F O R M 2

THE PATENTS ACT, 1970
(39 of 1970)
&
The Patents Rules, 2003
COMPLETE SPECIFICATION
(See section 10; rule 13)

1. Title of the invention. –

A NOVEL BIOPROCESSING TECHNOLOGY OF ENZYMATIC RESISTANT STARCH PRODUCTION FROM STARCHY RICH AGRO PRODUCE

2. **Applicant(s)**

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3. **PREAMBLE TO THE DESCRIPTION**

The following specification particularly describes the invention and the manner in which it is to be performed :

FIELD OF THE INVENTION

The present invention relates to a novel bio-processing method for increasing the resistant starch content of native starch granule using a novel biocatalyst amylopullulanase from *Lactobacillus amylophilus* GV6

BACKGROUND OF THE INVENTION

Starch, commonly known as "amylum" is a polysaccharide mostly used as an important biological product throughout the world for various purposes. Every starch molecule produced by the different plant species possesses variation in structure and composition (glucose chain length (or) amylose/amylopectin ratio). The differences in the structure and composition of starches from different sources determine the properties and the mode of interactions with the other food constituents for the desired product taste with fine texture. The stable nature of the native starch varies under different pH and temperature conditions. Hence, native starch has limited functional property. To enhance properties and functionality such as solubility, texture, viscosity and thermal stability, which are necessary for the desired role in the industry, native starches can be modified. The widening vista of application possibilities of starches with different properties has made research in non-conventional starches and other native starches more imperative. Recent studies on the relationship between the structural characteristics and functional properties of starches from different sources have continued to provide important information for optimizing industrial applications [Shuren J. Production and use of modified starch and starch derivatives in China. In: Howeler RH, Tan SL,editors. Cassava's Potential in Asia in the 21st Century: Present Situation and Future Research and Development Needs. In: Proceedings of 6th Regional Workshop; 21–25 February 2000; Ho Chi Minh City, Vietnam; 2000. pp. 553-563;] Santana AL, Meireles MAA. (2014). New starches are the trend for industry applications: A review. Food

and Public Health. 4(5), 229-241; Agrosynergie. Evaluation of Common Agricultural Policy Measures Applied to the Starch Sector Final Report—AGROSYNERGIE—November 2010 [Internet]. 2010. Available from: https://ec.europa.eu/agriculture/sites/agriculture/files/evaluation/market-and-income-reports/2010/starch/fulltext_en.pdf [Accessed: 13 March 2019].

On the basis of digestible property, starches are broadly classified as Digestible Starches and Resistant Starch. Digestible starches are readily digestible by the enzymes present in the body, namely RDS (Rapidly Digestible Starch) and SDS (Slowly Digestible Starch). RDS are mostly present in the starch-rich foods like breads and potatoes in higher amounts which primarily consists of amorphous and dispersed starch. SDS and RDS has the similarity of complete digestion in the small intestine but the major difference is that SDS digest more slowly. Resistant starch (RS)cannot be digested by the pancreatic enzymes of the body. It has been broadly categorized into five categories-RS_1, RS_2, RS_3, RS_4 and RS_5. RS_1 represents the physically inaccessible form of resistant starch like partly milled grains and dense type of processed starchy foods. It is measured quantitatively by difference between glucose release of homogenized food sample and non-homogenized food sample by enzymatic digestion. RS_1 is widely used as an ingredient in conventional foods due to its heat stable nature. RS_2 represents the granular form of resistant starch measured quantitatively by difference between glucose release of boiled homogenized food sample and un-boiled non-homogenized food sample by enzymatic digestion. The resistant nature of the starch is due to the compact structure of the raw starch in a radial pattern which is relatively dehydrated so that it limits the accessibility of various digestive enzymes that are present in the body.RS_1 and RS_2 represent residues of starch forms, which gets digested very slowly and sometimes incompletely in the small intestine.RS_3 represents the most resistant starch fraction present in most of the moist heated foods that are retrograded amylose formed during gelatinized starch cooling. It is measured quantitatively as the fraction, which resists both dispersion by boiling and enzyme digestion. It can only be dispersed with potassium hydroxide or dimethyl sulphoxide. RS_3 shows

absolute resistance to pancreatic amylase digestion. RS_4 is the RS where novel chemical bonds other than α-(1-4) orα-(1-6) are formed. Modified starches obtained by various types of chemical treatments are included in this type. RS_5 is the type mostly results due to amylose- lipid complex formation [AACC (2000). Method 10-50D, Baking Quality of Cookie Flour. Approved Methods of the American Association of Cereal Chemists, 10th ed. AACC, St. Paul; Fuentes-Zaragoza E, Riquelme-Navarrete MJ, Sánchez-Zapata E, Pérez Álvarez JA. (2010). Resistant starch as functional ingredient: A review. Food Research International, 43: 931–942.].

