Isolation and Characterization of Indigenous Bacteria Producing Amylase Enzymes from Sago Starch with Potential for Liquid Sugar Production

Marthenci Imbraiseri Wanggai [1], *Maria* Massora[2*], and *Rina* A Mogea[2]

[1]Master Biology Program, Wageningen University and Research, Wageningen, The Netherlands.
[2]Faculty of Mathematics and Natural Sciences, Universitas Papua, Manokwari, Indonesia

Abstract. Sago (Metroxylon sp.) is a carbohydrate producing plant that important in eastern Indonesia, and its starch can be hydrolysed to produce alternative sweeteners. The objective of this research is to isolate and characterize indigenous bacteria producing amylase enzymes from sago starch and to test the ability of indigenous bacteria producing amylase enzymes to hydrolyze sago starch into liquid sugar. The methods used to characterize indigenous bacteria producing amylase enzymes from sago starch and their ability to hydrolyze sago starch into liquid sugar was tested based on the results of sago starch hydrolysis, gelatinase liquid sugar, and the Seliwanoff and Benedict tests. The research results from the sago samples yielded 11 isolates producing amylase enzymes and showing potential for producing liquid sugar, particularly in IA 5, IA 9, IA 10, and IA 11, which have high reducing sugar concentration values. Among the 11 indigenous bacterial isolates producing amylase enzymes that were tested, the characteristics of isolates IA 1, IA 3, and IA 7 were identified as belonging to the genus Clostridium, while isolates IA 2, IA 4, IA 5, IA 6, IA 8, IA 9, IA 10, and IA 11 were identified as belonging to the genus Bacillus.

1 Introduction

Sago (*Metroxylon* sp.) is one of the most essential local carbohydrates in Eastern Indonesia, especially in Papua, because of its abundant availability. The high starch content makes sago a potential raw material for products, such as liquid sugar [1]. Previous research in Manokwari agricultural fields also showed high populations of functionally important native bacteria such as Bacillus and Pseudomonas, indicating that the local environment supports metabolically active microbial communities that can be utilized for biotechnology processes [2]. This ecological background encourages efforts to explore bacteria associated with sago due to their hydrolytic potential.

Liquid sugar can be produced from starch through microbial or enzymatic hydrolysis and is relevant as an alternative sweetener when sugar cane production cannot meet demand [3]. In starch rich environments, many indigenous bacteria produce extracellular amylase that breaks down starch into reducible simple sugars, so isolating these bacteria from their original substrate (sago starch) is a rational approach. Standard microbiological procedures and biochemical characterization enable the selection of promising isolates.

Therefore, the objectives of this study were to isolate indigenous amylase-producing bacteria from sago starch, to characterize the isolates morphologically and biochemically, and to evaluate their ability to hydrolyse sago starch into liquid sugar. The results are expected to provide baseline information for developing sago-based liquid sugar using local microbial resources

2 Material and methods

2.1 Sample and dilutions

Sago starch was purchased from Sanggeng market, Manokwari, and brought to the laboratory. Serial dilutions were made up by using 10 grams of sago starch, which were suspended in 90 ml of sterile NaCl and were used for isolation.

2.2 Isolation of bacteria

A 0.5 ml aliquot from each selected dilution was spread on Nutrient Agar (NA) plates and incubated at 30-37°C for 24-48 h. Colonies with different morphology were picked and purified on NA slants to obtain single isolates. In total 15 isolates were obtained.

2.3 Screening for amylase-producing bacteria

All isolates were streaked on NA with 2% soluble starch and incubated at 35-37 °C for 48 h. After incubation, plates were flooded with Lugols's iodine solution. Isolates that produced clear zones around the growth were considered amylase positive and

*Corresponding author: m.massora@unipa.ac.id

were given codes IA1-IA11. This simple plate assay is commonly used to select starch hydrolysing bacteria [4].

2.4 Preparation of sago starch medium and fermentation

Sago starch extract was prepared by mixing equal parts of sago starch and distilled water. For the hydrolysis test, 5 ml of sago starch extract were mixed with 5 ml of the bacterial suspensions. Tubes were tightly closed to create low oxygen conditions and incubated at 37 °C for 72 h. After incubation, cultures were centrifuged twice at 1500 rpm for 30 min to obtain clear supernatants, which were treated as crude liquid sugar. This step follows the approach used in local starch to sugar studies [3,4].

2.5 Qualitative Sugar test

Two qualitative tests were used to confirm sugar formation:

- Benedict test: 1ml sample + 2 ml Benedict reagent, boiled 5-0 min. Colors from green to brick red were interpreted using the usual Benedict scale to estimate reducing sugar content.

