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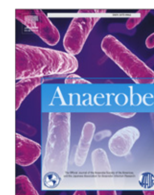

Molecular Biology, Genetics and Biotechnology

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

Q1 Endang Yuli Purwani [a,b], Tresnawati Purwadaria [c], Maggy Thenawidjaja Suhartono [b,*]

[a] Indonesian Center for Agricultural Postharvest Research and Development, Jln. Tentara Pelajar No.12, Bogor 16114, Indonesia
[b] Department of Food Science and Technology, Bogor Agricultural University, PO BOX 220, Darmaga Campus, Bogor 16002, Indonesia
[c] Indonesian Research Institute for Animal Production, PO BOX 221, Bogor 16002, Indonesia

A R T I C L E I N F O

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A B S T R A C T

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 wih amylase; RSRP, resistant starch type 3 derived from rice treated wih pullulanase; RSRAP, resistant starch type 3 derived from rice treated with amylase and pullulanase; SCFA, short chain fatty acid.

* Corresponding author. Department of Food Science and Technology, Bogor Agricultural University, PO BOX 220, Darmaga Campus, Bogor 16002, Indonesia. Tel.: +62 2518620517.
E-mail addresses: endangyp@yahoo.com.sg (E.Y. Purwani), tpurwadaria@yahoo.co.uk (T. Purwadaria), mthenawidjaja@yahoo.com (M.T. Suhartono).

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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 Philippines. 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 μmol 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 pullunase 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 Vaterinary 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; $CaCl_2$, 0.01; $MgSO_4$, 0.02; K_2HPO_4, 0.04, KH_2PO_4, 0.04, $NaHCO_3$, 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 CO_2. 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 10^9 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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2.6. Determination of resistant starch (RS)

RS content was analyzed according to the method described by Goni et al. [25] with modification. Briefly, the procedure is as following: 50 mg of sample was dispersed in 5 mL of KCl-HCl solution pH 1.5 and incubated with 4400 unit of pepsin solution at 40 °C for 60 min with constant shaking to remove the proteinous substances. Tris maleate buffer 0.1 M, pH 6.9 (4.5 mL) was added to the sample mixture and incubated with 100 unit of amylase at 37 °C for 16 h with constant shaking to hydrolyze the digestible starch, then centrifuged (1000 g for 15 min) twice, to discard the supernatant. The residue was moistened with 1.5 mL of distilled water and solubilized with 1.5 mL of KOH 4 M. RS solution was mixed with HCl 2 M and Na-acetate 0.4 M buffer, pH 4.75, then incubated with 100 unit of amyloglucosidase solution at 55 °C for 45 min. Glucose formed in the supernatant (collected after centrifugation at 1000 g for 15 min), was determined using phenol-sulfuric acid method [26]. RS content was calculated as glucose (g) $\times$ 0.9 and was expressed as percent of RS in sample analysis.

2.7. Amylase activity on agar plates

The assay employed a solid medium prepared with resistant starch (2 g/L). After 24 h of anaerobic incubation in the basal medium, bacterial culture (30 μL) was transferred onto the well (0.003 cm^2) of the starch agar plate. The inoculated plates were incubated in the anaerobic chamber for 48 h at 37 °C. Further visualization was conducted by adding I_2-KI solution (0.15% I_2 in 1.55 KI), and the area of clear zones around the well was measured.

2.8. Gas production and pH measurement

Gas production was measured by allowing the gas in the serum bottle to expand into glass syringe and thus can be measured. The pH of the cultures was determined using a pH meter.

2.9. Short chain fatty acid analysis

Culture medium was centrifuged at 3000 g for 10 min. The supernatant was filtered through a 0.22 μm filter into a 1.5 mL eppendorf tube for storage at 4 °C until use. Samples of 1 μl were injected into a high-resolution gas chromatography (Agilent Technologist, 7890A GC System) equipped with a flame ionization detector and an HP Innowax 19091–136 column (60 m $\times$ 0.250 mm). The carrier gas was helium with a flow rate of 1.8 ml/min, and the split ratio was 40:1. The oven temperature was maintained at 90 °C for 0.5 min, and then increased to 110 °C at a rate of 10 °C/min, increased to 170 °C at a rate of 5 °C/min and finally increased to 210 °C at a rate of 20 °C. Injector and detector temperatures were 275 °C. SCFA mixture containing acetate, propionate and butyrate at specific concentration were used as standard.