The present invention primarily focuses in increasing the resistant starch content of any native starch granule using a novel biocatalyst *amylopullulanase*, which was outsourced from *Lactobacillus amylophilus* GV6.

Resistant starch is generally increased by enhancing the apparent levels of amylose via debranching of amylopectin molecules. Following retrogradation, this will permit the amylose to form a more tightly packed crystalline structure, which is difficult for enzymes to hydrolyse. Typically, debranching enzymes such as pullulanase or isoamylase can be used to manipulate RS levels in a positive fashion. Both pullulanase and isoamylase exclusively act on the α-(1,6) -glycosidic bonds found at branch points on amylopectin molecules, which in turn increases the apparent amylose content while maintaining a constant total starch level. Since the aim of enzymatic treatment of starch is typically to hydrolyse the amylopectin branch chains, and not the amylose, pullulanase and isoamylase are more commonly used. Therefore, when starch is undergoing enzymatic debranching, the amylopectin content and structure will heavily influence the final RS content. When debranching is undertaken, the amylose content will increase, which upon retrogradation will produce higher levels of RS due to amylose forming tightly packed crystals of RS3, resulting in an overall increase in the degree of crystallinity. Additionally, the amylose strands may realign to form double helices stabilized by hydrogen bonds, which can lead to further formation of RS3. Although increased

amylose content within a starch from a given botanical source is indicative of increased crystallinity, no such trend exists between starches from various sources. However, crystallinity (as measured by x-ray diffraction) and double helix content (as measured by nuclear magnetic resonance) are highly correlated.

Hickman BE, Janaswamy S, Yao Y. (2009). Autoclave and β-Amylolysis Lead to Reduce in Vitro Digestibility of Starch. Journal of agricultural and food chemistry. 57, 7005-12 discloses autoclave treatment (3 × 121 °C for 30 min) and 20 h of β-amylolysis and the RS content of the corn starch increased from 11.4% to 29.9%, and from 9.1% to 23.1% for the wheat starch, whereas the autoclave treatment alone only increased RS levels by less than 2% over native RS levels. Hung PV, Phi NTL, Vy TTV. (2012). Effect of debranching and storage condition on crystallinity and functional properties of cassava and potato starches. Starch - Starke. 64(12), 964-971 relates to effect of debranching and storage condition on crystallinity and functional properties of cassava and potato starches and discloses pullulanase treatment on cassava starch and normal potato starches such that RS levels were increased from 3% to 35% on cassava starch and 5% to 48% on normal potato starches. The process includes autoclave treatment. Milašinović-Šeremešić M, Radosavljević MM, Dokic L. (2010). Effects of autoclaving and pullulanase debranching on the resistant starch yield of normal maize starch. Journal of the Serbian Chemical Society. 75(4). DOI: 10.2298/JSC1000027M relates to effects of autoclaving and pullulanase debranching on the resistant starch yield of normal maize starch and discloses Pullulanase treatment on precooked normal corn starch such that RS levels were increased from 5% to 48% on precooked normal corn starch. Reddy CK, Suriya M, Haripriya S. (2013) Physico-chemical and functional properties of resistant starch prepared from red kidney beans (Phaseolus vulgaris.L) starch by enzymatic method. CarbohydrPolym. 95(1), 220- 226 relates to Physico-chemical and functional properties of resistant starch prepared from red kidney beans (*Phaseolus vulgaris.* L) starch by enzymatic method and discloses Pullulanase to debranch gelatinized red kidney bean starch. Debranching for 10 h and followed by autoclaving (121 °C for 30 min) and retrogradation (4 °C, 24 h)

such that RS levels were increased from 21.3% to 31.5% on debranching and 42.3% on autoclaving and retrogradation. Amylose content also increased from 25.3% to 67.7%, reinforcing the fact that debranching enzymes cause the apparent amylose content to increase. Increases in RS have been attributed to the autoclave treatment regelatinizing the starch, which allowed the amylose chains to better realign during retrogradation, thus forming RS3.Simsek S, El SN. (2012). Production of resistant starch from taro (Colocasia esculenta L. Schott) corm and determination of its effects on health by in vitro methods. CarbohydrPolym. 90 (3). 1204- 1209relates to production of resistant starch from taro (*Colocasia esculenta* L. Schott) corm and determination of its effects on health by in vitro methods and discloses Pullulanase treatment on taro corm such that RS levels were increased from 2.2% to 35.1% on taro corm. Zhang B, Huang Q, Luo Fa, & Fu X, Jiang H, Jane J. (2011). Effects of octenyl succinylation on the structure and properties of high-amylose maize starch. CarbohydrPolym. 84(4), 1276- 1281 relates to effects of octenyl succinylation on the structure and properties of high-amylose maize starch and discloses α- amylase in conjunction with pullulanase treatment such that the RS content in normal corn starch increased to 58.9% from initial levels of about 45% when measured using the Goñi method. Cai L, Shi Y. (2010). Structure and digestibility of crystalline short-chain amylose from debranched waxy wheat, waxy maize, and waxy potato starches. CarbohydrPolym*, 79 (4),* 1117-1123 relates to Structure and digestibility of crystalline short-chain amylose from and discloses that 1% iso amylase for 24 h followed by storage at 25 °C for 24 h such that RS levels were increased from 0.4% to 67.7% and 4.3% to 68%.

