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Agro-Wastes as Substrates for Extracellular *Beta-D-Fructofuranosidase* production from Soil Aspergillus Sp.

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ABSTRACT

This study reports optimization of medium fermentation parameters for extracellular invertase production by Aspergillussojae sp. PRK-2 under solid-state fermentation. Tremendous studies have been carried out on yeast species but over the recent years molds, bacteria, and other non-yeast strains are now being globally investigated for invertase biosynthesis. Fungal microorganisms hydrolyzing the disaccharide sucrose was isolated from soil regions growing cash crops to obtain the most potent fungal strain releasing the enzyme. Morphologically identified strain was molecular sequenced by 18S rRNA sequencing. Furthermore, emphasis was given to optimize and enhance enzyme production under solid-state fermentative conditions. Aspergillussojae sp. produced extracellular invertase enzyme at pH 7.0 and temperature 37 °C, with maximum activity on 5th day. 50% moisture level was most favourable for enhanced invertase productivity. Production of enzyme was surplus with orange peel than soyabean meal and rice bran. Invertase production was elevated by supplementation with nitrogen source beef extract (1.5%). The optimized medium with agro-waste as substrate was found to be in higher titres 1561.35 (U/g) than un-optimized medium. The utilization of agro-wastes for external invertase production significantly elevated enzyme titres. The work represents use of agro-wastes as potent alternatives in medium formulations for enhancement of invertase production.

Keywords: Aspergillus sp., extracellular invertase, orange peel, solid-state fermentation

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INTRODUCTION

Invertases are officially known as beta-D-fructofuranoside fructohydrolase and generally referred as invertase. The enzymecatalyzes detachment ofthe terminal non-reducing beta-D-fructofuranoside residuein to monosaccharide units and was one of the earliest known enzymes to be studied [1]. Yeasts, bacteria, molds, plants and animals are reported for production of the hydrolytic enzyme[2-5].

Invert mixture has a nature of hygroscopicity and lower crystallinity than sucrose which enables the food products to remain moist, fresh and soft for longer duration. The prevention of crystallization can be achieved by the use of external invertases especially in the production processes of high fructose syrup, glucose syrup and also fluid filled chocolates or cake centres. Enzymatic hydrolysis contributes to colourless version of food products which is a boon to the food sectors[6,7].

Extracellular invertases are of huge importance in food and pharmaceutical sectors as these enzymes are highly desired due to their hygroscopicnature.Extensive research and emphasis has been given to invertase from yeasts particularly from *Saccharomyces cerevisiae* sp. which are mostly intracellular. However, the invertases from *S.cerevisiae*sp. are commonly used in food industriesdue to non-pathogenicity and non-toxicity(Food and Drug Administration,2001).Whereas emphasis must be given to least studied invertases especially fromfungal moldsor bacteria that are least explored and deserves much attention[8].As per Alegre*et al*. (2009) [9], there is a demand for alternative invertase sources which is capable to resist vigorous environmentalconditions of industries and thereby a need to explore wide range of microbes which possesses suitable properties.

SSF technology is governed by minimal water utilization. The technique involves growth of microbes on moist solids in near absence of water,the fermentation medium are closer to natural habitats of microorganisms and efficiently produces specific enzymes.The diversified use of various agro-industrial residues as substrates has made fermentation techniques popular today. Cost-effective bio-conversion of substrates as energy sources is beneficial for biosynthesis of extracellular enzyme production. Solid-state fermentation (SSF) approach has been used for production of various external enzymes.In addition, the use of agro-wastes reduces handling problems to greater extent[10]. There are few reports on external invertase production from filamentous fungi. *Aspergilluscaespitosus* was found to be a good producer of intracellular and extracellular invertase using wheat bran as substrate[9].

Optimization of fermentative parameters improves microbial efficiency to attain higher enzyme titres. Researchers performed theoptimization studies have demonstrated good yield in invertase productivity[9, 11-13].An enhanced level of their mostable invert as was produced from *Aspergillusochraceus* using sugarcane bagasse[14]. Uma *et al*. (2010) [15] has demonstrated the use of various fruit peel wastes as substrates which promoted external invertase production. The work reports use of agro-wastes as potent alternatives in medium formulations for enhancement of invertase production from the least explored microorganism.

