the ITS4, ITS5, NL-1 and NL-4 primers using either Amersham Pharmacia ALF Express II or ABI 310 (capillary) automated DNA sequencer, following the manufacturer's instructions. For identification, the obtained sequences were compared with those of all known yeast species, available at the GenBank database (<http://www.ncbi.nlm.nih.gov/BLAST/>) [13].

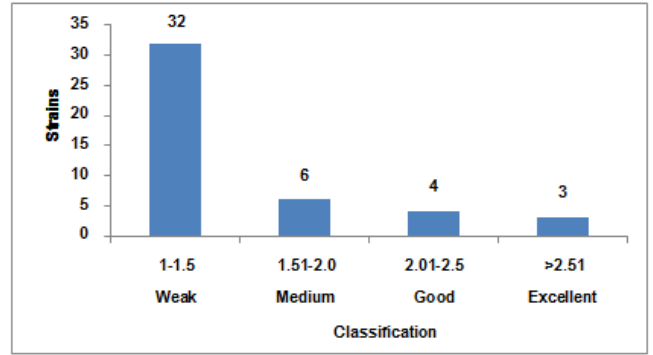
## 3. Results and Discussion

### 3.1. Screening of Cellulase Producing Yeast

For screening of cellulase producing yeast, a total of 82 yeast strains were grown in Petri dishes in order to identify those able to degrade carboxymethyl cellulose (CMC). After incubation, the plates were revealed with a Congo red solution and halo formation was analyzed. The formation of a clear halo surrounding the colony indicates hydrolytic activity (Figure 1). The enzymatic activity index (EAI) is a semi-quantitative parameter commonly used to assess enzyme production by microorganisms in solid medium [7, 14]. The result of cellulase producing yeast screening showed that 45 strains performed cellulose hydrolysis (Figure 2). Figure 2 shows values of enzyme activity recorded for the 45 selected strains when grown on solid medium containing carboxymethyl cellulose as a carbon source. Some authors recommend an EAI  $\geq 2.5$  for considering a microorganism as a producer of enzymes in solid medium [5, 14, 15]. Since colonies with the highest EAI are those with higher extracellular enzyme activity [15, 16, 17]. Among them, 3 strains namely UBU-SK6, UBU-JK8 and UBU-JK9/1 exhibited the EAI 3.5, 2.67 and 3, respectively. Therefore, all three were considered as cellulase producing strains and were assayed in liquid medium fermentations.



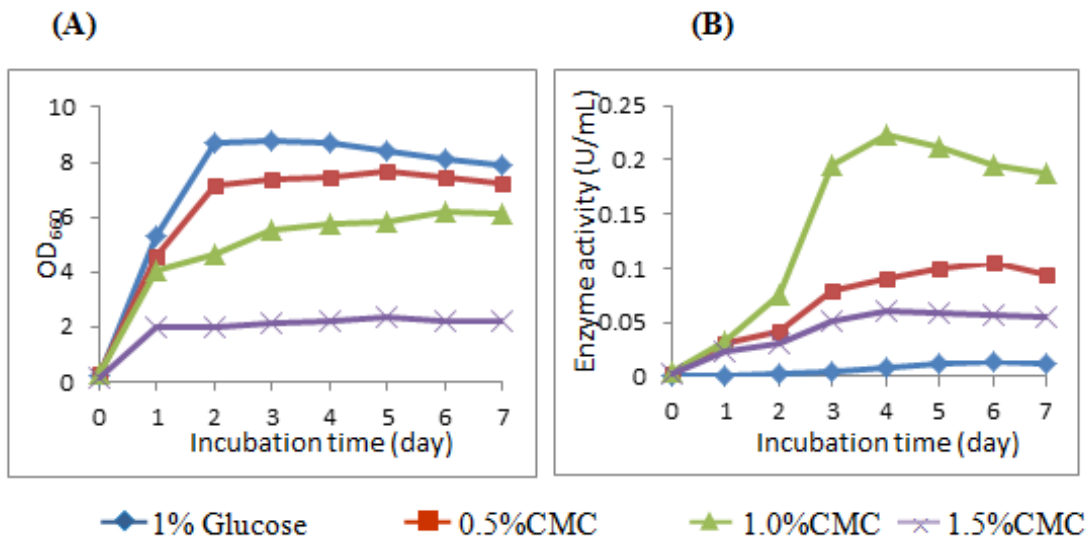
**Figure 1.** Halo of hydrolysis formed around the colony when grow for 48 h at 30°C