2.10. Statistical analysis

Chemical data on sago and rice starch were subjected to descriptives analysis. Data were presented as mean ± SD. Data on in vitro fermentation were analyzed by factorial analysis, kind of bacteria as a main factor and RS3 type as subfactor. Least significant different test (LSD-t) was carried out to compare the data between treatments, $p < 0.05$ was considered a significant different. SPSS 10.0 software was applied to analyze the data.

3. Result

3.1. Product derived from sago starch demonstrated higher RS content than those derived from rice starch

The moisture content, fat, carbohydrate, protein, ash and amylose content of sago and rice starch are shown in Table 1. Sago starch contained higher amylose and less protein than those of rice starch. Both starch showed high purity and appropriate to be processed for RS3.

Starch was completely gelatinized and kept at low temperature, prior to enzyme hydrolysis. Gelatinization caused disruption of starch granule and made it more susceptible to the enzyme. Incubation of gelatinized starch at low temperature is necessary to induce re-association and re-crystalization of the starch fraction (amylose, amylopectin). These processes were needed for enzyme application in RS3 production. The residue of the enzyme hydrolysis was separated and spray dried. The product was referred as resistant starch type 3 (RS3). Based on the enzyme treatments, the product was named as following: RSSA (RS3 derived from sago starch and treated with amylase), RSSP (RS3 derived from sago starch and treated with pullulanase), RSSAP (RS3 derived from sago starch and treated with enzyme cocktail composed of amylase and pullulanase), RSRA (RS3 derived from rice starch and treated with amylase), RSRP (RS3 derived from rice starch and treated with pullulanase), RSRAP (RS3 derived from rice starch and treated with enzyme cocktail composed of amylase and pullulanase).

When amylase was applied, the liquid present in the flask appeared brown and sweet smelling. Following pullulanase digestion, the liquid present in the flask appeared clear and odorless. Brown and sweet smelling liquid was also resulted when amylase and pullulanase were applied. The product derived from sago starch contained higher RS (31–38%) than those derived from rice starch (21–26%). Fig. 1 presents RS content of RS3 derived from sago and rice starch. The native sago or rice starch as starting material contained 11.43% and 13.96% of RS, respectively.

3.2. Degradation RS3 by bacterial strain

Clear zones were clearly observed in agar plate assay, implying that all tested RS3 could be well degraded by *C. butyricum* BCC B2571 or *E. rectale* DSM 17629.Clear zones indicated that the starch degrading enzymes excreted by the tested bacteria were active in the presence of RS3. The largest clear zone was formed by *C. butyricum* BCC B2571 on the plate containing RSSP (Table 2). On the other hand, *E. rectale* DSM 17629 appeared to excrete low activity of starch degrading enzyme.

3.3. Production of short chain fatty acids during in vitro fermentation

During the fermentation period, gas production and pH of the culture medium were recorded (Table 2). Gas composition was not

Table 1
Amylose content and major chemical component of sago and rice starch.[a]

Chemical component	Rice starch	Sago starch
Amylose (%)	29.68 ± 0.13	32.87 ± 0.19
Moisture (%)	12.72 ± 0.10	12.52 ± 0.14
Ash (%)	0.29 ± 0.01	0.07 ± 0.00
Protein (%)	2.23 ± 0.01	0.30 ± 0.00
Crude fat (%)	0.24 ± 0.00	0.28 ± 0.01

[a] Values represent the mean of duplicate measurements ± STD, except value for amylose represents the mean of triplicate measurement.

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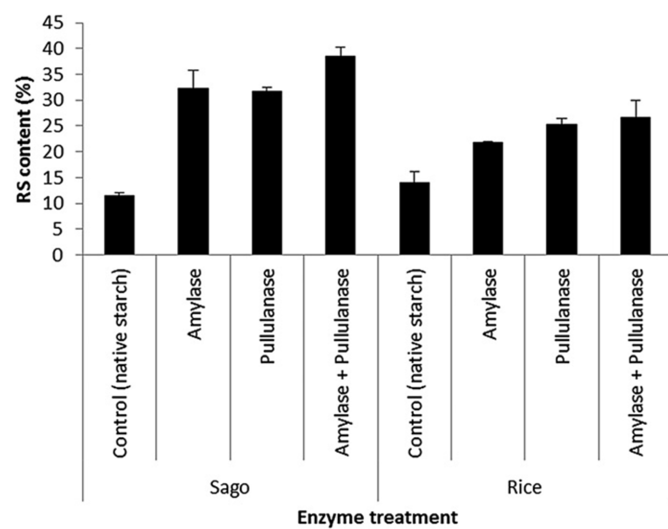

