Selection of Amylase-Producing Filamentous Fungi to Hydrolyze Cassava Starch

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Abstract

Due to the current crisis of increasing petroleum fuel prices and problems concerning global warming, cassava starch is used as a low-cost carbon source for ethanol production. Several studies suggested that fungal amylase could be the biochemical solution for transforming polysaccharides into simple sugar. Therefore, the objective of this study was to investigate and characterize the highly efficient amylase-producing fungal isolates that hydrolyze cassava starch to sugar. The four hundred twenty-one (421) isolates of filamentous fungi were tested for their ability to digest 2% soluble starch using an agar plate assay. Among these 421 isolates, 72 fungal isolates exhibited a clear zone diameter in 7 days. All 72 fungal isolates were also tested for their ability to digest 2% cassava starch on agar plates. Four isolates, including 1-5, 13-3, 21-3, and 5-3, had the largest clear zone sizes (≥ 10 mm). They were identified as *Taralomyces macrosporus*, *Penicillium* sp., *T. flavus*, and *Penicillium* sp., respectively. The partial sequencing of internally transcribed spacer (ITS) regions was performed for the selected fungal isolate. The resulting *T. flavus* 21-3 gene sequences were deposited in GenBank under the accession number OQ184710. The sugar liberation was performed in a medium containing 2% cassava starch broth. The results showed that *T. flavus* 21-3 demonstrated the highest efficiency, with a maximum reducing sugar concentration of 5.14 g L^{-1} and an enzyme activity of 0.37 U mL^{-1}. After 7 days of incubation at 30 ºC and growth in a medium containing cassava starch with a pH of 7.5, the culture exhibited high levels of enzymatic activity. These results implied that *T. flavus* 21-3 has the ability to produce amylase and could digest cassava starch into sugar, which is advantageous for industrial applications and warrants further research.

Keywords : Amylase; Cassava starch; Filamentous fungi; Hydrolysis; Selection

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1. Introduction

Thailand is a tropical country with a rich biodiversity of plants, animals, and microorganisms. Since the 1928 discovery of penicillin from *Penicillium notatum*, fungi have been regarded as a goldmine among microorganisms. The study of fungal bioactive compounds was conducted. The most common producers of bioactive compounds are ascomycetes (*Aspergillus*, *Penicillium*, and *Fusarium*) and imperfect fungi [1]. Many researchers reported that fungi are able to produce many bioactive substances such as alkaloids, antibiotics, camptothecin, enzymes, podophyllotoxin, and taxol [2]-[7], which have valuable potential applications in the chemical, pharmaceutical, food, agricultural, and biofuel industries.

Cassava (*Manihot esculenta*) is thought to be Thailand's most important economic crop. Planting has increased in Northeastern, Central (including Eastern), and Northern Thailand. In 2020, Thailand's total cassava plantation area was 9.4 million rai, and its annual production was approximately 29.0 million tons [8]. Cassava roots are utilized commercially for food, paper, glue, and ethanol production. Ethanol has demonstrated the ability to substitute traditional fossil fuels with the added benefits of renewability, non-toxicity, and clean consumption. Currently, cassava starch is used as a renewable carbon source alternative to sustain ethanol production. In addition to allowing enzymes to digest starch into sugar, cassava starch also enables filamentous fungi, which can produce α-amylase and glucoamylase, to digest starch into sugar. The filamentous fungi *Aspergillus niger*, *A. oryzae*, *A. kawachii*, *Curvularia lunata*, and *Thermomucor indicae-seudaticae* have been studied for their ability to produce α-amylase and glucoamylase [9]-[13]. Screening filamentous fungi that could effectively produce α-amylase and glucoamylase has greater industrial benefits.