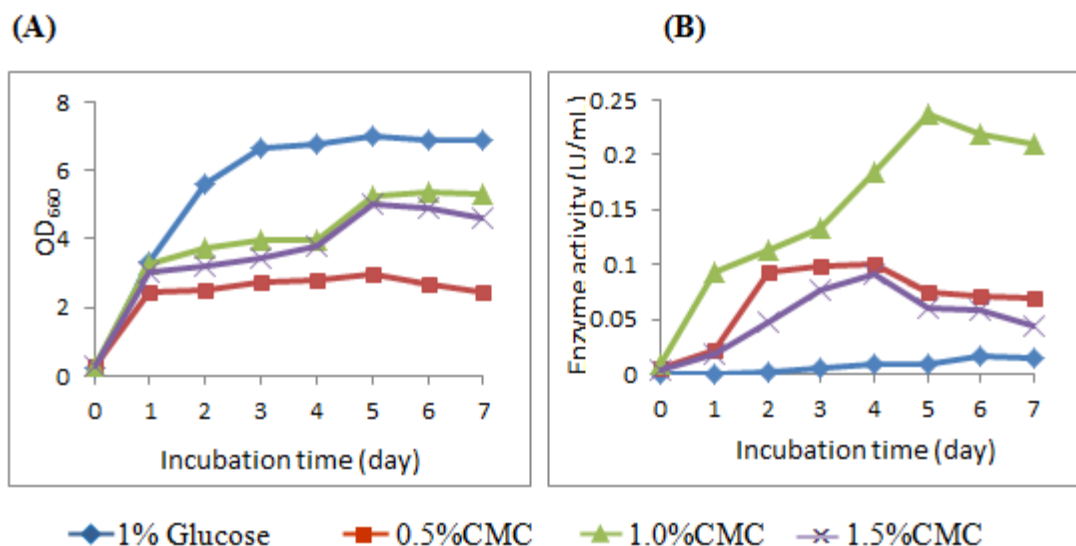
**Figure 2.** Classification of enzymatic activity indices (EAI) for 45 cellulase producing strains after 48 h at 30°C on CMC solid medium

### 3.2. Production of Cellulase in Liquid Medium

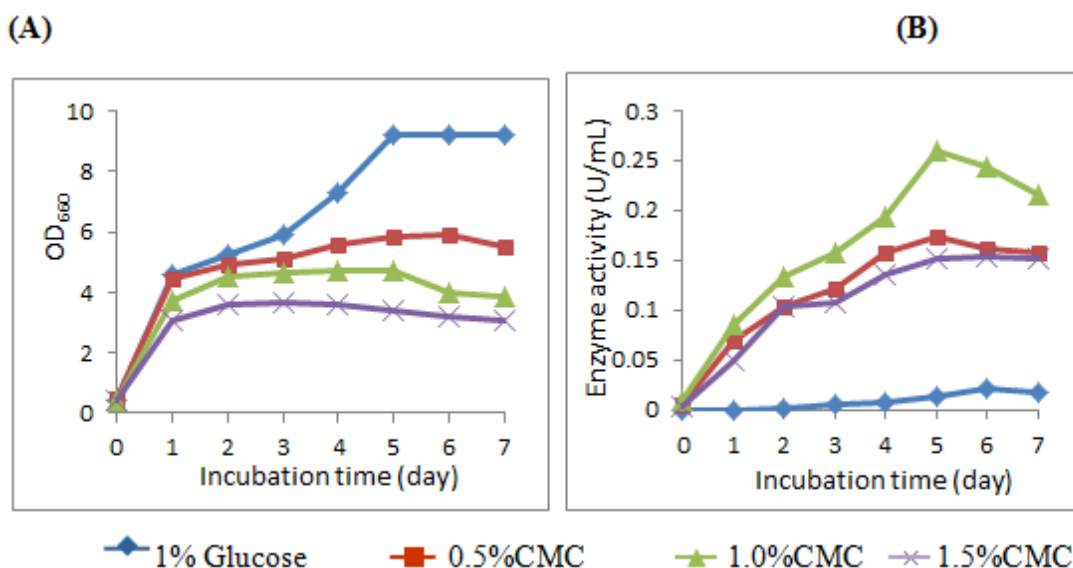
The result of cellulase production of three strains selected (UBU-SK6, UBU-JK8 and UBU-JK9/1) showed that culture medium containing 1.0% CMC is the most effective induction of the enzyme production; this value is quite close to the results for cellulase production by *Pseudomonas* sp. [18] and *Alternaria* sp. MS28 [19]. The highest cellulase activity of UBU-SK6 was observed at 96 h of fermentation ( $0.224 \text{ UmL}^{-1}$ ) (Figure 3) while the highest enzyme activity of yeast strains UBU-JK8 and UBU-JK9/1 were observed at 120 h of fermentation ( $0.238$  and  $0.26 \text{ UmL}^{-1}$ , respectively) as shown in Figures 4 and 5. When comparing cellulase production in our study to different earlier yeast strains, the activities of yeast named UBU-SK6, UBU-JK8 and UBU-JK9/1 were lower than those of *Candida stellata* ( $30.6 \text{ UmL}^{-1}$ ) [20], *Aureobasidium pullulans* strain PJF-4b ( $0.44 \text{ UmL}^{-1}$ ), *Candida glabrata* strain PJS-4a ( $0.38 \text{ UmL}^{-1}$ ) and *Candida litsaeae* strain PJB-3 ( $0.36 \text{ UmL}^{-1}$ ) [6]. However, the yields of cellulase from our yeasts isolates were two-fold higher than those of the most active *Trichosporon* spp. ( $0.12$  and  $0.14 \text{ UmL}^{-1}$ ) [21].