MATERIALS AND METHODS

Microorganism and in oculum preparation

Fungal colonies from fields growing crops such as sugarcanewere screened for extracellular invertase production. The serially diluted soil samples (1 g)were spread on tomedium containing sucrose as the sole carbon source (pH 6.5) and incubated at 28 ± 2 °C. The culture was maintained on potato dextrose agar (PDA) and sub-cultured every four weeks. The fungal colonies were inoculated in sucrose brothto test for invertase productivity. Potent positive isolates were morphologically identified by Lacto phenol cotton blue (LPCB) stain and 18S rRNA molecular sequencing was performed to determine the genus and the strain of the most potent isolate. Fungal inoculum was prepared from 4 - 5 days PDA slant culture. The sporulatedslants were scrapped under aseptic conditions with sterile distilled water (10 ml) containing 0.1% tween-80 to obtain homogenous spore suspension and wereadjusted to 10^8spores/ml which served as inoculum.

Collection and processing of agro-wastes

Agro-wastes as substrates such as corn cob (CC), sugarcane bagasse (SB), orange peel (OP), soyabean meal (SBM), rice bran (RB), oat meal (OM), wheat bran (WB), pomegranate peel (PP)were collected from the local market areas. The substrates were ground and sieved to obtain a uniform particle size of 0.5 mm.

Production of extracellular invertase under solid-state conditions

5 g of agro-wastes was suspended into 250 ml Erlenmeyer conical flasks and moistened with 10 ml of salt solution, autoclaved at 121 °Cfor 20 min and after cooling to room temperature the medium was inoculated with3% inoculum and the contents were mixed evenly. The flasks were incubated at 28 ± 2 °C for 4-5 days. Post incubation, crude form of external invertase which was released into the medium was extracted with 40 mlof sodium acetate bufferand shaken for 1 h at 100 - 150 rpm. The supernatant was obtained by centrifugation for 10min (10,000 rpm, 4°C) and used for further analysis.

Determination of invertaseactivity and protein quantification

Extracellular invertase activity was determined according to Miller (1959) [16] with slight variations. The reaction mixture contained sucrose (1%) in 0.5 ml sodium acetate buffer (0.1 M, pH 4.5) and enzyme solution (0.1 ml) and incubated at room temperature (28 ± 2 °C) for 30 min. Determination of reducing sugars was carried out by the addition of DNS (1.0 ml) with incubation for 10 min at 100 °C and absorbance was measured at 540 nm. One unit of enzyme activity was defined as amount of enzyme that released 1 µmol of glucose per min under the assay conditions mentioned. Enzyme activity was expressed as units per gram (U/g). At 660 nm the protein content was quantified as per Lowry *et al*. (1951) [17] using bovine serum albumin as standard. Absorbancewas recorded using UV-Visible Spectrophotometer (Systronics Double Beam 2202).

Effect of influencing process parameters under solid-state fermentation

Selection of substrate and time course study for optimal invertase production

Incubation time for invertase production by *Aspergillus* sp. was carried out at room temperature (28 ± 2 °C) at initial pH 6.5. Time course study progressed by inoculating the humidified culture medium of various substrates with 3% (v/w) inoculum and incubated for 7 days. Control samples were devoid of inoculum. The samples were withdrawn at 24 h intervals and tested for invertase activity and protein content.

Effect of moisture content

To determine the most suitable moisture level for maximum invertase production, the medium was moistened from 20 to 80%and the invertase activity was estimated on the optimum day of incubation.

Effect of temperature

The solid-state fermentation medium of pH 6.5 was incubated at different temperatures viz. roomtemperature 28, 37, 45 and 55 ± 2 °C. Atthe end of the incubation period of 4 days, theculture filtrate was used for the invertase assay.

Effect of inoculum size

The influence of inoculum levels on extracellular invertase fermentative medium was carried out with different inoculum concentrations of fungal spore suspensions ranging from 3 to 15%(v/w).

Effect of pH

An initial pH of 6.5 served as control, while the effect of pH of culture medium was examined by adjusting the medium pH to 4.5to8.5 with a digital pH meter (LI 120 ELICO). The pH was adjusted prior to the sterilization of the solid-state medium.

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Effect of beef extract concentration

Beef extract was tested at 0.5 to 3.0% (w/w). The extract was added individually to the cultivation medium to study the effect of nitrogen as a nutritional component to enhance invertase production.

