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Screening and Characterization of Raw Starch Degrading Amylase (RSDA) from Locally Isolated Fungal Strains in UNIMAS Fungal Collection

Voon Siew Fung

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Voon Siew Fung (28588)

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Supervisor: Assoc. Prof. Dr. Awang Ahmad Sallehin Awang Husaini

Co-supervisor: Assoc. Prof. Dr. Mohd Hasnain Md Hussain

Resource Biotechnology
Department of Molecular Biology

Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

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Declaration

I hereby declare that the thesis is based on my original work except for quotation ad citation, which have been duty acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UNIMAS or other institutions.

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Voon Siew Fung
Resource Biotechnology Programme
Department of Molecular Biology
Faculty of Resource Science and Technology
University Malaysia Sarawak

Table of contents

List of Abbreviations

cm	centimetre
DNS	Dinitrosalicylic acids
ml	Millilitre
mm^2	Millimeter square
MSA	Minimal salt agar
nm	Nanometre
PDA	Potato Dextrose Agar
rpm	Revolutions per minute
RSDA	Raw starch degrading amylase
µl	Microlitre
%	Percent
(v/v)	Volume per volume
(v/w)	Volume per weight
(w/v)	Weight per volume
^oC	Degree Celsius

SCREENING AND CHARACTERIZATION OF RAW STARCH DEGRADING AMYLASE (RSDA) FROM LOCALLY ISOLATED FUNGAL STRAINS IN UNIMAS FUNGAL COLLECTION

VOON SIEW FUNG

Resource Biotechnology Programme
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

Raw starch degrading amylase (RSDA) is the enzyme that catalyst the breakdown of starch into simple sugar by cleaves the bonds between the adjacent glucose units which lead to the formation of linear and branched oligosaccharides. Six isolated fungal strains namely *Bionectria ochroleuca*, *Trichoderma harzianum, Trichoderma virens*, *Aspergillus versicolor, Aspergillus niger* and *Aspergillus flavus* were screened for their ability to hydrolyze raw sago starch by producing the RSDA through qualitative and quantitative test. Characterizations were carried out and the strains were compared for their degradative ability on raw and gelatinized sago starch, substrate specificity and preferred pH for the reaction medium. Results showed that *A. flavus* was the best performing strain that produced the highest amount of RSDA and it has the broad activity toward both small and large granule of raw starches.

Keywords: Raw starch degrading amylase (RSDA), raw sago starch, *A. flavus*, characterization, degradative ability

ABSTRAK

Kanji mentah degradasi amilase adalah sejenis enzim yang digunakan untuk memecahkan kanji kepada gula dengan memecahkan ikatan antara unit-unit gula bersebelahan dan menyebabkan pembentukan oligosakarida. Enam strain kulat yang dinamakan Bionectria ochroleuca, Trichoderma harzianum, Trichoderma virens, Aspergillus versicolor, Aspergillus niger *dan* Aspergillus flavus *telah digunakan untuk skrin kemampuan mereka untuk mengeluarkan kanji mentah degradasi amilase untuk hydrolise kanji mentah melalui kajian kualitatif dan kuantitatif. Karakterisasi telah dijalankan dan strain-strain telah dibandingkan dengan kemampuan mendegradasikan kanji sagu mentah dan kanji sagu, kekhususan substrak dan optimum pH. Keputusan dari penyelidikan menunjukkan* A. flavus *adalah strain yang terbaik dalam menghasilkan kanji mentah degradasi amilase.* A. flavus *mempunyai activiti yang luas terhadap kedua-dua butir kanji yang kecil dan besar.*

Kata kunci: Kanji mentah degradasi amilase, kanji mentah, A. flavus, *karakterisasi dan kemampuan degradasi*

1.0 Introduction

Raw starch degrading amylases (RSDA) are enzymes used for hydrolysis of starch into glucose by the breakdown of the bonds between the simple glucose units which lead to the formation of linear and branched oligosaccharides (Mamatha *et al.*, 2012). They can act below the gelatinized temperature of starch by degrade directly on the raw starch granules. RSDA can be obtained from different sources and it is produced from various living organisms such as plant, animals and microorganism. Tripathy *et al.* (2011) stated that RSDA is easily extracted from fungi sources as the fungal mycelium can be easily removed from the enzyme production medium. Moreover, the fungi isolated from the soil is very good source in the production of the raw starch degrading amylase enzymes.