Fig. 1. RS content of RS3 derived from sago and rice starch treated with enzyme addition. Rice or sago starch (50 g) was suspended in 200 mL of water, boiled and stirred for 10 min, removed from heat and cooled down to 30 °C. The gel was vacuum sealed in a retort pouch and autoclaved at 121 °C under 15 psi for 1 h, and then 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. Retrograded starch was hydrolyzed with: (a) 1 mL of amylase for 3 h at 85 °C, (b) 1 mL of pullulanase for 3 h at 55 °C, (c) 1 mL of amylase for 3 h at 85 °C continued with 1 mL of pullunase for 3 h at 85 °C. Hydrolyzed starch was centrifuged, for 10 min at room temperature. The supernatant was discarded and the residue was stored in cool room (10 °C) overnight, suspended in water, homogenized and loaded into spray drier with inlet temperature of 160 °C.

determined, however, in a simple qualitative test hydrogen gas was not detected, which indicated that probably the major gas production was CO_2.

The SCFA content produced by *C. butyricum* BCC B2571 or *E. rectale* DSM 17629 during fermentation of RS3 varied, as shown in Table 3. Overall, total SCFA produced by *C. butyricum* BCC B2571 was comparable with those produced by *E. rectale* DSM 17629. The data showed that acetate was found as the major product. In this study *C. butyricum* BCC B2571 produced butyrate at concentration up to 46 mM in the medium supplemented with RSSA. It was higher ($p < 0.05$) than butyrate produced in the medium supplemented with other type of such as RSSP, RSSAP, RSRA, RSRP and RSRAP. On the other hand, butyrate at concentration > 40 mM was produced by *E. rectale* DSM 17621 not only in the medium supplemented with RSSA but also in the medium supplemented with RSSP and RSSAP.

Table 2
Clear zone area, gas and pH formed by *C. butyricum* BCC B2571 or *E. rectale* 17629 incubated in the media containing RS3 produced by different enzymes.

Treatment	Clear zone (cm^2)	Gas (ml)	pH
C. butyricum BCC B2571			
RSSA	2.83b	12.20b	4.35
RSSP	4.87c	9.30b	4.57
RSSAP	0.61a	6.75a	4.28
RSRA	0.64a	8.17ab	4.31
RSRP	5.28c	11.93b	4.36
RSRAP	2.75b	8.70ab	4.43
E. rectale 17629			
RSSA	0.90a	5.90a	4.64
RSSP	1.00a	8.20ab	4.74
RSSAP	1.32a	5.60a	4.68
RSRA	0.80a	10.25b	4.67
RSRP	1.35a	9.65b	4.77
RSRAP	0.72a	10.60b	4.65

Mean values in a column followed by the different letter are significantly different ($p < 0.05$).

Table 3
SCFA produced by *C. butyricum* BCC B2571 and *E. rectale* 17629 grown in media containing RS3 produced by different enzymes.

Treatment	Total SCFA (mM)	Acetate (mM)	Propionate (mM)	Butyrate (mM)
C. butyricum BCC B2571				
RSSA	177.97^{b}	83.70^{b}	47.57^{b}	46.70^{b}
RSSP	82.31^{ab}	46.71^{ab}	8.90^{a}	17.65^{a}
RSSAP	52.56^{ab}	18.04^{a}	11.90^{a}	11.14^{a}
RSRA	112.83^{b}	57.76^{ab}	17.80^{a}	18.14^{a}
RSRP	98.32^{ab}	52.19^{ab}	24.37^{a}	21.76^{a}
RSRAP	83.89^{ab}	48.88^{a}	7.95^{a}	27.05^{a}
E. rectale 17629				
RSSA	151.48^{b}	66.74^{ab}	38.70^{ab}	46.05^{b}
RSSP	205.45^{b}	93.76^{b}	52.30^{b}	59.39^{b}
RSSAP	150.99^{b}	74.53^{ab}	31.97^{ab}	44.93^{b}
RSRA	169.70^{b}	108.08^{b}	27.63^{a}	33.99^{a}
RSRP	29.96^{a}	21.12^{a}	5.64^{a}	6.03^{a}
RSRAP	77.52^{ab}	40.12^{a}	14.95^{a}	22.46^{a}

Mean values in a column followed by the different superscript letters are significantly different ($p < 0.05$).