The process in US005849090 A defines resistant starch production by swelling the native starch granules with water, then treating with pullulanase to debranch the amylopectin present. The debranched starch is treated under thermal conditions to retrograde the amylose and dried to form granular resistant starch powder.

CN110172104A discloses high- temperature- resistant modified starch prepared by using a neutral protease, a high- temperature amylase and a high- temperature liquefaction enzyme.

The present invention addresses the shortcomings in the art and need in the art to provide a chemical free process for resistant starch production which is cost-effective, eco-friendly and achieves high amount of resistant starch in a range of starchy substrate subjected to the process of the present invention.

OBJECTS OF THE INVENTION

It is an object of the present invention to provide a novel cost-effective, eco-friendly process involving *Amylopullulanase*, a hyperactive bifunctional enzyme isolated from *Lactobacillus amylophilus* GV6 (a GRAS microorganism) for Resistant Starch production.

The enzyme has been used to obtain high amount of resistant starch in a range of starchy substrate subjected to the process of the present invention.

It is a further object of the present invention to provide a chemical free process for resistant starch production from a range of starchy substrate.

It is yet another object of the present invention to provide a process where the entire reaction is completed under mild reaction conditions.

It is an object of the present invention to provide a process where the entire reaction is completed within comparatively less incubation time.

It is an object of the present invention to provide a process where the no physical treatment process has been applied.

It is an object of the present invention to provide a process where no pre-gelatinization treatment is used.

It is an object of the present invention to provide a process where the entire reaction is completed using direct native starchy flour system.

It is an object of the present invention to provide a process where the entire reaction is completed using a *3-sequential enzymatic* treatment with reducing enzyme concentration and time at each step.

SUMMARY OF THE INVENTION

According to an aspect of the present invention there is provided a process for increasing the Resistant Starch content of raw starchy substrates, the process comprising the following steps:

a. Adding distilled water and *amylopullulanase* to the starch substrate, and incubating by keeping for about 120 min in a water shaker at about 200 rpm/ min at about 50-60 ℃.
b. Centrifuging the slurry, followed by discarding the supernatant to obtain the pellet;
c. Drying the pellet till dry followed by grinding till powder;
d. Incubating the powder with a lower dose of *amylopullulanase than that in step (a),* for about 60 min in a water shaker at about100 rpm/ min at about 50-60 ℃;
e. Centrifuging, the slurry, followed by discarding the supernatant to obtain the pellet;
f. Drying the pellet till dry followed by grinding till powder;
g. Incubating the powder with a lower dose of *amylopullulanase, than that in step (d),* for about 30 min in a water shaker at about 50 rpm/ min at about 50-60 ℃;
h. Centrifuging the slurry, followed by discarding the supernatant to obtain the pellet;
i. Drying the pellet till dry;
j. Optionally grinding the dried pellet to a powder.

The *amylopullulanase* according to the present invention is isolated from *Lactobacillus amylophilus* GV6 (a GRAS microorganism). The amount of *amylopullulanase* at each relevant step is decreased.

DETAILED DESCRIPTION OF THE INVENTION

An aspect of the present invention relates to a bioprocessing method for increasing the Resistant Starch content of starchy substrates by using *Amylopullulanase*. The method is an innovative, chemical free, enzymatic bio-processing system for Resistant Starch production.

The method includes use of Amylopullulanase, a novel hyperactive bifunctional enzyme has been employed for Resistant Starch production. The enzyme has been isolated from *Lactobacillus amylophilus* GV6. *Lactobacillus amylophilus* GV6 [Bioprocess Engineering volume 23, pages155–158 (2000)], (a GRAS microorganism), which makes the bioprocessing system absolutely hazardous free. "GRAS" is an acronym for the phrase *Generally Recognized As Safe* in respect of food safety such as under FDA.

