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Comparative kinetics evidence of a novel 1, 4-α-D-glucan glucohydrolase from *Aspergillus oryzae* IIB-6 as a raw starch-degrading enzyme

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Abstract

Raw starch-digesting enzymes (RSDEs) have industrial applications, where they eliminate the need for pre-gelatinization/-liquefaction in glucose syrup production. This syrup is then used as feedstock for cost-effective manufacturing of various value-added industrial products, including biofuels, biochemicals, biomaterials, and pharmaceutical products. The study aimed to produce a novel raw starch-digesting 1,4-α-D-glucan glucohydrolase (RSD-GGH) from a GRAS strain, *Aspergillus oryzae* IIB-6, via solid-state fermentation. Partially purified RSD-GGH at 50-70% $(NH_4)_2SO_4$ saturation was used to saccharify raw starches extracted from different botanical sources, to evaluate their extent (R_s) and degree of hydrolysis (*DE*, %) compared to soluble starch. The amylose (AM) and amylopectin (AMP) contents of raw starches from rice, wheat, maize, sweet potato, and potato were 16.56, 20.53, 25.13, 30.56, and 51.65 % (LSD ~0.753), and 83.44, 79.47, 74.87, 69.44, and 48.37 % (LSD~1.202), respectively. The R_s and *DE* values of potato-based soluble, rice, wheat, corn, sweet potato, and potato raw starches were calculated as 3.75, 2.65, 1.6, 0.7, 0.3, 0.1 % and 0.0375, 0.0265, 0.016, 0.007, 0.003, and 0.001 %, respectively, while the K_m and V_{max} values at 60 °C, pH 5, were 14.851, 36.875, 40.671, 57.192, 113.23, and 453.01 $mg.ml^{-1}$, and 34.488, 1250.2, 833.34, 476.19, 370.37, and 454.02 $mg.ml^{-1}.min^{-1}$, respectively. The extent of saccharification (R_s) and dextrose equivalent (*DE*) values were also calculated. Significant variations ($p \leq 0.05$) in AM and AMP contents, as well as the botanical origin of the raw starches, influenced the enzyme's kinetics accordingly. The enzyme has great potential for the industrial production of fuel ethanol and other biotechnological products.

Keywords: Raw starch digesting enzyme, 1, 4-α-D-glucan glucohydrolase, raw starch extraction, dextrose equivalent, extent of saccharification, amylose, amylopectin, enzyme kinetics.

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1. INTRODUCTION

In recent times, the bioprocessing of agro-based biomass into value-added metabolites has been a trendy research area in biotechnology [1]. Among the plant carbohydrates, starch is the most imperative carbon and energy source, and with regard to total biosynthesis, it is second to cellulose. It is a cost-effective substrate for producing maltose, glucose, and fructose syrups, which have extensive applications as feedstock for the production of food and pharmaceutical products, and also for glucose fermentation, producing bio-ethanol in the fuel industry. However, *Saccharomyces cerevisiae* is unable to utilize starch-based substrates; therefore, amylases are needed to hydrolyze the starchy materials (Fig. 1). However, *S. cerevisiae* has been engineered for a consolidated bioprocessing of raw starch degradation and to ferment the resulting sugar into ethanol in a single step [2,3]. Conventionally, starch-to-glucose conversion requires three steps, namely gelatinization, where the starch slurry is gelatinized by heating up to 100-105 °C. This process is energy-intensive and requires additional equipment, thus increasing the cost of production of starch-based products (Fig. 1). The subsequent two steps are enzymatic reactions known as liquefaction, catalyzed by 1, 4-α-D-glucan glucanohydrolase (GGNH) (E.C. 3.2.1.1), and saccharification, catalyzed by 1, 4-α-D-glucan glucohydrolase (GGH) (E.C. 3.2.1.3) [4]. GGNH is an endo-enzyme that mainly produces maltose by randomly hydrolyzing the gelatinized starch slurry, while Exo-GGH catalyzes the polymer chain from the non-reducing end, hydrolyses both α-l, 4 and α-1, 6 glycosidic linkages, liberating glucose in the β-anomeric form in a successive manner as the sole product. GGH showed greater affinity for amylopectin, while GGNH has greater affinity for maltodextrin. Therefore, raw starch-digesting GGH (RSD-GGH) is especially useful in saccharifying the raw starch for commercial fuel alcohol production [5–7].