RSDA can be divided into α-amylase, glucoamylase, α-glucosidases, isoamylases, β-amylases and maltogenic β-amylases (Robertson *et al.,* 2006). They can be further classified into endo-amylase and exo-amylase. Lately, there are many studies have been done to screen and characterize fungi which capable of producing RSDA. The studies mainly carried out to document the potential of the fungi in producing RSDA to degrade the raw starch and its application in various industries.

According to Kumari *et al.* (2012), raw starch degrading amylases produced from microorganisms has been successfully replaced the starch hydrolysis by using the chemical route in most of the starch processing industries. The application of chemical catalyst in various industrial processes has disadvantages in term of commercial and environmental values as compared to when using biology catalyst, RSDA (Tripathy *et al*., 2011). The bioconversion of starch by using microbial RSDA has been increasingly applied in industrial processes as it has many advantages over the hydrolysis of starch by chemical catalysts. The advantages by using the microbial RSDA in industrial processes are it is economic benefits,

less energy consumption, more time efficiency and easy in process of modification and optimization (Bozic et al., in press). Liu and Xu (in press) reported that RSDA can be used in various biotechnology industrial processes ranging from food, brewing, baking, and textile to detergent industry.

There are many methods for the production of RSDA whereby the agro industrial waste such as sago pith waste, corn, cereal grain, rice bran and sorghum are used as the substrates in the production of the RSDA by using the microbial sources. The application of these agroindustrial wastes as the resources and substrates has solved the environmental pollution problem due to disposal of these agroindustrial wastes.

In order to reveal the full potential of various fungi to produce RSDA by using the raw starch as substrate, characterization of RSDA need to be done. Therefore, the objectives of this research study are to screen for the raw starch degrading amylase (RSDA) from locally isolated fungal strains and characterization of RSDA enzymes produced from different fungal strains.

2.0 Literature Review

2.1 Raw starch degrading amylase (RSDA)

Starch is the most important sources in the food industries for the production of syrups containing the glucose, maltose or fructose. Raw starch degrading amylase (RSDA) is one of the enzymes that used for the conversion of these starches into the simple sugars. RSDA can act directly on raw starch granules below the gelatinization temperature (Sun *et al*., 2008). RSDA can be produced from various sources including plants, animals and microorganisms and the microbial sources of RSDA are the most important.

Microbial RSDA have many advantages for the industrial production as compared to other sources of RSDA production. According to Sun *et al*. (2008), the industrial production of microbial RSDA is cost effectiveness, the time and space required for production is less, more consistency and the process of modification and optimization is easy. Sun *et al*. (2008) reported that the microorganisms that involved in the production of RSDA included the fungi, yeast and bacteria. The RSDA produced from different microorganisms may produce different end products from the enzymatic hydrolysis of the substrate although the same substrate is used. For example, glucose is produced as the end product by the RSDA produced from *Gibberella pulicaris*, *Acremonium* sp. and *Nodilusporium* sp. whereas glucose and maltose are produced as the end products by the RSDA produced from '*Synnematous*' sp. (Marlida *et al*., 2000b).

Raw starch degrading enzymes can be divided into α-amylases, glucoamylase, α-glucosidases, isoamylases, β-amylases and maltogenic β-amylases (Robertson *et al.,* 2006). RSDA can be further classified into endo-amylase and exo-amylase. Examples of endo-amylase are α-amylase and isoamylase while examples of exo-amylase are glucoamylase, α-glucosidases, β-amylases and maltogenic β-amylases (Robertson *et al*., 2006).