Amylase is a group of hydrolases capable of digesting starch by cleaving the glycosidic bond. This enzyme is divided into two important groups, namely glucoamylase and α-amylase. Glucoamylase (α-1,4-glucan glucohydrolase, amyloglucosidase, EC 3.2.1.3) produces glucose residues by continuously cleaving α-1,4 glycosidic bonds from non-reducing ends of amylose and amylopectin, whereas α-amylase (endo-1,4-α-D-glucan glucohydrolase) randomly cleaves α-1,4 glycosidic bonds within the linear amylose chain [14]. In general, they are used extensively in the food, bakery, brewing, detergent, and textile industries, particularly in alcohol production factories [15]. Recently, Beltagy *et al*. [16] reported that *A. flavus*, an amylase-producing fungus, was selected according to the formation of a clear zone. The enzyme produced from this fungus was utilized in bioethanol production. In addition, Kalia *et al*. [17] reported that *Trichoderma reesei* was able to produce α-amylase from starch effluent obtained from the textile industry. This enzyme can also be utilized during the desizing process.

The main objective of the present study was to identify and characterize filamentous fungal isolates with a high ability to produce amylase enzymes for digesting cassava starches into sugar using plate assays. Based on the zone of inhibition, potential isolates were selected for assessment of enzyme production using cassava starch as the raw material. The optimization of enzyme production was investigated.

2. Research Methodology

2.1 Preparation of materials

Fresh cassavas, a 12-month Kasetsart University (KU) strain, were washed, peeled, and cut into small pieces before being exposed to sunlight for 1–3 days to allow them to dry thoroughly. The cassava chips were then ground down and sifted through a 150 µm mesh screen. Cassava starches had to be kept in a clean and dry container for further study.

The cassava starches were prepared for moisture analysis according to Thai Industrial Standards TISI No. 52, method number 6.4. The percentage of starch was then determined using the color method [18]. This flour was used as the substrate.

2.2 Cassava starch hydrolysate from fungi

The 421 isolates of filamentous fungi were preserved in the Pathogenics Department, Faculty of Agriculture, Kasetsart University. All of them were isolated from plant debris in different locations in Thailand. The soil dilution plate method, soil plate method, alcohol treatment method, and heat treatment method were used to separate the fungi. All active fungal isolates were placed on potato dextrose agar (PDA) plates, incubated at room temperature for 7 days, and then pierced with a 5 mm sterile steel borer. Each mycelial plug was placed in the middle of a 2% soluble starch agar plate, incubated at room temperature for 7 days, and then flooded with iodine solution (2 g iodine, 2 g ammonium sulfate and 300 ml deionized water). Only the clear zone size was measured with the vernier caliper, excluding the fungal colony.

The ability of fungal isolates to hydrolyze soluble starch was evaluated using cassava starch. The mycelial plug of each selected fungus was placed in the middle of a 2% cassava starch agar plate and then incubated at room temperature for 7 days. Only the clear zone size was measured using a vernier caliper. All experiments were done in triplicate, and the results were expressed as the average ± SD.

Fungi capable of producing a clear zone on the cassava starch were collected for the starch digesting test with a 2% cassava starch broth medium. Each fungus was cultured on a PDA at room temperature for 7 days. Five mycelial plugs of each fungus were taken using a 5 mm sterile steel borer and inoculated into a cassava starch broth medium. The culture broth was placed on the rotary shaker with a shaking speed of 130 rpm at room temperature. After 7 days, the samples were collected, and the enzymatic activity was analyzed. An amount of reducing sugar was determined using the Nelson-Somogyi method [19]. Each experiment was performed in triplicate.

In the current study, the effects of important factors on amylase production in shake flask cultivation were evaluated using the one-factor-at-a-time technique. These factors were pH, temperature, and incubation period. The experiments were done in a cultivation medium consisting of 2% cassava starch with an initial pH of 7.0, which was then sterilized by autoclaving at 121 °C for 15 min. To facilitate the digestion of cassava starch, each selected fungus was cultivated in sterile media for 7 days at room

temperature with a shaking speed of 130 rpm. In the specific experiments of one-factor-at-a-time optimization, various levels of each target factor were considered, such as pH (5.0, 5.5, 6.0, 7.0, 7.5, and 8.0), temperature (26, 28, 30, 32, and 34 °C), and incubation period (6, 7, 8, 9, 10, 11, 12, 13, and 14 days). Each experiment was performed in triplicate.

