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Fermentation RS3 derived from sago and rice starch with *Clostridium butyricum* BCC B2571 or *Eubacterium rectale* DSM 17629

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ABSTRACT

Resistant starch type 3 (RS3) is retrograded starch which is not digested by human starch degrading enzyme, and will thus undergo bacterial degradation in the colon. The main fermentation products are the Short Chain Fatty Acid (SCFA): acetate, propionate and butyrate. SCFA has significant benefit impact on the metabolism of the host. The objectives of this research were to study the SCFA profile produced by colonic butyrate producing bacteria grown in medium containing RS3. RS3 was made from sago or rice starch treated with amylase, pullulanase and the combination of amylase and pullulanase. Fermentation study was performed by using *Clostridium butyricum* BCC B2571 or *Eubacterium rectale* DSM 17629, which has been identified as capable of degradation of starch residue and also regarded as beneficial bacteria. Experimental result revealed that enzyme hydrolysis of retrograded sago or rice starch was beneficial to RS formation. RS3 derived from sago contained higher RS (31–38%) than those derived from rice starch (21–26%). This study indicated that *C. butyricum* BCC B2571 produced acetate, propionate and butyrate at molar ratio of 1.8 : 1 : 1, when the medium was supplemented with RSSA at concentration 1%. In the medium containing similar substrate, *E. rectale* DSM 17629 produced acetate, propionate and butyrate at molar ratio of 1.7 : 1 : 1.2. High levels of acetate, propionate and butyrate at molar ratio of 1.8 : 1 : 1.1 was also produced by *E. rectale* DSM 17629 in medium supplemented with RSSP at concentration 1%. The results showed that both bacteria responded differently to the RS3 supplementation. Such result provided insight into the possibility of designing RS3 as prebiotic with featured regarding SCFA released in the human colon with potential health implication.

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1. Introduction

Starch and starch component which are not digested in the small intestine and which passes into the large intestine is referred as resistant starch. Resistant starch is fermented by the bacteria in

the large intestine, which will ferment the resistant starch into short chain fatty acids (SCFA), particularly: acetate, propionate and butyrate as well as producing gasses such as hydrogen, carbon dioxide and methane [1].

The SCFA have significant benefit impact on the metabolism of the host. In gastrointestinal tract of mammalian species, it is directly absorbed in the production sites. Unabsorbed SCFA enter the hepatic portal blood. Acetate is metabolized by the liver where it is converted into acetyl-CoA, which can act as precursor for lipogenesis and also stimulates gluconeogenesis. Propionate has been shown to inhibit gluconeogenesis and increase glycolysis in rat hepatocytes [2]. Butyrate is the main energy substrate for the colonocytes and has been implicated in the prevention of colitis and colorectal cancer [3].

Many foods which are rich in carbohydrate contain varying amounts of starch which may resist to the digestion for various reasons. Different types of resistant starch occur naturally in the human diet. Resistant starch has been classified into four general

Abbreviations: RS1, resistant starch type 1; RS2, resistant starch type 2; RS3, resistant starch type 3; RSSA, resistant starch type 3 derived from sago treated with amylase; RSSP, resistant starch type 3 derived from sago treated with pullulanase; RSSAP, resistant starch type 3 derived from sago treated with amylase and pullulanase; RSRA, resistant starch type 3 derived from rice treated with amylase; RSRP, resistant starch type 3 derived from rice treated with pullulanase; RSRAP, resistant starch type 3 derived from rice treated with amylase and pullulanase; SCFA, short chain fatty acid.

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subtypes called RS type 1 (RS1), RS type 2 (RS2), RS type 3 (RS3) and RS type 4 (RS4) [4,5]. RS1 is physically inaccessible, because the starch molecules are trapped in the structural carbohydrate and this, difficult to be digested. RS2 refers to native granula starch with highly dense crystalline structure that prevents enzymatic digestion. RS3 refers to non-granular starch-derived materials that resist digestion. RS3 is generally formed during the retrogradation of starch granules, and can be produced by autoclaving and cooling in the presence of water. When starch is heated, amylose is solubilized and a starch gel is formed. Upon cooling, the gel undergoes transformations leading to a partially crystalline structure (retrogradation). During this retrogradation, amylose is reassociated to form strong crystallization. RS4 is chemically modified starch. In this regard, chemical bonds other than α -(1,4) or α -(1,6) such as starch phosphate ester are included in this group.

Several methods have been developed to prepare the readily fermentable RS [6–8]. In the current study, starch hydrolyzing enzymes (amylase and pullulanase) were applied to produce readily fermentable resistant starch. Sago and rice starches were selected due to their great potential to be developed into valuable products such as functional food ingredient. In Indonesia, there are a number of rice varieties. However, there are only few varieties which are popular for direct consumption. Other varieties may have excellent agronomics traits but not preferred usually due to inferior in their taste/aroma. Thus, they may be used for valuable food ingredient development such as RS. The world estimated area of sago palm is about 2.25 million ha of wild stand and 0.2 million ha of semicultivated [9]. Indonesia has the largest sago palm area followed by Papua New Guinea, Malaysia and Phillipine. Sago produces higher starch compared to other crops; it gives around 2–3 tons starch per ha, compared to cassava which is 2 tons and maize ton [10]. In addition, there are completely different amylose content and molecular properties between sago and rice starch [11–13].