Statistical analysis

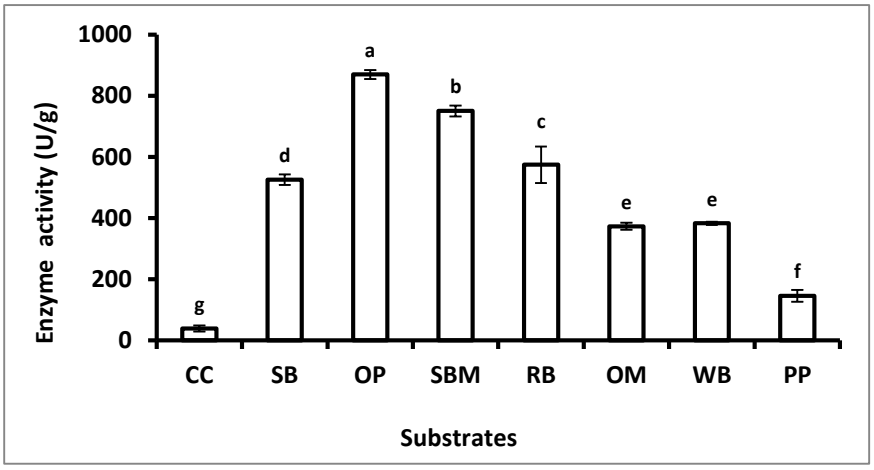
All the experiments conducted were carried out independently in triplicates (n = 3). The results are expressed as mean ± standard deviation (SD). Results were analysed by analysis of variance (ANOVA) and homogeneous subsets were given by Duncan's multiple range test (DMRT) at 5 % significance level. The *P* values less than 0.05 were considered significantly different. The SPSS statistical package (IBM SPSS Statistics 20) was used for statistical evaluations.

RESULTS AND DISCUSSION

Several reports have shown the extracellular invertase production from filamentous fungus such as *A.ochraceus*[14], *A. caespitosus*[9], *A. niger*[11, 18], *A. flavus*[15] and *Paecylomicesvariotii*[12]. Globally, enormous amounts of agro-wastes or industrial residual products are being efficiently put to use for enzyme production.Utilization of agroindustrial residues as substrates for SSF are an attractivechoiceas it brings down the cost in industrial processes,enables good recovery of products and accessibility of variety of substrates [19].Substrates such as organic waste matter facilitate good microbial growth and enzyme production. Selection of substrates is crucial as they behave as facilitators for microbial anchorage, supporting growth and sufficient enzyme production.

*A.sojae*sp. invertase produced highest invertase activity with orange peel (869.94 U/g) compared to soyabean meal (750.81 U/g) and rice bran (574.55 U/g) (Figure1).Food processing wastes are excellent sources of nutrient sources, *A. ochraceus* was cultured in a medium supplemented with sugar cane bagasse as carbon source[14], *S. cerevisiae* NRRL Y-12632 was cultivated in red carrot residues with optimum growth on fourth day. Mona et al. [20] and Uma with her co-workershas demonstrated the use of fruit peel wastes as substrates such as peels of pineapple, orange, and pomegranate [15]. Consequently, the agro-wastes influence invertase production differently as the enzyme production by *P. variotii* was about twice higher than *A. caespitosus* with wheat bran as substrate under the same solid-state fermentative conditions [9, 12]. The particle size of 0.5 mm was maintained throughout the experiments as a larger particle size reduces the contact surface between particles and growing fungus thereby affecting invertase production [21]. Therefore the agro-wastes as substrates have a varied impact on the microbial invertase production.