The method of the present invention allows the making of a customized dietary supplement for a broader range of diabetic patients. The method of the present invention is applicable for a range of starchy substrates; therefore, the system will not have to depend on the availability of any seasonal starchy vegetable source. The raw starchy substrate source includes from agro produce like rice, wheat, barley, corn, green banana, taro and other abundantly available starch agro residues like sweet potato, conch potato etc. The present invention results in an ingredient (product) which is designed as a result of the present invention in such a manner that the biological system of the consumer will receive glucose at a control level, which will provide continuous energy and at the same time will increase the satiety of any individual.

According to an aspect of the present invention there is provided a process for increasing the Resistant Starch content of raw starchy substrates, the process comprising the following steps:

a. Adding distilled water and *amylopullulanase* to the starch substrate, and incubating by keeping for about 120 min in a water shaker at about 200 rpm/ min at about 50-60 ℃.
b. Centrifuging the slurry, followed by discarding the supernatant to obtain the pellet;
c. Drying the pellet till dry followed by grinding till powder;
d. Incubating the powder with a lower dose of *amylopullulanase than that in step (a),* for about 60 min in a water shaker at about 100 rpm/ min at about 50-60 ℃;
e. Centrifuging, the slurry, followed by discarding the supernatant to obtain the pellet;
f. Drying the pellet till dry followed by grinding till powder;
g. Incubating the powder with a lower dose of *amylopullulanase, than that in step (d),* for about 30 min in a water shaker at about 50 rpm/ min at about 50-60 ℃;
h. Centrifuging the slurry, followed by discarding the supernatant to obtain the pellet;
i. Drying the pellet till dry;
j. Optionally grinding the dried pellet to a powder.

The *amylopullulanase* according to the present invention is isolated from *Lactobacillus amylophilus* GV6 (a GRAS microorganism).

The powder so prepared can be used as an ingredient for further use.

The present invention has immense use in the Food industries and Pharmaceutical industries.

The amount of distilled water in the process of the present invention is preferably in the range of 6– 8 mL/g of substrate.

The amount of *amylopullulanase* in step (a) of the process of the present invention may be in the range of 70µL- 90 µL, v/w.

The amount starch substrate in step (a) of the process of the present invention would depend on -the need of the production process and may be in the range of -1g – 10kg. The enzyme titer may be changed accordingly by a person skilled in the art.

Centrifugation of the method of the present invention can be standard centrifuge machines (such as 5804 R, Benchtop Centrifuge, Eppendorf, India). Centrifugation may be carried out at about 7000-9000 rpm for about 15-30 min.

The pellet may be dried in standard dryers such as Contemporary Hot Air Oven, Bioinstrumentation India. Drying may be carried out at about 60-80 ℃.

The amount of *amylopullulanase* in step (d) of the process of the present invention may be in the range of 50µL- 70 µL, v/w.

The amount of *amylopullulanase* in step (g) of the process of the present invention may be in the range of 30µL- 50 µL, v/w.

The pellet is ground into powder in grinder, for e.g., Bajaj Pluto, 500 W Mixer Grinder, Bajaj Electrical Limited, Himachal Pradesh- 173205, India can be used.

The present invention has various advantages such as -

a. A chemical free and enzyme based process for Resistant Starch production thus eco-friendly and green technology based.

b. The process is enzyme mediated and thus the non- specific interactions in the reaction medium has been drastically reduced.
c. Being enzyme mediated reaction, the time for conversion is drastically reduced.
d. The glucose release pattern has been studied, where a reduction approximately 30 - 50% reduced glucose profile has been found out.
e. AI- ML based optimization has revealed a reduced glucose release profile.
f. The production of RS is eco- friendly and cost effective.
g. The microbial enzyme is from GRAS origin.
h. No physical, chemical or physico- chemical is required for the RS starch production, thus the entire reaction can be completed under mild reaction conditions.
i. The Resistant Starch% increased up to 76-90% generally for various starchy substrates.
j. The incubation time for the enzymatic processing is about 210 min and it is applicable for a range of starchy substrates.

The present invention is now being illustrated by way of non-limiting examples.

Examples:

Example 1

A. Species taken as substrates:

Serial no.	General name	Also commonly known as	Plants parts used	Scientific name	Outsourced from
1.	Taro	Elephant ear, Eddoe	Corm	*Colocasia esculenta*	Cultivated in Agricultural and Food Engineering

					Department, IIT Kharagpur
2.	Cassava	Manioc, Yuca, Tapioca	Tuber	*Manihot esculenta*	Cultivated in Agricultural and Food Engineering Department, IIT Kharagpur
3.	Banana	Plaintain	Fruit	*Musa paradisiaca* sub. *sapientum* L. Kuntze	Cultivated in Agricultural and Food Engineering Department, IIT Kharagpur
4.	Maize	Corn	Kernel	*Zea mays* L.	Cultivated in Agricultural and Food Engineering Department, IIT Kharagpur
5.	Rice	Basabhog	Seed	*Oryza sativa*	Cultivated in Agricultural and Food Engineering Department, IIT Kharagpur
6.	Potato	---	Tuber	*Solanum tuberosum*	Cultivated in Agricultural and Food Engineering