Major butyrogenic species of the human colonic microbiota fell within the XIVa cluster of gram-positive bacterium. They have been identified in the genera *Clostridium*, *Eubacterium* and *Fusobacterium* [14,15]. In vitro fermentation study of RS3 from potato, corn starch and their derivatives products has been reported. They were performed in a batch or continuous system with pure or mixed culture of human feces extract [16–19]. However, limited reports are available on the capability of butyrate producing bacteria to ferment RS3. The objective of this research was to investigate the (in vitro) utilization of RS3 derived from sago and rice starches to produce short chain fatty acids (SCFA) by *Clostridium butyricum* BCC B2571 or *E. rectale* DSM 17629, which have been identified as starch degrader residue and regarded as beneficial colon bacteria [20,21].

2. Material and methods

2.1. Rice, starch and chemicals

Rice grain (Cisokan) was obtained from the Indonesian Center for Rice Research, Sukamandi, Indonesia. Sago was purchased from the sago processing unit at Sukabumi area. Starch degrading enzyme was obtained from Novo Nordisk through the distributor at Jakarta. Two types of enzyme were applied, they were amylase (Teramyl 120 L) and pullulanase (Promozyme D2), respectively. Teramyl 120 L had standard activity of 120 KNU-T/g and a density of approximately 1.26 g/mL, while standard activity and density of Promozyme D2 were 1350 NPUN/g and 1.20 g/mL, respectively. The unit of 1 KNU-T amylase was equivalent with 5.9×10^5 U, and the unit of 1 NPUN pullulanase was equivalent with 140 U. Unit definition is as following: one unit of amylase liberate 1.0 mg of maltose from starch in 3 min at 6.9 at 20 °C, while one unit of pullulanase

liberate 1.0 μ mol of maltotriose (measured as glucose) from pullulan at 5.0 at 25 °C.

2.2. Preparation of RS3

Sago starch was washed with tap water three times, sun-dried and sieved at 100 mesh and stored in plastic bag until used. Rice starch was isolated by using alkaline steeping, according to the method described by Wang and Wang [22].

The following procedure was applied to prepare RS3. Rice or sago starch (50 g) was suspended in 200 mL of water, boiled and stirred for 10 min, removed from heat and cooled down to 30 °C. The gel was vacuum sealed in a retort pouch and autoclaved at 121 °C, 15 psi for 1 h, and stored at 4 °C for 12–14 h, to enhance retrogradation. Retrograded starch was suspended in 1 L of water and blended in a waring blender at high speed for 2 min. The retrograded starch suspension was enzymatically hydrolyzed. The following treatments were applied for starch hydrolysis: (a) 1 mL of amylase (3.0 KNU-T/g substrate) for 3 h at 85 °C, (b) 1 mL of pullulanase (32.0 NPUN/g substrate) for 3 h at 55 °C, (c) 1 mL of amylase for 3 h at 85 °C continued with 1 mL of pullulanase for 3 h at 85 °C.

Hydrolyzed starch was centrifuged for 10 min at room temperature. The supernatant was discarded and the residue was collected. The residue was stored in cool room (10 °C) overnight, suspended into water and homogenized for 2 min by using homogenizer. The suspension was loaded into the spray drier. The inlet temperature of the dryer was 160 °C.

2.3. Bacterial strains and culture media

Pure culture of *C. butyricum* BCC B2571 was obtained from Culture Collection of Indonesian Research Center for Veterinary Sciences (IVETRI), Indonesia. The basal medium for maintaining *C. butyricum* BCC-B2571 consisted of the following (in g/L): yeast extract, 3; beef powder, 10; peptone 10; glucose, 5; soluble starch, 1; NaCl, 5; Na-acetate, 3; cysteine hydrochloride, 0.5. The pH was adjusted to 6.8. *Eubacterium rectale* DSM 17629 was obtained from DSMZ, Germany and it was maintained in medium composing of (in g/L): tryptone, 5; bacteriological peptone, 5; yeast extract, 10; beef extract, 5; glucose, 5; Tween 80 1 mL, resazurin 0.001; CaCl₂, 0.01; MgSO₄, 0.02; K₂HPO₄, 0.04, KH₂PO₄, 0.04, NaHCO₃, 0.4, NaCl, 0.08, Vitamin K1, 0.0002. The pH was adjusted to 7.0.

2.4. In vitro fermentation

Growth medium was distributed into serum bottle flushed with CO₂. Every bottle contained 20 mL of medium. The bottle was sealed with a butyl rubber septum and sterilized at 121 °C for 15 min. It was then inoculated with 1 mL of 24 h pre-cultured bacterial strain (at about 10⁹ CFU/mL), and incubated under anaerobic condition at 37 °C in water bath. Various type of RS3 (1%) and glucose (0.5%) were added into the medium. Fermentation was carried out for 48 h and performed at three replications. In another in vitro fermentation, glucose as the only carbon source (concentration 1.5%) was also run.

2.5. Analysis of rice and sago starch

Amylose content of the starch was analyzed according to the method of Juliano [23]. Chemical component (moisture content, ash, proteins and fat) was analyzed according to the standard method of AOAC [24].

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