This is higher ($p < 0.05$) than butyrate produced in the medium supplemented with RSRP. Butyrate was found approximately from 19% to 33% of the total SCFA.

Fermentation of RS3 was directed to produce high concentration of butyrate. As expected, fermentation of RSSA by *C. butyricum* BCC B2571 resulted 84 mM of acetate, 48 mM of propionate and 46 mM of butyrate or molar ratio around 1.8 : 1 : 1. Fermentation of RSSA by *E. rectale* DSM 17629 resulted 66 mM of acetate, 38 mM of propionate and 46 mM of butyrate or molar ratio around 1.7 : 1 : 1.2. High concentration of acetate (93 mM), propionate (52 mM) and butyrate (59 mM) or molar ratio approximately 1.8 : 1 : 1 was also observed in the fermentation of RSSP by *E. rectale* DSM 17629.

SCFA production either by *C. butyricum* BCC B2571 or *E. rectale* DSM 17629 was much higher in the fermentation medium with glucose as the only carbon sources. *C. butyricum* BCC B2571 produced acetate, propionate and butyrate at concentration of 115 mM, 47 mM and 53 mM, respectively or molar ratio about 2.4 : 1 : 1.1, while *E. rectale* DSM 17629 produced 594 mM of acetate, 291 mM of propionate and 287 mM of butyrate or molar ratio 2 : 1 : 1.

4. Discussion

Starch hydrolysis with alpha-amylase produces mixture of branched α-dextrin, short linear oligosaccharide, maltose and glucose. The short linear oligosaccharide, maltose and glucose were removed during preparation of RS3. Thus, the remaining material was branched α-dextrin. Pullulanase hydrolyzes the α–(1–6 glycosidic) bond of amylopectin and release free linear chains fraction. Part of this linear fraction seemed to be unable to form RS structure. Slightly different RS content among all enzyme treatments was found. High product recovery (approximately 13–18%) was obtained from production of RS by hydrolyzing starch with pullulanase (Fig. 1). Compared to the control, which was not subjected to enzyme hydrolysis, enzymes treatment of sago or rice starch resulted in a clear increasing of the RS content. This result indicated that enzymes hydrolysis process would be beneficial in the RS formation.

The product derived from sago starch demonstrated higher RS content than that derived from the rice starch. Amylose content and intrinsic properties of starting material seem to have significant contribution to the RS formation. Interaction between amylose chain could easily occurred and the double helical structure will thus be formed [27]. This double helix structure is likely more resistant to the enzyme digestion compared to the linear structure. Higher amylose content could produce more double helical structure.

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Sago and rice starch showed different molecular amylopectin structure as reported by Srichuwong et al. [28]. Long chain unit with degree of polymerization (DP) of 13 in sago starch was higher (approximately 63%) than those found in rice starch (approximately 57%). Long chain unit would create long helices and strengthen hydrogen bond between the chain, and this will spin the entire crystalline region [29]. Consequently, this structure will be more resistant towards enzyme hydrolysis. Susceptibility of the starch to the enzyme hydrolysis is also affected by granule size [27]. Larger granule size would have a smaller surface area to volume ratio and this resulted in less enzyme binding or potential hydrolysis. However, this feature was likely not applicable in our study as the starch granule was completely disrupted by gelatinization and autoclaving treatment.

RS content in our products were comparable with product prepared from high amylose maize starch with citric acid hydrolysis [19]. It was higher than RS content in debranching sago starch which was about 5–7% [8]. The difference could have been caused by several factors such as starch source, enzyme used, and heating-cooling condition.

At the present study, we applied *C. butyricum* BCC B2571 or *E. rectale* DSM 17629 as strain model instead of other well known beneficial microorganism. In addition as starch degrader and butyrate producer, both strains were commonly found in animal including human colon. They are not included as pathogenic bacteria. *E. rectale* was found as abundant groups [15].