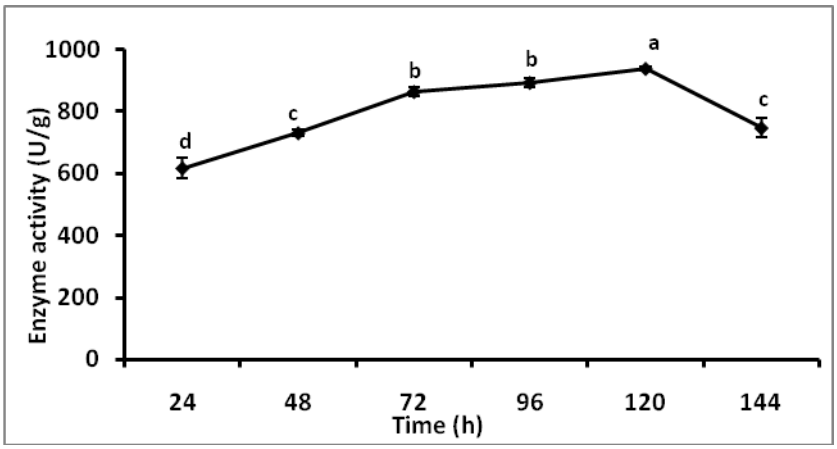
Figure 1: Effect of substrates



Enhanced invertase activity was observed with *A.sojae*sp. invertase on 5thday (938.1 U/g) at 37 °C but prolonged incubation and higher temperatures reduced activity (Figure 2). The optimum time for thermostable invertase release was observed at 96 h and a temperature of 40 °C favoured high levels of invertase

production[14]. 37 °C was more suitable for extracellular invertase production (1103.65 U/g)(Figure4). *A. flavus*invertase failed to be active beyond 30 °C[15]which was similarly seen in case of *A.caespitosus*[9] and*A. niger*[13]. Decline in activity could be attributed to unavailability of substrates in a prolonged incubation condition and an increased temperature could have led to denaturation of the active site of the enzyme. A reduced invertase production is attributed to carbohydrate content of the substrates which was not determined, as the sugars may have repressed enzyme productivity as explained by catabolite repression. Some studies have demonstrated this phenomenon[8, 12, 13].

Figure 2: Effect of incubation time



Microbial activity is greatly influenced by the water activity and moisture contents. Invertase production was highestwith50% moisture levels (940.05 U/g) as elevated levels declined activity (Figure3).An intracellular invertase from *A. niger*LBA-02also desired 50% moisture content[13]. Initial moisture levels of 65% favoured *A. niger*Aa-20 [22], *A. niger* was influenced with 80% [11]and90%moisture content was ideal for*S. Cerevisiae* invert as production [20], whereas lower moisture levels was found to be better for *A. sojae*sp. for enzyme release.An inoculum level of 3% was optimum for *A. flavus* with enzyme titre of 25.8 (U/ml) [15] whereas 9% maximally enzyme productivity (Figure5).

*A. sojae*invertase exhibited optimum activity at pH range 6.0 – 7.0 facilitated optimum enzyme release (Figure6). Production of invertase was optimum at pH 5.5 for *A. niger*Aa-20 [22], favourable growth was observed for *A. flavus*at an optimum pH 5.0 [15]. Neutral invertases (NIs) are regarded to be highly superior than invertases from *S. cerevisiae*or other yeast strains. NIs are ideal for many of the industrial processes such as food and alcohol fermentation, and also desirable for biosensor applications [23].

Figure 3: Effect of moisture content

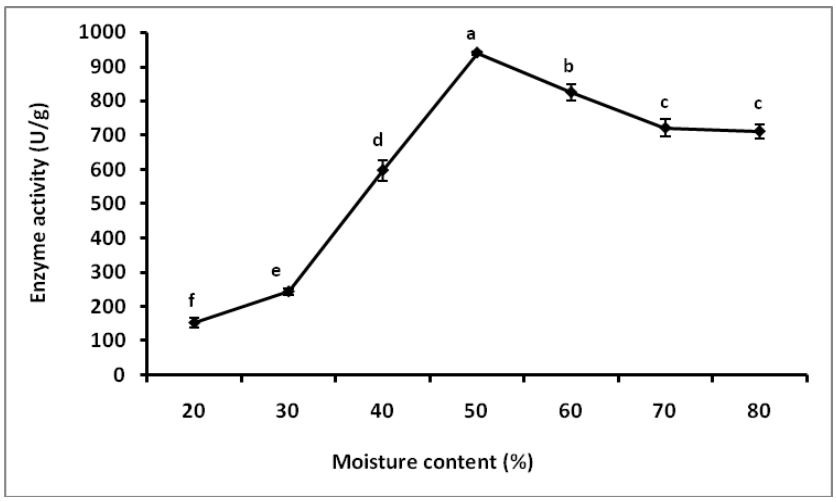


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Figure 4: Effect of initial temperature