The hydrolysis of the α-1,4-linkages by endo-amylases is restricted in the region of α-1,6-branching. According to Robertson *et al.* (2006), α-amylases are able to catalyze the hydrolysis of α-1,4-linkages between the adjacent glucose units in the amylose and amylopectin polymers in order to produce dextrins by the endo-action of this type of enzyme. For example, α-amylase from Archaeon *Thermococcus profundus* DT 5432 produced both the maltose and maltotriose of α-configuration from the hydrolysis of soluble starch, glycogen, amylose and amylopectin (Marlida *et al*., 2000b). Isoamylases are involved in the hydrolysis of α-1,6-linkages at the branch points in amylopectin.

On the other hand, exo-amylases play important role in hydrolyzing the terminal or the next-to-terminal linkage from the non-reducing ends of starch chains by exo-action (Robertson *et al.,* 2006). Glucoamylases are able to catalyze the hydrolysis of both α-1,4-linkages and α-1,6-linkages in order to produce β-glucose. For example, glucoamylase produced from *Rhizopus neivus* will produce glucose from hydrolysis of starch granules of maize and amylomaize-7 (Marlida *et al*., 2000b). Glucosidases are involved in the hydrolysis of only α-1,4-linkages to produce α-glucose whereas β-amylase and maltogenic α-amylase play their role in the production of β-maltose and α-maltose respectively (Robertson *et al*., 2006).

RSDA is used in variety of industrial processes for the production of valuable products. For example, RSDA is used in food industry in starch liquefaction process for conversion of starch into maltose, glucose and high fructose containing syrups. Besides that, RSDA is also used for the production of high molecular weight branched dextrins (Aiyer, 2005). Other than that, the RSDA can also be used in other industrial processes such as in brewing industry and textile industry. There are many important components that are required for the production of RSDA. These components include the carbon sources, nitrogen sources

and metal ions. The pH, moisture and temperature need to be maintained on optimum condition in order to produce RSDA

2.2 Fungi producing RSDA

Starch is the most important sources and substrate in the food industries for the production of syrups containing the glucose, maltose or fructose. Raw starch degrading amylase (RSDA) is starch hydrolyzing enzyme that catalyzes the degradation of raw starch into simple sugars by breaking down the bonds between the simple glucose units. RSDA can hydrolyzed the raw starch into simple sugars in single step without involvement of gelatinization process which is usually the energy consumption step required in normal starch processing.

RSDA used in food industrial can be produced from different fungal species. According to Sun *et al*. (2009), the used of fungi sources for production of RSDA has many advantages such as it is cost effectiveness, time and space required for the production is less, more consistency and the process of modification and optimization is easy. The fungal species that produced the fungal strains included *Aspergillus awamori, Aspergillus foetidus, Aspergillus niger, Aspergillus oryzae, Aspergillus terreus, Mucor rouxians, Mucor javanicus, Neurospora crassa, Rhizopus delmar, Rhizopus oryzae and Arthrobotrys amerospora* (Norouzian *et al*., 2005). Among the different fungal species that produced the RSDA, the *Aspergillus niger* is the most often used fungi that is used in producing the RSDA used for starch processing industries. The application of RSDA produced from *Aspergillus niger* is used in starch processing industries because they have good thermostability and high activities at near neutral pH values (Norouzian *et al.,* 2005).

2.3 Starch

Starch is the major form of the carbohydrate where it is found as granules in plant tubers and seed endosperm. Starch is made up of several million amylopectin molecules and larger number of smaller amylose molecule (Chaplin, 2012). Amylopectin is polymer that contain short chain of 1,4-linked-α-D-glucose and 1,6-α-linked branches whereas amylose is the polymer that contain linear 1,4-linked-α-D-glucan (Ahmad *et al*., 1999). Amylopectin is usually consist of 17-26 D-glucosyl units separate the α-1,6 branch points while amylose consist of almost 6000 D-glucopyranose units (Ahmad *et al*., 1999). These two polysaccharides made up all of the starches with the ratio of amylose to amylopectin vary for different starch sources. For example, sago starch contains 26% of amylose, corn starch contains 28% amylose and sweet potato starch contains 18% of amylose.