					Department, IIT Kharagpur
7.	Wheat	---	Seed	*Triticum aestivum* L.	Cultivated in Agricultural and Food Engineering Department, IIT Kharagpur
8.	Yam	---	Tuber	*Dioscorea alata*	Cultivated in Agricultural and Food Engineering Department, IIT Kharagpur
9.	Sweet potato	---	Tuber	*Ipomoea batatas*	Cultivated in Agricultural and Food Engineering Department, IIT Kharagpur
10.	Conch potato	Jicama, sakh aloo	Tuber	*Pachyrhizus tuberosus*	Cultivated in Agricultural and Food Engineering Department, IIT Kharagpur

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B. Methodology/Process Description

The invention proposes the development of a bioprocess for increasing the Resistant Starch content of starchy substrates using amylopullulanase, isolated from *Lactobacillus amylophilus* GV6.

[Lactobacillus amylophilus GV6 is the strain donated by Osmania University].

Bioprocessing system involves the following steps enumerated below in points:

1. Firstly, to quantified amount of the substrate (10 g)**,** calculated amount of distilled water (varies from 6– 8% v/w) and calculated amount of amylopullulanase (varies from 70µL- 90 µL, v/w) is added in Erlenmeyer flask and kept for 120 min in a water shaker (200 rpm/ min) at 50 ℃. **(First enzymatic treatment step)**
2. After the incubation, the slurry was centrifuged at 7000 rpm for 15 min.
3. Thereafter, the supernatant was discarded and the pellet is obtained.
4. The pellet is then dried at 80 ℃ till dry and grinded.
5. Secondly, the powder was again incubated with a lower dose of amylopullulanase (varies from 50µL- 70 µL, v/w) and incubated for 60 min in a water shaker (100 rpm/ min) at 50 ℃. **(Second enzymatic treatment step)**
6. After the incubation, the slurry was centrifuged at 7000 rpm for 15 min.
7. Thereafter, the supernatant was discarded and the pellet is obtained.
8. The pellet is then dried at 80 ℃ till dry and grinded.
9. Lastly, the powder was again incubated with a further lower dose of amylopullulanase (varies from 30µL- 50 µL, v/w) and incubated for 30 min in a water shaker (50 rpm/ min) at 50 ℃. **(Third enzymatic treatment step)**
10. After the incubation, the slurry was centrifuged at 7000 rpm for 15 min.
11. Thereafter, the supernatant was discarded and the pellet is obtained.
12. The pellet is then dried at 80 ℃ till dry.
13. The dried pellet is then grinded and used as an ingredient for further use.

Centrifugation of the method of the present invention is by standard centrifuge machines (such as 5804 R, Benchtop Centrifuge, Eppendorf, India).

The pellet is dried in standard dryers such as Contemporary Hot Air Oven, Bioinstrumentation India.

The pellet is ground into powder in grinder, Bajaj Pluto, 500 W Mixer Grinder, Bajaj Electrical Limited, Himachal Pradesh- 173205, India.

Incubation is carried out in a water bath incubator shaker which is commercially available.

C. Results

a. It has been demonstrated that the sole use of amylopullulanase at different steps and at different low enzymatic titers in concurrent stages, significantly increased the content of RS in all the below mentioned starchy agro-produces. The range of RS% increment after each enzymatic treatment step has been given below in Table 1 and Table 2 is the range of RS% increment from native to final after the process of the invention.

b. It is to be noted that the environment for cultivation, the climacteric period, the variety of the crop and the time of harvest are crucial factors in determining the native starch content and type.

For measuring the starch content, the methodology as described by Megazyme (2008), *Resistant starch assay procedure*. http://secure.megazyme.com/ downloads/en/data/K-RSTAR.pdf, has been followed.