2.3 Identification of fungi

The morphological characteristics of the colonies were determined using the method described by Manoch *et al*. [20] and Domsch *et al*. [21]-[22]. Growth pattern, color, and texture on different media, such as malt extract agar (MEA), Czapek's agar (CZA), Czapek yeast extract agar (CYA), and oatmeal agar (OA), were observed for 7 to 14 days at room temperature. The diameter of colonies was measured in millimeters, most effectively by transmitted light and from the reverse side. Microscopic characters were examined on a slide preparation using sterile distilled water and lactophenol as mounting media and observed under a light microscope. *Camera lucida* drawings were employed. Photomicrographs of the fungal structure were taken under stereo, light, and scanning electron microscopes.

The identification of a selected fungus was confirmed using molecular techniques and ITS primers. DNA was extracted from young mycelia using a modified Murray and Thompson method [23]. The universal primer pairs ITS1 and ITS4 were used for ITS gene amplification [24]. The PCR reactions were conducted on a thermal cycler, and the amplification process consisted of initial denaturation at 95 °C for 5 min, 34 cycles at 95 °C for 1 min (denaturation), at 55 °C for 1 min (annealing), and at 72 °C for 1.5 min (extension), followed by a final extension at 72 °C for 10 min. The PCR products were examined by agarose gel electrophoresis (1% agarose with 1X TBE buffer) and visualized under UV light after staining. DNA sequencing analyses were performed using the dideoxyribonucleotide chain termination method by Macrogen Inc. (Seoul, South Korea). The DNA sequences were edited using the FinchTV software, submitted to the BLAST program for alignment, and compared to those of fungal species in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/).

2.4 Analytical methods

The sugar concentration was determined using the Nelson-Somogyi method. The high sugar concentration was diluted with distilled water until the optical density was in the range of 0.1 to 1.0, measured at a wavelength of 520 nm. The sample sugar concentrations were compared to the glucose standard.

The analytical enzymatic activity was described by Saleem and Ebrahim [14] with modifications. The reaction mixture, consisting of 1 ml of 2% soluble starch in sodium acetate buffer (pH 6.0) and 1 ml of crude extract, was incubated at 40 °C and left to stand for 30 min. The amount of reducing sugar was determined using the Nelson-Somogyi method and

spectrophotometrically measured at a wavelength of 520 nm. The results were compared to the glucose standard curve. One unit of amylase activity is defined as the amount of enzyme necessary for releasing 1 µmol of glucose under optimized assay conditions.

3. Results and Discussion

3.1 Soluble starch hydrolysate from fungi

The best of the 421 isolates of filamentous fungi, including 23 genera and 32 species, were tested with 2% soluble starch by inoculating the center of a soluble starch agar medium and incubating it at room temperature for 7 days. The results showed that 72 isolates were able to digest soluble starch, with three isolates (21-3, 13-3, and 5-3) producing a clear zone larger than 20 mm, 10 isolates producing a clear zone between 10 and 20 mm, and the rest producing a clear zone smaller than 10 mm. The isolate 21-3 produced the largest clear zone at 42 ± 2 mm. The clear zone after flooding with iodine solution is shown in Fig. 1. Yuwa-Amornpitak [25] reported that the largest clear zone of 0.1% starch agar at pH 4 and subjected to the culture filtrate of *Rhizopus* spp. isolated from look-pang (a traditional Thai fermenter) measured 57 mm. In addition, 166 fungal isolates obtained from 12 look-pang-khao-mak samples were screened for their ability to hydrolyze starch. Among these, 37 isolates of filamentous fungi showed a hydrolysis capacity within the 10–11 mm range. The results revealed that these fungi were able to produce the starch-degrading enzyme [26]. The screening of endophytic fungi for amylase production was proposed by Sunitha *et al*. [27]. Out of 30 endophytic isolates, only seven isolates demonstrated amylolytic activity on solid media. Among these seven isolates, *Cylindrocephalum* sp. (Ac-7) showed the largest clear zone. In 2014, Saleem and Ebrahim [14] reported that out of 46 fungal isolates, eight isolates (17.39%) exhibited high amylase activity, 27 isolates (58.70%) exhibited moderate activity, and the rest (23.91%) exhibited low amylase activity. Among these fungal species, *A. niger* and *Rhizopus stolonifer* were the most active in producing amylase. The screening of amylase-producing microorganisms was also proposed by Zhou *et al.* [28]. After placing the crude enzyme solution on a soluble starch plate and allowing it to incubate overnight, the iodine solution was flooded. The largest clear zone size of the starch hydrolysis was selected to determine amylase activity. The results showed that the amylase-producing activity of fungal isolate M8 was not significantly different from that of bacterial isolate A-1, with an average of 1.50 U mL^{-1}.