RS3 was well degraded by *C. butyricum* BCC B2571 or *E. rectale* DSM 17629. It was verified by clear zone formation on the agar plate assay. Starch degrading activity in clearing zone formation (Table 2) of *C. butyricum* BCC B2571 or *E. rectale* DSM 17629 does not correlate with the origin of starch, nor with enzyme used in the RS3 preparation. Another word, specific preferences of the above mentioned bacteria strain on the RS3 was not found. Organization of enzyme system for starch utilization of butyrate producer bacteria possesing a gram-positive cell wall ultrastucture was studied by Ramsay et al. [30]. It was reported that the enzyme was anchored onto the bacterial cell wall, thus ensuring that the site of hydrolysis is close to the system for transporting hydrolysis products into the bacterial cell. This hydrolysis product was further metabolized to produce SCFA.

As the study result showed, the level and proportion of SCFA is dependent on the bacteria strain and kind of RS3. Scientific study supported the view that retrograded starch (RS3) is known as powerful butyrate producing substrate. Butyrate level produced in our study is higher than those produced in the RS3 fermentation by bacterial mixed culture of human gut as reported by other authors. Lesmes et al. [17] reported that varying acetate, propionate and butyrate were produced from the fermentation of RS3 (derived from high amylose corn starch treated with amylase) by mixed bacterial culture of human feces. Acetate ranged from non detected level to 17.14 mM, propionate ranged from non detected to 5.29 mM and butyrate ranged from non detected to 0.75 mM. Other study showed that fermentation of RS3 (derived from high amylose corn starch treated with acid hydrolysis) by mixed culture originated from different human feces also resulted in acetate, propionate and butyrate at low concentration levels. The highest level of acetate, propionate and butyrate was observed at approximately 17 mM, 18 mM and 16 mM, respectively [19].

High concentration of SCFA was resulted from the fermentation of RS3 by pure culture of *C. butyricum* NCIMB 7423. Reid et al. [16] reported that fermentation of RS3 (derived from potato starch treated with pancreatin amylase) resulted in acetate and butyrate at concentration of 42 mM and 78 mM, respectively. Maximum concentration of acetate and butyrate at 21 mM and 54 mM, were produced by fermentation of *C. butyricum* NCIMB 7423 on RS3 derived from different maize starch treated with pancreatin amylase [31]. They were comparable with our study. Based on the previous published report and our study, we assumed that high level of SCFA during in vitro fermentation of the pure culture is related to the non-competation condition between bacteria.

Production of SCFA by the tested bacterial strain in the medium supplemented with RS3 was observed to be lower than those produced in the medium without RS3 supplementation (with glucose as the only carbon source). Glucose as simple sugar is regarded as a more ready to be metabolized substrate by bacterial strain compared with the complex RS3. In vivo environment may completely be different with that of the in vitro. Glucose availability was limited at the lower gut. Therefore, the provision of fermentable carbohydrate such as RS3 is needed. It is important to enhance the ability of the butyrate producing bacteria to compete with other species. Our result indicated that RS3 is likely to be effective, since the bacterial strains are capable to utilize the starch to produce butyrate.

It was of interest to us to find that butyrate concentration produced by *C. butyricum* BCC B2571 in the medium supplemented with RS3 derived from sago starch treated with amylase (RSSA) was higher than those in the medium supplemented with RS3 derived from sago starch treated with pullulanase (RSSP), combination of amylase-pullulanase (RSSAP) or RS3 derived from rice. Significant high butyrate concentration was also found in the medium of *E. rectale* DSM 17629 supplemented with RS3 derived from sago starch. Enzyme used in the preparation of RS3 derived from rice starch did not affect the butyrate production by *E. rectale* DSM 17629. This may relate to the difference of RS3 polymorphism (physical characteristic in X-ray diffraction pattern). According to Lesmes et al. [17], difference polymorphisms induce difference colonic bacteria strains such that this produce different SCFA (including butyrate) levels. This study was designed as part of study on RS3 as functional food ingredient with capability to prevent colorectal cancer diseases. The high concentration level of SCFA containing butyrate found in our study was useful to make a number of dilution to obtain various concentrations to treat cancer cell.