Table 1

Serial no.	General name	Resistant starch content (%)			
		Native starch	**After 1st step**	**After 2nd step**	**After 3rd step**
1.	Taro	**3.96 – 6.92**	**38.36 – 41.32**	**63.36 – 66.31**	**79.28 – 82.02**
2.	Cassava	**8.28 – 12.67**	**40.26 – 44.24**	**62.37 – 64.34**	**76.22 – 78.16**
3.	Banana	**35.23 – 38.59**	**61.28 – 64.59**	**77.66 – 80.02**	**85.56 – 88.25**
4.	Maize	**2.23 – 7.21**	**37.43 – 42.21**	**62.32 – 67.54**	**77.59 – 82.22**
5.	Rice	**3.68 – 6.55**	**40.82 – 44.26**	**67.22 – 71.21**	**85.12 – 89.03**
6.	Potato	**3.27 – 6.56**	**39.26 – 42.26**	**64.66 – 68.56**	**81.25 – 85.52**
7.	Wheat	**3.21 – 5.26**	**40.14 – 43.12**	**66.26 – 69.65**	**84.02 – 87.54**
8.	Yam	**2.67 – 6.23**	**39.2 – 42.82**	**65.26 – 69.89**	**82.26 – 86.56**
9.	Sweet potato	**3.16 – 7.52**	**40.18 – 44.25**	**67.22 – 71.21**	**84.23 – 88.52**
10.	Conch potato	**2.69 – 5.56**	**38.8 – 41.2**	**64.8 – 67.85**	**81.03 – 84.56**

- **For taro**, after 1st step enzymatic treatment the RS content increased by ~34%, after 2nd step enzymatic treatment the RS content increased by ~25% and after 3rd step enzymatic treatment the RS content increased by ~15%. As a result of effective enzymatic treatment, the RS content increased by ~75%, in comparison to its counter native parts.
- **For cassava**, after 1st step enzymatic treatment the RS content increased by ~31%, after 2nd step enzymatic treatment the RS content increased by ~22% and after 3rd step enzymatic treatment the RS content increased by ~14%. As a result of effective enzymatic treatment, the RS content increased by ~67%, in comparison to its counter native parts.
- **For banana**, after 1st step enzymatic treatment the RS content increased by ~26%, after 2nd step enzymatic treatment the RS content increased by ~16% and after 3rd step enzymatic treatment the RS content increased by ~8%. As

a result of effective enzymatic treatment, the RS content increased by ~50%, in comparison to its counter native parts.

- **For maize**, after 1st step enzymatic treatment the RS content increased by ~35%, after 2nd step enzymatic treatment the RS content increased by ~25% and after 3rd step enzymatic treatment the RS content increased by ~15%. As a result of effective enzymatic treatment, the RS content increased by ~75%, in comparison to its counter native parts.
- **For rice**, after 1st step enzymatic treatment the RS content increased by ~37%, after 2nd step enzymatic treatment the RS content increased by ~26% and after 3rd step enzymatic treatment the RS content increased by ~18%. As a result of effective enzymatic treatment, the RS content increased by ~81%, in comparison to its counter native parts.
- **For potato**, after 1st step enzymatic treatment the RS content increased by ~36%, after 2nd step enzymatic treatment the RS content increased by ~25% and after 3rd step enzymatic treatment the RS content increased by ~16%. As a result of effective enzymatic treatment, the RS content increased by ~78%, in comparison to its counter native parts.
- **For wheat**, after 1st step enzymatic treatment the RS content increased by ~37%, after 2nd step enzymatic treatment the RS content increased by ~26% and after 3rd step enzymatic treatment the RS content increased by ~17%. As a result of effective enzymatic treatment, the RS content increased by ~80%, in comparison to its counter native parts.
- **For yam**, after 1st step enzymatic treatment the RS content increased by ~36%, after 2nd step enzymatic treatment the RS content increased by ~26% and after 3rd step enzymatic treatment the RS content increased by ~17%. As a result of effective enzymatic treatment, the RS content increased by ~79%, in comparison to its counter native parts.
- **For sweet potato**, after 1st step enzymatic treatment the RS content increased by ~37%, after 2nd step enzymatic treatment the RS content increased by ~27% and after 3rd step enzymatic treatment the RS content

increased by ~17%. As a result of effective enzymatic treatment, the RS content increased by ~75%, in comparison to its counter native parts.

- **For conch potato**, after 1st step enzymatic treatment the RS content increased by ~36%, after 2nd step enzymatic treatment the RS content increased by ~26% and after 3rd step enzymatic treatment the RS content increased by ~16%. As a result of effective enzymatic treatment, the RS content increased by ~78%, in comparison to its counter native parts.

Table 2

Serial no.	General name	Resistant Starch (%)	
		Native	**Modified**
1.	Taro	**3.96 – 6.92**	**79.28 – 82.02**
2.	Cassava	**8.28 – 12.67**	**76.22 – 74.16**
3.	Banana	**35.23 – 38.59**	**85.56 – 88.25**
4.	Maize	**2.23 – 7.21**	**77.59 – 82.22**
5.	Rice	**3.68 – 6.55**	**85.12 – 89.03**
6.	Potato	**3.27 – 6.56**	**81.25 – 85.52**
7.	Wheat	**3.21 – 5.26**	**84.02 – 87.54**
8.	Yam	**2.67 – 6.23**	**82.26 – 86.56**
9.	Sweet potato	**3.16 – 7.52**	**84.23 – 88.52**
10.	Conch potato	**2.69 – 5.56**	**81.03 – 84.56**

c. It is to be noted that till date the process is unique because no such 3 step sequential application of *amylopullulanase* has been demonstrated for resistant starch production. The enzyme has been used solely without any combination techniques. The process is highly effective, since the bioprocess technology is capable of enhancing the RS content in all the demonstrated starchy agro-produces.

d. For better understanding and interpretation, a comparative chart has been given to understand the uniqueness of the process. **(Table 3).**

Table 3 - A comparative chart showing different strategies adopted for Resistant Starch production using Pullulanase.