On the other hand, by classifying the fungi above using the method of Manoch *et al.* [20] and Domsch *et al*. [21]-[22], the 72 isolates that produced clear zones on 2% soluble starch and enzymes capable of digesting starch into sugar were the following: 16 isolates of *A. niger*, three isolates of *C. lunata*, seven isolates of *T. flavus*, 10 isolates of *Scytalidium thermophilus*, five isolates of *Rhizomucor pusillus*, and 31 isolates of unidentified species due to being sterile fungi. Sattar Qureshi *et al.* [29] proposed that amylase production was carried out by various fungal species, including *Mucor geophillus*, *A. niger*, *A. fumigatus*, *P. lilacinum*, and a mixed culture (*A. niger* +

A. fumigatus). The results showed that the maximum production of α-amylase (1.36 U mL^{-1}) was produced by *A. fumigatus* when grown at 31 ± 2 °C for 168 h in the medium without glucose. The fungal species, namely *Aspergillus* sp., *Chaetomium* sp., and *Penicillium* sp., also showed high amylolytic activity when the clear zone diameter on the plate was evaluated [30].

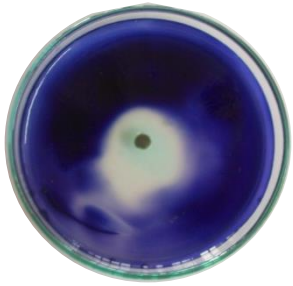

Fig. 1 Clear zone on 2% soluble starch of filamentous fungi, after incubation at room temperature for 7 days and then flooded with iodine solution.

3.2 Selection of cassava starch hydrolyzing fungi

Seventy-two isolates of fungi that can digest soluble starch were tested for their ability to digest 2% cassava starch on an agar medium incubated at room temperature for 7 days. The test was repeated three times before measuring the clear zone size. It was found that only 18 isolates were able to digest cassava starch and soluble starch (Table 1), whereas the remaining isolates could only digest soluble starch but not cassava starch. A reason for this might be that cyanogenic glucosides remaining in cassava starch dominated the digestion of fungi. Senn and Pieper [31] reported that cassava was toxic due to more than 2.80 g kg^{-1} of the cyanogenic glucoside.

Table 1 The fungal isolates produced a clear zone in the 2% cassava starch agar medium.

Fungal Isolation	Identification	Clear zone (mm)
21-3	*Talaromyces flavus*	35 ± 3
5-3	*Penicillium* sp.	13 ± 3
13-3	*Penicillium* sp.	12 ± 1
1-5	*Talaromyces macrosporus*	10 ± 1
2.2-1	*Aspergillus fumigatus*	6 ± 1
5-2	*Aspergillus flavus*	6 ± 1
10-1	*Eupenicillium* sp.	6 ± 1
5-2-8	*Aspergillus* sp.	6 ± 1
13-4	*Humicola* sp.	6 ± 1
12-1	Mycelium hyphae	5 ± 1
10-3	*Aspergillus terreus*	5 ± 2
16-3	*Penicillium* sp.	5 ± 1
2-1	*Aspergillus niger*	4 ± 0
2.1-8	*Aspergillus* sp.	4 ± 1
1-1	*Talaromyces flavus*	4 ± 0
15-2	*Eupenicillium parvum*	3 ± 0
11-2	*Penicillium* sp.	2 ± 0
11-2-18	*Aspergillus* sp.	1 ± 0

It was found that the following filamentous fungi, *A. niger*, *A. oryzae*, *A. kawachii*, *C. lunata*, and *T. indicae-seudatica*, could produce α-amylase enzyme and glucoamylase [9]-[13]. *A. niger* was also found in the 2-1 isolate; however, the 2-1 isolate could only produce a clear zone of 4 ± 0 mm during cassava starch digestion. It was determined that fungi capable of producing a clear zone larger than 10 mm had a high level of performance. The 21-3 isolate of *T. flavus* (Fig. 2) produced a clear zone that was greater than 35 ± 3 mm, outperforming all other isolates in this experiment. The gene sequence was deposited in GenBank under the accession number OQ184710. The 5-3 isolate of *Penicillium* sp. could produce a clear zone of 13 ± 3 mm. The 13-3 isolate of

Penicillium sp. could produce a clear zone of 12 ± 1 mm, and the 1-5 isolate of *T. macrosporus* could produce a clear zone of 10 ± 1 mm.