The pathway of acetate, propionate and butyrate formation by human fecal microflora has been reported. According to the study of Miller and Wolin (1996), acetate is formed from CO_2 through Wood Ljungdahl pathway; propionate is formed by a CO_2 fixation pathway and butyrate is formed by condensation of two molecules of the acetyl-Co enzyme A (acetyl-CoA). At the final step of butyrate synthesis, there are two alternatives pathway. First, the butyrate kinase pathway. The enzymes phosphotransbutyrylase and butyrate kinase convert butyryl-CoA to butyrate with intermediate formation of butyryl-phosphate. Second, a butyryl-CoA:acetate CoA-transferase transfer the CoA moiety to the external acetate, which lead to the formation of acetyl-CoA and butyrate. It is also known as butyryl-CoA transferase pathway [1]. Phosphotransbutyrylase and butyrate kinase pathway is found in the solventogenic bacterium *Clostridium acetobutylicum* [32]. Butyryl-CoA transferase pathway is dominant route for human colonic bacterium for butyrate synthesis [33].

In our study, the butyrate was likely formed by *C. butyricum* BCC B2571 via butyryl-CoA transferase upon accumulation of sufficient acetate in the medium. We also observed the metabolic shift of *C. butyricum* BCC 2571 when it was grown at different conditions (data not shown). Similar pathway appeared to be found in *E. rectale* 17629. It was consistent with the study reported by Duncan et al. [34]. The authors reported that in vitro study has shown that in the presence of carbohydrate *E. rectale* consumed large amount of acetate for butyrate formation.

Surface structure of substrate such as RS3 could enhance viability of bacteria strain by providing a location for physical adhesion [21]. The surface structure of RS3 is different from that of native starch. Various grooves and fissures on the exterior surface of the granules are noticed in the RS3 molecule due to enzyme attack. Enzyme molecules influence the starch granule surface in different ways. Five patterns of enzymes attack have been identified: pin-holes, sponge-like erosion, numerous medium-sized holes, distinct loci leading to single holes in individual granules [35]. Planchot et al. [36] stated that the mode of enzymatic degradation depend on the starch and enzyme used. Physical interaction of bacterial cell and surface of the substrate often becomes the initial step for degradation by bacterial enzyme. Close physical relation between bacterial cell and the starch granule ensured that the hydrolytic products were accessible to the organism and that the enzyme secretion was advantageous. It is in agreement with the result of Sharps and Macfarlane (2000). The author reported that the bacteria colonized resistant starch granule and formed rosette-like structures.

The in vivo fermentative metabolism is affected by the changes in microbial population. In the case of RS3, the role of *C. butyricum* BCC B2571 and *E. rectale* DSM 17629 as primary starch degrader and butyrate producer was confirmed. It can be potentially useful to direct the strategy for increasing population of butyrate producing species.

Production of short chain fatty acids (SCFA), mainly butyrate, has been deemed a favorable trait of colon-target functional foods due to their proven beneficial physiological effects on the gut and differentiation of colonic epithelial cells [21,37]. Currently, there is an increasing interest in the use of foods that are capable of modulating the composition of human colonic microflora in a way that is beneficial to health. Prebiotics have been defined as “non digestable” food ingredients that selectively stimulate a limited number of bacteria in the colon, to improve the host health [38]. The capability of RS3 as prebiotic was supported by fact that RS3 was degraded by tested bacterial strain (*C. butyricum* BCC B2571 or *E. rectale* DSM 17629) as shown in amylase activity test on agar plate. The hydrolitic capability was used for their growth in colon. Both bacteria will produce the SCFA including butyrate. This is an important traits, as butyrate, is the preferred energy source for colonocytes and has protective effect against colon diseases as state previously. Therefore RS3 is a substrate for beneficial colon bacteria or as a prebiotic. Further information on the roles of supernatant resulted from RS3 fermentation in preventing colorectal cancer diseases will be reported later in our accompanying paper.

Our study demonstrates that enzyme hydrolyzed starch can be considered as potential prebiotics from the point of view of fermentation capability to generate butyrate acid which is regarded as potential to combat cancer.

5. Conclusion

RS type 3 (RS3) could be developed from sago and rice starch through enzyme hydrolysis with amylase, pullulanase or cocktail enzyme composed of amylase and pullulanase. RS content in the product ranged from 21% to 38%; they were degraded by *C. butyricum* BCC B2571 or *E. rectale* DSM 17629, and lead to SCFA production at different levels. Our study demonstrated that RS 3 derived from sago starch treated with amylase or pullulanase were considered as potential products from the point of view of fermentation capability to generate butyrate.

Author disclosure

Authors have no conflict of interest.

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