Starch can range from 1 to 60 μm in size with example of sago starch with granule size of 15-50 μm , corn starch with granule size of 5-25 μm and sweet potato with granule size of 4-40 μm (Satin, n.d.). Different sources of starch is different in overall structure through distribution of granule size, shape, amylose and lipid content, distribution of length of chain in amylopectin and crystalline structure (Ahmad *et al*., 1999).

According to Robertson *et al*. (2006), the digestion of raw starch granules by RSDA is occurred through several routes. The first route is the local or distributed digestion at the surface pores. The second route is the centripetal digestion along starch polymer chains. The third route is by artifactual cracks digestion and the fourth route is the digestion at the susceptible site by diffusion through starch structure.

2.3.1 Sago starch

Sago starch is made up of linear polymer of amylose and branch polymer of amylopectin, which comprised of 27% of amylose and 73% of amylopectin (Flach, 1997). Sago starch is usually present in the sago pith which has to be separated from the cellulosic materials in order to obtained and extracted good quality and quantity sago starch. Sago starch granules are different in the size ranging from 10 μm to 50 μm in diameter with the average diameter of 32 μm in the granule size (Karim *et al*., 2008). Granules of sago starch are bigger than that of rice, corn and wheat but smaller than that of the potato. The sago starch granules are oval or polygonal-shaped with some truncated oval granules. Sago starch consists of 10.6% to 20.0% of moisture content; 0.06% to 0.43% of ash; 0.10% to 0.13% of crude fat; 0.26% to 0.32% of fibre and 0.19% to 0.25% of crude protein (Karim *et al*., 2008).

There are generally two methods of starch extraction which is by mean of traditional method and modern method. In the traditional method of starch extraction, it can be divided into two levels, that are domestic level and the small-scale processing plant level. Domestic level is usually practiced by the individual farmers in the garden without the need for the transportation of the heavy trunk. According to Karim *et al*. (2008), the process of domestic level of starch extraction will be carried out as described. The trunk is split lengthwise and the pith is rasped by the chopper or small hoe made from bamboo. After that, the leaf sheath of the sago palm is used to put with the rasped mixture of fibre and pith with the sieve placed at its lowest end. The mixture is kneaded by hand after it is added with water. The water together with the starch granules in suspension passes through the sieve and is flow into an old dugout canoe or any suitable container will the fibres remain on the top of the sieve. The bottom of the canoe or container is settled with the starch and the excess water will flow over the sides. The fibrous remnants are removed after kneading and the wet starch is taken out from the canoe or container.

According to Karim *et al.* (2008), small-scale processing plant level of starch extraction is carried out as described. The shorter length of sago trunks about 1 to 1.2 m are cut and tied into rafts to be transported to the plant by the rivers or water system. The pith is dug out of the split trunk and then the pith is rasped by using engine-powered rasps. The rasped pith is then trampled by foot on a platform. The starch and coarse fibre are separated by a rotating mesh washer made of metal. The settling pond made of boards is used to store the starch slurry and finally the wet starch is dried under the sun.

In modern method of starch extraction, some modifications are made to the small processing plant to extract the sago starch. According to Karim *et al*. (2008), the process of modern method of starch extraction is carried out as described. The storage pond with 30cm long is split into eight segment by which each of the segment are fed into slicers that slice the pith from the bark. In some factories, the bark of the sections of the logs is first removed. The 80 to 100cm long debarked sections are fed into the mechanical rasper equipped with chrome nails. The pith will rasped into finer pieces to be fed into the hammer mill through a conveyer belt. The coarse fibres are separated by passed the starch slurry obtained through a series of centrifugal sieves. Nozzle separator is used for further purification of starch through sieve bends. In order to obtain very pure starch, a series of cyclone separators has also been used. Rotary vacuum drum dryer is used for the process of dewatering of the sago starch and this process is followed by the hot air drying.