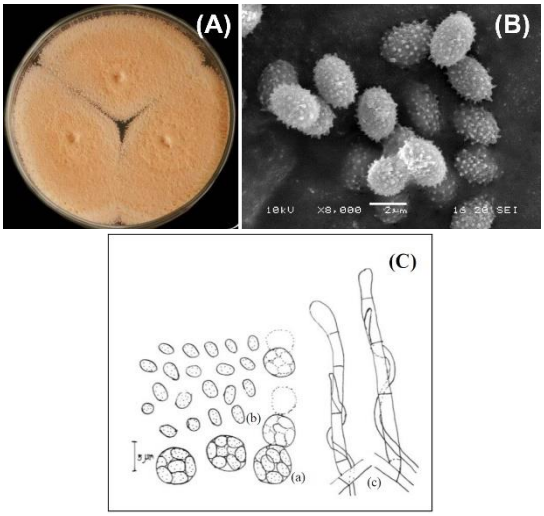

Fig. 2 *Taralomyces flavus* 21-3, (A) Growing on PDA medium for 14 days; (B) Spores pictured under a scanning electron microscope and (C) Camera lucida drawings of *T. flavus*, (a) asci and ascospores, (b) ascospores and (c) ascomatal initials.

3.3 Selection of fungi digested cassava starch to sugar

Four fungal isolates, 21-3, 5-3, 13-3, and 1-5, were tested for their ability to digest 2% cassava starch broth with a shaking speed of 130 rpm at room temperature for 7 days. The Nelson-Somogyi method was used to determine the amount of sugar and enzymatic activity in the aliquot. The results showed that *T. flavus* 21-3 was able to produce a reducing sugar of 5.14 g L^{-1} from the digestion of cassava, and its enzymatic activity was 0.37 U mL^{-1} (Table 2). As a result, the 21-3 was selected for further studies. In 2003, Peixoto *et al.* [32] studied the effect of various carbon sources on the growth and amylase activity of *R. microsporus* var. *rhizopodiformis*. The results revealed that this fungus produced high levels of amylolytic activity at 45 °C in a liquid medium supplemented with starch, sugarcane bagasse, oatmeal, or cassava flour. Cassava starch and other carbon sources, such as soluble starch, potato starch, and corn starch, have also been reported as effective substrates for glucoamylase production. At the end of fermentation, glucoamylase production by the yeast *Saccharomyces cerevisiae*, which contained wild-type glucoamylase cDNA from *A. awamori*, was 4.90 U mL^{-1} in cassava starch medium, 8.30 U mL^{-1} in potato starch medium, 7.00 U mL^{-1} in soluble starch medium, and 2.70 U mL^{-1} in corn starch medium [33].

Table 2 Digestion of 2% cassava starch broth to sugar by fungal isolates.

Fungi	Clear zone (mm)	Enzymatic activity (U mL^{-1})	Reducing sugar (gL^{-1})
T. flavus 21-3	35 ± 3	0.37 ± 0.01	5.14 ± 0.04
Penicillium sp. 5-3	13 ± 3	0.24 ± 0.01	2.88 ± 0.02
Penicillium sp. 13-3	12 ± 1	0.21 ± 0.00	2.38 ± 0.02
T. macrosporus 1-5	10 ± 1	0.13 ± 0.00	1.00 ± 0.01