Table 1. Qualitative Benedict scale for reducing sugar determination

Colour observation	Score	Concentration
Blue – turbid green	-	-
Yellowish green	+1	< 0.5%
Yellow to yellowish green/turbid yellow	+2	0.5-1.0%
Orange	+3	1.0-2.0%
Red	+4	>2%

- Seliwanoff test: 0.5-1 ml sample + 1 ml Seliwanoff reagent, heated 1-5 min Red to cherry color indicated ketose sugars, while a weak pink color indicates aldose sugars [6].

2.6 Morphological and biochemical characterization

Colony morphology (shape, margin, elevation, color) were recorded from NA plates. Gram staining and cell shape were observed microscopically. Biochemical test included indole, methyl red (MR), Voges–Proskauer (VP), citrate utilization, motility, urease, triple sugar iron agar (TSIA), catalase and gelatin hydrolysis, carried out according to Cappuccino and Sherman [4]. The pattern of positive/negative reactions was compared with descriptions in *Bergey's Manual of Determinative Bacteriology* to suggest likely genera [5].

3 Result and discussion

3.1 Isolation and screening

Fifteen bacterial isolates were successfully obtained from the sago starch sample, showing that the material still carried a diverse microbial population even though it was a marketed product. When these isolates were screened on starch agar, 11 of them produced clear hydrolysis zones after flooding with iodine, indicating extracellular amylase activity. This proportion is in line with previous observations that starchy or agricultural substrates from the Manokwari area can harbour functionally active bacteria [2].

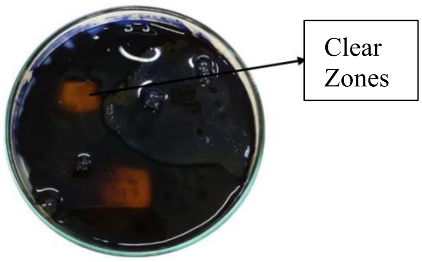

Fig. 1. Selection Results of Amylase Enzyme-Producing Bacteria Isolate IA 7

3.2 Ability to hydrolyse sago starch into liquid sugar

All 11 amylase-positive isolates were able to grow in the sago starch extract and produced clear supernatants after 72 h. Benedict's test showed that every supernatant contained reducing sugars, confirming that the bacteria did not only break down starch on plates but could also hydrolyse it in liquid form. Most isolates produced green to yellowish precipitates (<1% sugar), but four isolates (IA5, IA9, IA10 and IA11) produced yellow to orange precipitates corresponding to approximately 1.0–2.0% reducing sugar. These four isolates can therefore be considered the most promising for further work on sago-based liquid sugar. Seliwanoff reactions were positive (red) for only some of the hydrolysates, indicating the presence of ketose-type sugars in addition to aldoses. This suggests that different isolates may produce slightly different sugar profiles, which could be due to different amylase types or secondary metabolism. For food applications, this information is useful because sugar profile affects sweetness and downstream processing [6, 7,11].

The sugar levels obtained here are lower than those reported for fully enzymatic or multistep hydrolysis processes on other starches [8,11], but the method used in this study was deliberately simple (single inoculation, no added commercial enzyme, short time). For community-level or small-scale utilisation of local sago, using indigenous bacteria as enzyme sources can still be attractive, especially if the bacteria are later cultured specifically to produce crude amylase that is then applied to sago starch under optimised conditions.

3.3 Morphological and biochemical characteristics

Most isolates were Gram-positive, rod-shaped and catalase positive.

Fig. 2. Gram staining Results of Amylase Enzyme-Producing Bacteria Isolate IA 1

Table 2. Biochemical characteristics of amylase-producing bacterial isolates.

Isolate	Lac	Glu	Suc	Mot	H2S	Gas	Ind	MR	VP	Cit	Ure	Cat	Gel
IA 1	+	+	+	+	–	–	+	+	+	–	–	+	+
IA 2	+	+	+	+	–	–	+	+	+	+	–	+	+
IA 3	+	+	+	+	–	–	+	–	+	–	–	+	+
IA 4	+	+	+	+	–	–	+	–	+	+	–	+	+
IA 5	+	+	+	+	–	+	+	+	+	+	–	+	+
IA 6	+	+	+	+	–	+	–	+	+	+	+	+	+
IA 7	+	+	+	+	–	–	+	+	+	–	–	+	+
IA 8	+	+	+	+	–	–	+	+	+	+	+	+	+
IA 9	+	+	+	+	–	–	+	+	+	+	+	+	+
IA 10	+	+	+	+	–	–	+	+	+	+	+	+	+
IA 11	+	+	+	+	–	–	+	+	+	+	+	+	+

Lac = lactose; Glu = glucose/dextrose; Suc = sucrose; Mot = motility; Ind = indole; MR = methyl red; VP = Voges–Proskauer; Cit = citrate; Ure = urease; Cat = catalase; Gel = gelatin.