3.0 Materials and methods

3.1 Sample collection

The locally isolated fungal strains were obtained from Molecular Biology Laboratory (UNIMAS Fungal Collection) and these fungal strains were *Bionectria ochroleuca*, *Trichoderma harzianum, Trichoderma virens*, *Aspergillus versicolor, Aspergillus niger* and *Aspergillus flavus.*

3.2 Stock culture preparation

Two hundred and fifty ml of Potato Dextrose Agar (PDA) was prepared and poured into the plastic Petri dish. The isolated fungal strains were cultured on agar for 7 days at 37^{o}C to be used as working culture. After 7 days, 5 mm^2 block of PDA with fungi grown on it was cut and transferred into a new PDA to be cultured at 37^{o}C for stock culture. The stock culture was prepared in 20% glycerol. Sixteen ml of distilled water and 4 ml of glycerol was mixed and autoclaved. One ml of the mixture solution was transferred into the eppendoft tube. Two 5 mm^2 of the agar block containing fungi was cut from working culture and transferred into the eppendoft tube. The culture stocks were then kept in -20^{o}C freezer.

3.3 Qualitative measurement of RSDA ability

The qualitative measurement of RSDA ability was carried out based on method proposed by Marlida *et al*. (2000b). Two hundred and fifty ml of minimal salt agar (MSA) with 1% (w/v) raw sago starch as carbon sources was prepared and poured into the Petri dish. The MSA comprised of 0.25 g K_2HPO_4, 2.5 mg $ZnSO_4.7H_2O$, 1.25 mg $CuSO_4.5H_2O$, 0.125 g $MgSO_4.7H_2O$, 2.5 mg $FeSO_4.7H_2O$, 0.125 g KCl, 0.75 g $NaNO_3$, 5 g agar and 2.5 g of raw sago starch in 250 ml of distilled water. After that, the isolated strains were grown onto MSA and after being cultivated for 2 to 3 days, RSDA ability was assessed by the formation of

clear zone around the colonies when flooded with iodine solution (1% potassium iodide and 0.3% iodide (w/v)). The diameter of clear zone was measured.

3.4 Quantitative measurement of RSDA activity

Quantitative measurement of RSDA activity was carried out based on method used by Marlida *et al*. (2000b). Three of 5 mm^2 in diameter agar blocks of the fungal strains from 7 days old culture were used as inoculums. The three agar blocks of fungal strains were transferred into the minimal salt broth medium with 1% of raw sago starch as carbon sources. The minimal salt broth medium comprised 1 g K_2HPO_4, 10 mg $ZnSO_4.7H_2O$, 5 mg $CuSO_4.5H_2O$, 0.5 g $MgSO_4.7H_2O$, 10 mg $FeSO_4.7H_2O$, 0.5 g KCl, 3 g $NaNO_3$ and 10 g raw sago starch in 1000 ml of distilled water. The raw sago starch was first autoclaved at 121^oC for 1 and half hours and was then added to the sterilized minimal salt broth medium with pH adjusted to 5.5. The minimal salt broth medium was then inoculated and incubated at 27^oC on rotary shaker at 140 rpm for 7 days. Centrifugation was carried out at 15000 g for 20 min at 4^oC to remove the mycelia and residual starch granules. Filtration was carried out by using Whatman filter paper No. 4 and the filtrate obtained was used as crude enzyme. The amount of activity present in the crude enzyme was determined. All strains were cultivated for 2-10 days for measurement of growth profiles. The amounts of enzyme secreted into the medium were determined.

3.5 Measurement of RSDA activity

Measurement of RSDA activity was carried out as proposed by Marlida *et al*. (2000b). The reducing sugar liberated by each fungus strains was measured by the Dinitrosalicylic (DNS) method of Miller (1959). In DNS test, 0.5 ml of 2% (w/v) raw sago starch in 0.l M acetate buffer and 0.5 ml of enzyme solution was used as the reaction mixture and the mixture was incubated with shaking at 37^{o}C for 30 min. After that, the reaction was stopped by adding 1.0 ml of DNS color reagent and boiled for 10 min in the boiling water. The mixture was allowed to cool to room temperature and 1 ml of Rochelle salt was added to the mixture and then vortex. The reading of spectrometer at absorbance of 540 nm was measured. 1 unit of raw starch degrading activity was defined as the amount of enzyme that liberated 1 µmol of reducing sugar per minute (as glucose)/min under the standard assay conditions