3.4 Optimal conditions for digesting cassava starch to sugar

T. flavus 21-3 was examined under various pH, temperature, and incubation conditions, and the results revealed that the most suitable conditions for starch digestion were insemination in cassava starch broth with an initial pH of 7.5, a temperature of 30 °C, and an incubation

period of 9 to 14 days (Table 3). However, the cassava starch broth may become dry if digestion is allowed to continue for too long. Yuwa-Amornpitak [26] used fungi isolated from look-pang and the yeast *S. cerevisiae* to produce ethanol from cassava chips, and it was found that *Rhizopus* fungi could be isolated from 10 isolates of look-pang. The study also discovered that after 72 h of incubation, *Rhizopus* sp. #3Su could produce the highest amount of sugar at 25.90% from a source containing 6% cassava. Regarding the incubation period of 24 h, a fermentation of the *Rhizopus* sp. #3Su co-culture with the *S. cerevisiae* yeast could produce 14.4 g L^{-1} of ethanol from a source containing 6% cassava. Kunamnemi *et al.* [34] discovered a suitable environment for the thermotolerant fungus, *Thermomyces lanuginonus*. Using wheat and allowing it to incubate for 120 h at 50 ^{o}C, 90% humidity, pH 6.0, a salt concentration of 1.5:10 (v/w), and a bran weight ratio of 1% (w/w) could facilitate the production of amylase enzyme at a maximum of 534 U g^{-1}. Moreover, Ray [35] reported the results of using two fungi, *Botryodiplodia theobromae* and *R. oryzae*, to digest a medium source containing 2% cassava incubated at 30 °C and pH 6. It was found that the amylase enzyme could be produced at 3.30 and 3.80 units, respectively. The results also demonstrated that adding ammonium acetate and ammonium nitrate to the medium source (subjected to a 2-L fermentation container) could produce a higher amount of enzymes at 4.80 and 5.10 units, respectively.

Table 3 Digestion of 2% cassava starch broth to sugar by *Taralomyces flavus* 21-3 at different factors.

Factors	**Enzymatic activity (U mL^{-1})**
pH	
5.0	0.02±0.00
5.5	0.04±0.01
6.0	0.04±0.01
6.5	0.03±0.00
7.0	0.04±0.00
7.5	0.58±0.02
8.0	0.04±0.01
Temperature (°C)	
26	0.01±0.00
28	0.18±0.01
30	0.47±0.00
32	0.17±0.01
34	0.18±0.01
Incubation period (Days)	
6	0.01±0.00
7	0.04±0.00
8	0.06±0.01
9	0.26±0.01
10	0.17±0.00
11	0.24±0.01
12	0.37±0.01
13	0.33±0.00
14	0.40±0.00

*pH and temperature of cassava starch hydrolysate were incubated for 7 days.

When exposed to a new fungal isolate with a high amylase production efficiency, *A. flavus*, isolated from soil in Syria, could produce a large number of enzymes at pH 5 and a temperature of 50 °C [36]. *Aspergillus* sp., isolated from seeds, is highly effective at producing amylase and could efficiently produce enzymes by fermenting a mixture of wheat,

soil, oil, and bran in a 1:2:2 ratio [37]. According to research on thermotolerant fungi with high efficiency, soil-isolated *Penicillium rugulosum* could produce a significant amount of amylase when incubated for 3 days in a carbon source containing galactose at pH 7.0 and a temperature of 57 °C [38]. *A. flavus*, isolated from Indian soil, could produce amylase when incubated at pH 6.0 and 30 °C on a *Cocos nucifera* substrate [39].

It is evident that screening and selecting amylase-producing fungi that are able to efficiently digest cassava starch into sugar will be beneficial to industries.

4. Conclusion

The screening and selection of 421 filamentous fungi capable of efficiently digesting cassava starch into sugar found that 18 isolates could digest 2% cassava starch. Among the 18 isolates, only four isolates produced a clear zone with a minimum of 10 mm. *T. flavus* 21-3 produced a larger clear zone of 35 ± 3 mm than other species. The maximum amount of reducing sugar was 5.14 g L^{-1}, and the enzymatic activity was 0.37 U mL^{-1}. When subjected to the 21-3 isolate, it was found that fermentation of 2% cassava starch for 9 to 14 days at 30 °C and pH 7.5 provided the most suitable conditions. For the digestion of cassava starch by this fungus, our findings suggest that nutrient components such as initial carbon concentration, nitrogen sources, and growth factors should be optimized. In addition, large-scale amylase production should be further studied to confirm the efficiency of *T. flavus* 21-3.

5. Acknowledgement

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