**Figure 3.** Effect of carboxymethyl cellulose (CMC) concentration from 0 to 1.5% (w/v) on cellulase production of yeast named UBU-SK6. Growth (A) and enzyme production (B) were measured at 24 h intervals for 7 days.



**Figure 4.** Effect of carboxymethyl cellulose (CMC) concentration from 0 to 1.5% (w/v) on cellulase production of yeast named UBU-JK8. Growth (A) and enzyme production (B) were measured at 24 h intervals for 7 days.



**Figure 5.** Effect of carboxymethyl cellulose (CMC) concentration from 0 to 1.5% (w/v) on cellulase production of yeast named UBU-JK9/1. Growth (A) and enzyme production (B) were measured at 24 h intervals for 7 days.

### 3.3 Molecular Identification

Molecular identification was based on ribosomal targets: the ITS1-5.8S-ITS2 and D1-D2 domains of the RNA operon. In recent years, the number of ITS sequences available in public databases has increased significantly, and the expanding database may improve the quality and accuracy of fungal identification [11, 22]. The D1/D2 recombinant deoxy-ribonucleic acid (rDNA) region has been sequenced for almost all known yeasts, including non-pathogenic species. As an identification tool, the sequencing of this region is relatively simple to apply and yields clear results [9, 11]. The BLAST searches, even as phylogenetic analysis, are used for identification by searching public databases for the closest known sequences to the unidentified yeast [23]. A BLAST search in the NCBI database

(<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) of the ITS1-5.8S-ITS2 and D1/D2 domains of the subunit (26S) ribosomal DNA of UBU-SK6, UBU-JK8 and UBU-JK9/1 showed 98-100% sequence identity with *Candida* sp. 05-7-186T, *Candida easanensis* and *Candida* sp. ST-390 respectively.

## 4. Conclusions

From a total of 82 yeast strains, 45 strains showed a halo of hydrolyzed substrate in the solid medium and three strains which were isolated from soil stood out for having high levels of enzyme activity. Considering production of cellulase from these yeast strains in liquid media, the result showed that the enzyme activity of these 3 strains, UBU-SK6, UBU-JK8 and UBU-JK9/1, exhibited 0.224, 0.238 and 0.26

UmL<sup>-1</sup>, respectively. The identification of UBU-SK6, UBU-JK8 and UBU-JK9/1 by sequencing the ITS1-5.8S-ITS2 and D1/D2 domains of the subunit (26S) ribosomal DNA showed 98-100% similarity as *Candida* sp. 05-7-186T, *Candida easanensis* and *Candida* sp. ST-390 respectively. Currently, little is reported in literature regarding the production of cellulases from *Candida* genus. However, our study describes cellulase production from this resource can contribute to development of microbial bank and provide data on enzyme characteristics for cellulose hydrolysis into fermentable sugars which could have potential for a wide range of industries such as animal feed and biofuel production.

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