3.6 Estimation of starch-degrading ability

The raw starch-degrading abilities (RDAs) were calculated by the following equation: RDA (%) = *B/A* x 100 by which *A* was gelatinized starch-degrading activity and *B* was the raw-starch degrading activity. According to Marlida *et al*. (2000b), estimation of starch-degrading ability was carried out as below. A and B was reacted in the mixture solution by which the solution contained 0.5 ml of enzyme solution, 1 ml of gelatinized or raw starch solution (2% (w/v) in 0.1 M acetate buffer at optimum pH) and 0.5 ml of 0.1 M acetate buffer at optimum pH. The mixture was incubated 30 min at 37^{o}C. Reducing sugar liberated into the supernatant was measured by the DNS method as described by Miller (1959).

3.7 Effect of pH on enzymatic raw sago starch hydrolysis

The effect of the pH of the reaction medium on enzymatic raw sago starch hydrolysis was carried out in 0.1 M acetate buffer as described by Marlida *et al.* (2000b). 0.5 ml of 2% (w/v) raw sago starch in acetate buffer at different pH (4.5, 5.0, 5.5 and 6.0) with 0.5 ml of enzyme solution was used as the reaction mixture. The reaction mixture was incubated at 37^{o}C for 24 h. After 24 h, 1 ml DNS color reagent was added into the reaction mixture and boiled in the boiling water for 10 min. The reaction mixture was allowed to cool at room temperature and 1 ml of Rochelle salt was added. The reducing sugar released was determined by the DNS method of Miller (1959).

3.8 Effect of different types of starches as substrate

According to Marlida *et al*. (2000b), the effects of different type of starches as substrate were determined by the reaction of the crude enzyme preparation in the presence of various starches which were tapioca, sweet potatoes, yam and raw sago starches. 0.5 ml of crude enzyme with 0.5 ml of 2% (w/v) raw starches in 0.1 M acetate buffer at optimum pH for each enzyme and incubated at 37^{o}C for 24 h. The reaction was stopped by boiled the mixtures in boiling water for 10 min. After that, centrifugation was carried out at 15000 g for 20 min and the DNS method of Miller (1959) was used to determine the reducing sugar liberated into the supernatant.

4.0 Results

4.1 Qualitative measurement of RSDA ability

The 6 strains of the fungi growing on the MSA were flooded with iodine solution after 3 days of cultivation and were compared with control to screen for their ability to secrete RSDA. The observation of the clear zone around the growing colony of all fungal strains indicated the ability to secrete RSDA into the growth medium and thus the presence of RSDA activity. The ability of fungal strains to hydrolyze raw sago starch granules was compared. As shows in Table 1, *A. flavus* was the best performing strain that produced the widest clear zone with diameter of 3.63 cm followed by the *B. ochroleuca* and *A. versicolor* that produced clear zone with more than 3 cm in diameter, which are 3.50 cm and 3.47 cm, respectively. *A. niger* produced clear zone with diameter of 2.47 cm. Both *T. virens* and *T. harzianum* produced clear zone with diameter not more than 1 cm, with the smallest clear zone produced by *T. harzianum* that was only 0.83 cm in diameter. From the result, it can be concluded that *A. flavus* was the best performing that produced RSDA.

Table 1: Comparison of activity of RSDA from six fungal strains using raw sago starch as substrate

	Diameter of clear zone formed (cm)				
Fungal Strains	R1	R2	R3	Mean	Standard deviation
B. ochroleuca	3.4	3.6	3.5	3.50	0.100
T. harzianum	0.8	0.8	0.9	0.83	0.058
T. virens	0.9	0.9	0.9	0.90	0.000
A. versicolor	3.5	3.4	3.5	3.47	0.058
A. niger	2.4	2.6	2.4	2.47	0.115
A. flavus	3.6	3.7	3.6	3.63	0.058