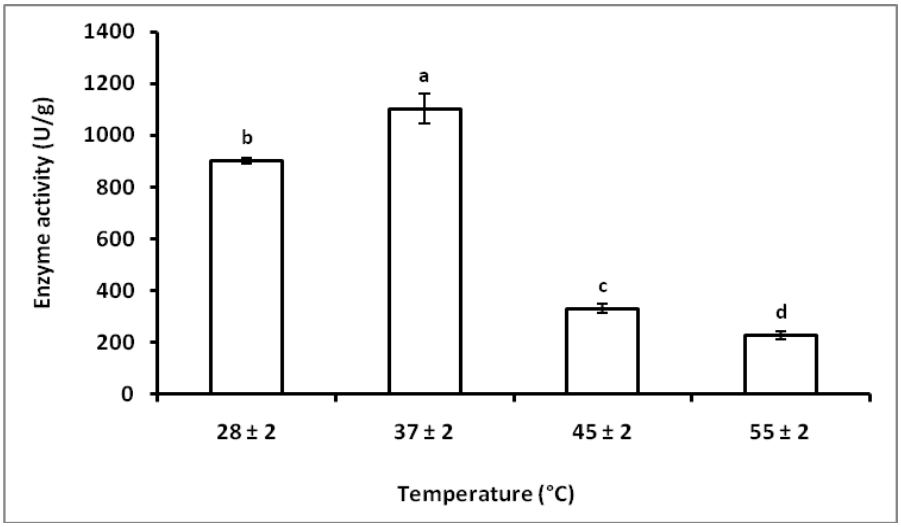


Figure 5: Effect of inoculum size

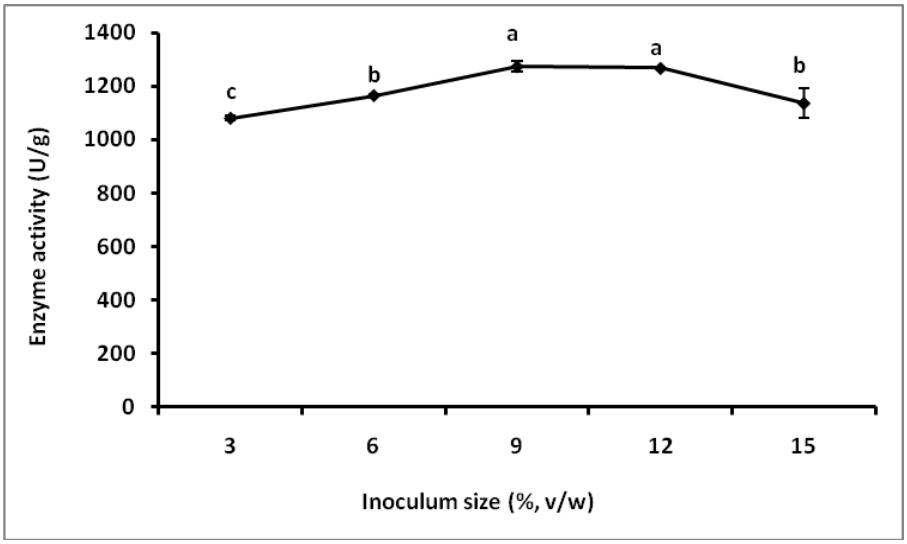
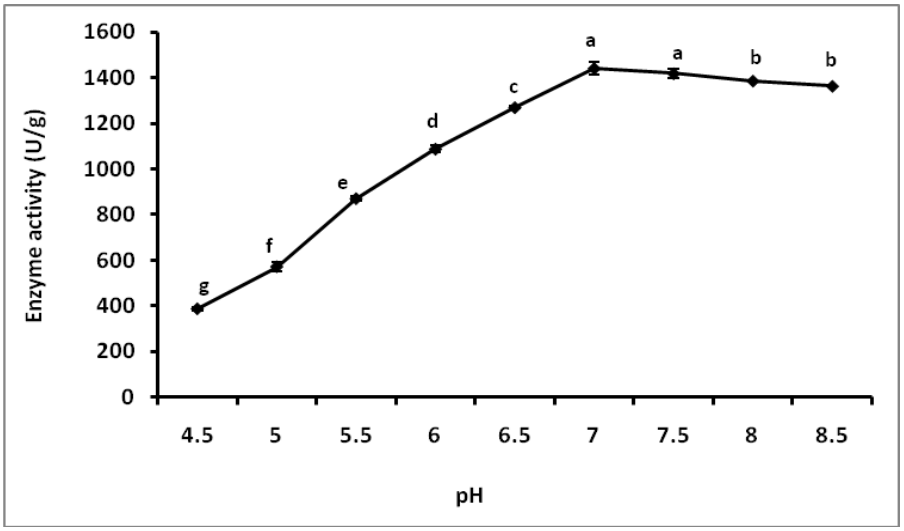