From the below table, it is clear that sole use of amylopullulanase from *Lactobacillus amylophilus* GV6 for resistant starch production has not been elaborated or even when used is able to achieve the substantial increase compared to that of the present invention. The present process of the present invention holds the uniqueness since the demonstrated technique is an unique 3 sequential enzymatic treatment technique using the same enzyme. The enzyme titre is comparatively low. The overall time for the enzymatic treatment is less, whereas the resistant starch yield is comparatively high as demonstrated above.

The below mentioned table elaborates different techniques for different agro-produces.

Treatment type	**Enzymes**	**Substrate**	**Reaction conditions**	**RS yield (g/kg)**	**Reference**
Single Enzyme treatment	Pullulanase type II (or Amylopullulanase)	Semolina	Enzyme treatment for 14 h at 50 °C, Autoclaving and Cooling (1 cycle)	**165**	P. Biswas, P. Jayaseelan, P., M. Das, A. Sikder, K. Chaudhury & R. Banerjee, Int. J. Biol. Macromol. doi: 10.1016/j.ijbiomac.2022.09.281.
	Pullulanase type II (or Amylopullulanase)	Green Banana Flour	Enzymatic treatment for 4 h at 37 °C	**690**	M. Das, N. Rajan, P. Biswas, R. Banerjee, A novel approach for resistant starch production from green banana flour using amylopullulanase, LWT 153

					(2022) 1–8, https://doi.org/10.1016/j.lwt.2021.112391.
	Pullulanase type II (or Amylopullulanase)	**Normal Rice Starch**	Enzymatic treatment for 7 h at 100 °C Cooling at 4 °C overnight Autoclaving for 30 min at 121 °C and subsequent cooling at 4 °C (two repeated cycles)	**405**	H. Peng, L. Qian, Z. Fu, L. Xin, Z. Hua, J. Woolf, et al., Using a novel hyperthermophilic amylopullulanase to simplify resistant starch preparation from rice starches, J. Funct. Foods 80 (2021) 1–6, https://doi.org/10.1016/j.jff.2021.104429.
	Pullulanase type II (or Amylopullulanase)	**Indian Fox nut**	Enzyme treatment for 12 h at 60 °C Autoclaving and Cooling (1 cycle)	**359**	P. Biswas, M. Das, S. Boral, G. Mukherjee, K. Chaudhury, R. Banerjee, Enzyme mediated resistant starch production from indian fox nut (Euryale ferox) and studies on digestibility and functional properties, Carbohydr. Polym. 237 (2020), 116158, https://doi.org/10.1016/j.carbpol.2020.116158

	Pullulanase type I	Maize Starch	Pre-gelatinized for 1 h at 100 °C Pressure-cooked using autoclave for 30 min Enzymatic debranching for 12h at 50 °C Cooling for 24 h at 4°C Autoclaved for 30 min at 121 °C Cooling for 24 h at 4 °C	**434**	J. Long, B. Zhang, X.F. Li, X.B. Zhan, X.M. Xu, Z.J. Xie, et al., Effective production of resistant starch using pullulanase immobilized onto magnetic chitosan/Fe3O4 nanoparticles, Food Chem. 239 (2018) 276–286, https://doi.org/10.1016/j. foodchem.2017.06. 117.
	Pullulanase type I	Rice and Maize Starch	Pressure-cooked using autoclave for 15 min Enzymatic treatment for 16 h at 50 °C Heated to 100 °C Autoclaving and Cooling (for 2 repeated cycles)	**108 for rice 114 for maize**	J.J. Liu, Y. Liu, F. Yan, Z.Q. Jiang, S.Q. Yang, Q.J. Yan, Gene cloning, functional expression and characterisation of a novel type I pullulanase from paenibacillus barengoltzii and its application in resistant starch production, Protein Expr. Purif. 121 (2016) 22–30, https://doi.org/10.1016/j.pep.2015.12.020