When biochemical patterns were compared with *Bergey's Manual*, most isolates (IA2, IA4–IA6, IA8–IA11) were close to the genus Bacillus, while a smaller group (IA1, IA3, IA7) showed characteristics compatible with Clostridium. This finding matches other work from Manokwari soils where *Bacillus* and other spore-forming bacteria were among the dominant functional groups [2]. *Bacillus* spp. is also well known as industrial α-amylase producers [9,10], so finding them on sago starch supports the idea of using local strains as starter material.

3.4 Implications

Taken together, the results show three important points: (i) sago starch sold locally still carries indigenous bacteria with useful enzymes, (ii) several of these bacteria can produce measurable levels of reducing sugar from sago starch without added commercial enzymes, and (iii) the dominant genera found are genera that are already recognized as safe and useful in food/biotech contexts. Follow-up work should focus on increasing sugar yield (by adjusting pH, temperature, substrate concentration and time) and confirming the identity of the best isolates using molecular methods.

4 Conclusion

Sago starch from Manokwari contained indigenous bacteria, 11 of which showed clear amylase activity on starch agar. All 11 isolates were able to hydrolyse sago starch in liquid culture and produce reducing sugars, with isolates IA5, IA9, IA10 and IA11 giving the highest Benedict scores (≈1–2% sugar). Morphological and biochemical tests indicated that most isolates belonged to the genus *Bacillus* and a few to *Clostridium*. These findings confirm that local, sago-associated bacteria can be used as sources of amylase for developing simple sago-to-liquid-sugar processes.

References

1. F. Asmuruf, J.F. Wanma, A. Rumatora, "Budidaya pemanfaatan sago (*Metroxylon* sp.) oleh sub-etnis Ayamaru di Kampung Sembaro," *J. Kehutanan Papuasia* **4**(2), 114–127 (2018)
2. I.A.F. Djuuna, S. Prabawardani, M. Massora, "Population distribution of phosphate-solubilizing microorganisms in agricultural soil," *Microbes and Environ.* 37 (1): ME21041 (2022). https://doi.org/10.1264/jsme2.ME21041
3. Yazid,E dan Nursanti,L. *Penuntun Biokimia untuk Mahasiswa Analis* (C.V Andi Offset, Yogyakarta, 2006)
4. J.G. Cappuccino, N. Sherman, *Microbiology: A Laboratory Manual*, 10th ed. (Pearson, London, 2014).
5. R.S. Holt, N.R. Krieg, P.H.A. Sneath, J.T. Staley, S.T. Williams (eds.), *Bergey's Manual of Determinative Bacteriology*, 9th ed. (Williams & Wilkins, Baltimore, 1994).
6. Kusbandari, A. "Analisis Kualitatif Kandungan Sakarida Dalam Tepung dan Pati Umbi Gayong (*Canna edulis* Ker.)," *J. Pharmaciana*, **5** (1): 35-42 (2015).
7. A. Hernández-López, A. Hernández-Rodríguez, L. Hernández-Rodríguez, "Quantification of reducing sugars based on the qualitative technique of Benedict," *ACS Omega* **5**, 31650–31657 (2020).

8. Saraswati, S., Rosidah I., Hapsari Y, "Pembuatan Glukosa Secara Enzimatik dari Bahan Baku Pati Sagu," *J. Teknik Kimia Indonesia*, **3**(1): 56-63 (2018). https://doi.org/10.26555/chemica.v3i1.4306
9. Melliawati, R., Suherman R. S., Subardjo B. "Pengkajian kapang endofit dari Taman Nasional Gunung Halimun sebagai penghasil glukoamilase," *Berkala Penelitian Hayati*, **12**(1):19-25(2006). https://doi.org/10.23869/393
10. Rahayu, M. A., Sulistyaningtyas A.R., Darmawati S. "Isolasi Bakteri Hidrofilik Penghasil Enzim Amilase dari Limbah Industri Tapioka," *in Proc. Semin. Nas. Unimus*. 147-155 (2019).
11. E. Betiku, O.O. Akindolani, A.R. Ismaila, "Enzymatic hydrolysis optimization of sweet potato (*Ipomoea batatas*) peel using a statistical approach," *Braz*. *J*. *Chem*. *Eng*. **30**(2), 337–345 (2013). https://doi.org/10.1590/S0104-66322013000200014