Figure 6: Effect of initial pH



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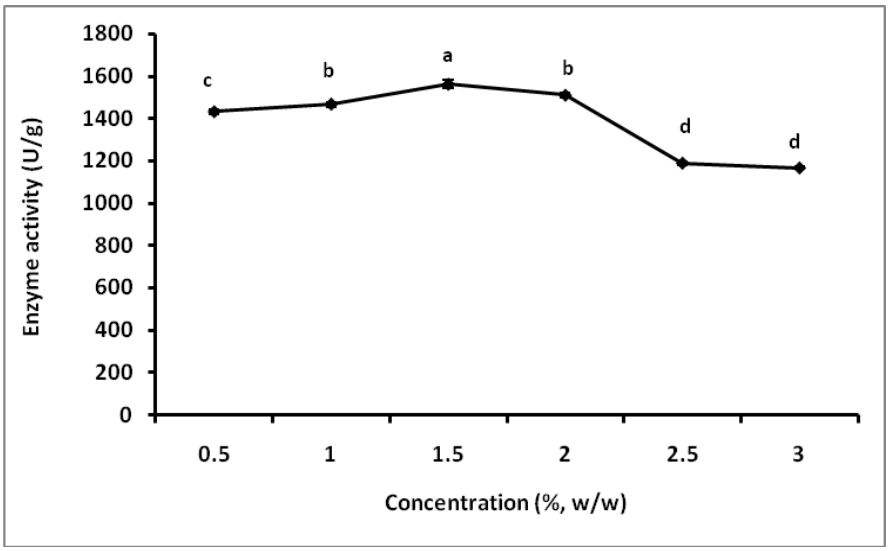
The usage of agro-wastes as nutrient source substrates in a fermentation process adds value to the waste matter. *A. sojae*sp. posed optimum invertase production at 1.5% (w/w) beef extract (1561.35 U/g) (Figure 7).Nitrogen sources are regarded to be factors which promote cell growth and eventually result in enzyme production. *S. cerevisiae*, *P. variotti* demonstrate use of organic nitrogen sources such as peptone[12, 24].

Figure 7: Effect of nitrogen source concentration



Enzyme production by SSF technology has good advantages in terms of high productivity, simple operation procedures and most importantly much lesser possibility of growth of contaminants[22].Several traditionally and non-traditionally used agricultural residues/products have been used as substrates for SSF[25]. For bulk production of enzymes the large amounts of food-processing waste mass can be used as they are rich in carbohydrates and other nutrients[26].

All the optimized factors promote the physiologicalpotential of *A. sojae* sp. influencing extracellular invertase production. The selected strain utilizes lesser moisture content compared to other fermenting strains demanding larger levels. Microbial invertases are more stable than plant invertases and preferred in food divisions. Additionally microbial enzymes are safer to use much more convenient [27].With regard to cost factor, the production of enzymes on the laboratory scale generally favours SSF over SmF [28].

CONCLUSION

Industrial sectors desire enzymatic hydrolysis of sucrose by invertase than acid treatments, as the products could be a sustainable supply of carbohydrates achieved by fermentation processes. The demand for sugar is on the rise today and it has been proposed that world sugar demand will mostly escalate up to 203 million tonnes by 2021. Invert sugars/syrup could be a replacement to table sugar which is an economic advantage and may reduce the tedious work of sugar growers. Utilization of agro-industrial wastes can reduce the operational costs for invertase production by SSF technology. Thus, *A. sojae*invertase can be economically and easily produced under optimized culture conditions as the enzyme poses to be a promising candidature capable to meet industrial need. Studies on purification and characterization of the isolated invertase are underway in order to unravel the prospects of the dynamic enzyme for industrial applicability.

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