	Pullulanase type I	Maize Starch	Enzymatic treatment for 32 h at 46 °C Autoclaving and Cooling (for 2 repeated cycles)	**447**	H.X. Zhang, Z.Y. Jin, Preparation of resistant starch by hydrolysis of maize starch with pullulanase, Carbohydr. Polym. 83 (2011) 865–867, https://doi.org/10.1016/j.carbpol.2010.08.066
	Pullulanase type I	Unripe Banana Starch	Gelatinized for 15 min using boiling water bath, Autoclaved, Enzymatic treatment at 50 °C for 22 h, Autoclaving and Cooling (1 cycle)	**185**	R.A. Gonzalez-Soto, E. Agama-Acevedo, J. Solorza-Feria, R. Rendon-Villalobos, L. A. Bello-Perez, Resistant starch made from banana starch by autoclaving and debranching, Starke 56 (2004) 495–499, https://doi.org/10.1002/star.200400283
	Pullulanase type I	Banana Starch	Gelatinized by autoclaving, Enzymatic treatment for 24 h at 50 °C	**506**	U. Lehmann, G. Jacobasch, D. Schmiedl, Characterization of resistant starch type III from banana (Musa acuminate), J. Agric. Food Chem. 50 (2002) 5236–5240, https://doi.org/10.1021/jf0203390

Dual Enzymatic Treatment	Pullulanase type II (or Amypullulanase) and Amyloglucosidase	Green Banana Flour	Amylopullulanase treatment for 4 h at 37 °C, Amyloglucosidase treatment for 2 h at 37 °C	**806**	M. Das, N. Rajan, P. Biswas, R. Banerjee, Dual enzyme treatment strategy for enhancing resistant starch content of green banana flour and in vitro evaluation of prebiotic effect, LWT Food Sci. Technol. 160 (2022), 113267, https://doi.org/10.1016/j.lwt.2022.113267
	α-Amylase and Pullulanase type I	Maize Flour	Pre-gelatinized for 20 min at 80 °C, α-amylase treatment at 90 °C for 15 min, Pullulanase treatment for 12 h at 46 °C, α-amylase treatment at 95 °C for 45 min	**588.7**	H.X. Zhang, Z.Y. Jin, Preparation of products rich in resistant starch from maize starch by an enzymatic method, Carbohydr. Polym. 86 (2011) 1610–1614, https://doi.org/10.1016/j.carbpol.2011.06.070

WE CLAIM :

1. A process for increasing the Resistant Starch content of raw starchy substrates, the process comprising the following steps:
    a. Adding distilled water and *amylopullulanase* to the starch substrate and incubating by keeping for about 120 min in a water shaker at about 200 rpm/ min at about 50-60 ℃.
    b. Centrifuging the slurry, followed by discarding the supernatant to obtain the pellet;
    c. Drying the pellet till dry followed by grinding till powder;
    d. Incubating the powder with a lower dose of *amylopullulanase than that in step (a),* for about 60 min in a water shaker at about 100 rpm/ min at about 50-60 ℃;
    e. Centrifuging, the slurry, followed by discarding the supernatant to obtain the pellet;
    f. Drying the pellet till dry followed by grinding till powder;
    g. Incubating the powder with a lower dose of *amylopullulanase, than that in step (d),* for about 30 min in a water shaker at about 50 rpm/ min at about 50-60 ℃;
    h. Centrifuging the slurry, followed by discarding the supernatant to obtain the pellet;
    i. Drying the pellet till dry;
    j. Optionally grinding the dried pellet to a powder.

2. The process as claimed in claim 1 wherein the amount starch substrate in step (a) of the process is 1g to 10 kg.

3. The process as claimed in claim 1 wherein the amount of distilled water is in the range of 6– 8 mL/g of substrate.

4. The process as claimed in claim 1 wherein the amount of *amylopullulanase* in step (a) of the process is in the range of 70µL- 90 µL, v/w.

5. The process as claimed in claim 1 wherein the amount starch substrate in step (a) of the process is 10g.

6. The process as claimed in claim 1 wherein the amount of *amylopullulanase* in step (d) of the process is in the range of 50µL- 70 µL, v/w.

7. The process as claimed in claim 1 wherein the amount of *amylopullulanase* in step (g) of the process is in the range of 30µL- 50 µL, v/w.

8. The process as claimed in claim 1 wherein the centrifuging is carried out at about 7000 – 9000 rpm for about 15 – 30 min.

9. The process as claimed in claim 1 wherein the drying is carried out at about 60 - 80 ℃.

Dated this the 28th day of March 2023

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ABSTRACT

A NOVEL BIOPROCESSING TECHNOLOGY OF ENZYMATIC RESISTANT STARCH PRODUCTION FROM STARCHY RICH AGRO PRODUCE

The present invention provides a novel cost-effective, eco-friendly process involving *Amylopullulanase*, a hyperactive bifunctional enzyme from *Lactobacillus amylophilus* GV6 (a GRAS microorganism) for Resistant Starch production.