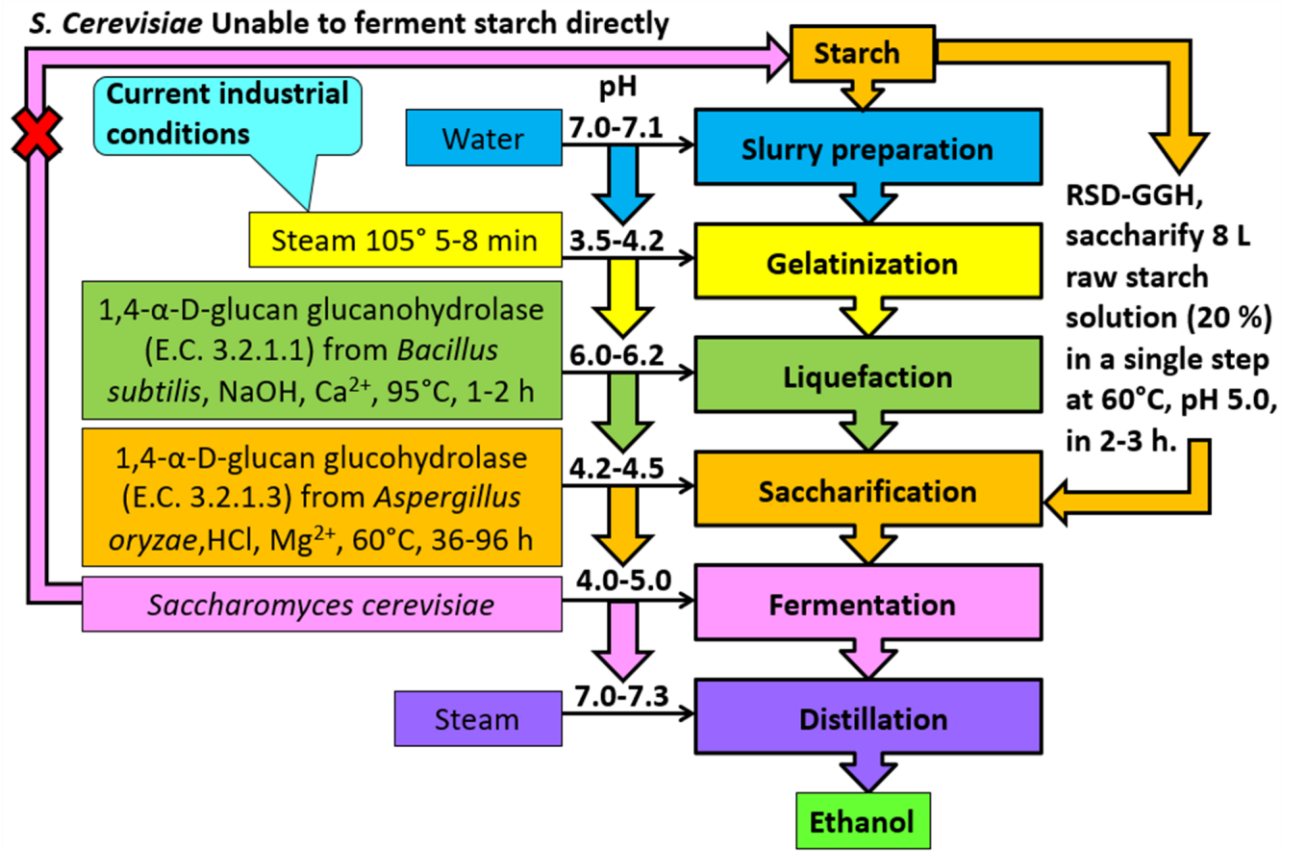

Fig. 1. Bio-catalytic de-polymerization of raw starch. RSD-GGH bypasses gelatinization and liquefaction steps and converts starch slurry into glucose syrup directly.

RSD-GGH from fungal sources hydrolyzes the relatively intact granules of raw starches below the temperature (100-105 °C) of gelatinization, which is highly desirable with a view to effective utilization of

natural resources, reducing energy costs, minimizing pollutants formation, and viscosity problems [8]. Recently, a five-membered starch saccharifying microbiota has been identified by solid-state bio-processing using agro-waste-biomass as the substrate. Among them, genus *Aspergillus* is the second that has a complete metabolic pathway for the degradation of starch to glucose [9]. *A. oryzae* releases large amounts of GGH titre in solid-state fermentation than in liquid culture, which have extremely strong hydrolyzing activities for granular raw starches compared to other GGHs. Structurally, the enzyme consists of a starch-binding domain (SBD) and a catalytic domain (CD), both of which are linked by a linker molecule. The SBD is responsible for catalytic activity on raw starch. These enzymes are different from other starch-degrading enzymes with respect to their special catalytic affinity (K_m) and interaction of their SBD with the microcrystalline structures of the granular raw starch molecules [6,10].

Regular starch preparations comprise approximately 70 to 80 % amylopectin (AMP) and 20 to 30 % amylose (AM), waxy preparations contain < 10 % AM, while high AM starches contain > 40 % AM [11]. AM and AMP composition greatly affect the rate of saccharification [12]. In increasing order, the rate of saccharification for starches containing high AM (100 %) < hybrid variety containing 64 to 66 % AM < waxy corn starches containing AMP 99 to 100 % [13]. Only waxy corn (AMP) starch has the highest rate of conversion into ethanol [14,15]. There is a lack of corn varieties with starch that have optimized AM and AMP compositions for maximum conversion into fuel ethanol [12].

Overall, the energy mix of Pakistan comprises oil (27.03 %), natural gas (24 %), coal (24.83 %), hydroelectricity (7.54 %), nuclear energy (5 %), and other resources (12.19 %). Pakistan's energy mingling mainly depends on thermal power, and it profoundly relies on the import of fossil fuels such as oil and gas because its domestic reservoirs are limited and getting depleted. This reliance results in numerous challenges for the country. It is exposing the country to external shocks, as approximately 30 % of Pakistan's foreign exchange is expended to import the fuel, which creates a significant economic burden (Transforming Pakistan's Energy Landscape: A Path To Sustainable And Secure Future). Therefore, fuel ethanol is a promising alternative that not only alleviates the energy crisis but this bio-based technology also reduces the CO_2 and other harmful substances in the environment.

In the present study, RSD-GGH from a newly isolated GRAS strain *A. oryzae* IIB-6 was employed for the direct saccharification of different cereal and tuber raw starches without pre-gelatinization and pre-liquification. The activities of the enzyme towards its different raw substrates were expressed in terms of K_m and V_{max}. The AM and AMP composition of the raw starches was also determined, which significantly affected the kinetics parameters of the enzyme. To our knowledge, it is the first report to conduct this type of comparative kinetics study of RSD-GGH for the direct saccharification of raw starches extracted from different botanical sources.

2. MATERIALS AND METHODS

2.1 Chemicals and materials

Rice, corn, wheat, potato, and sweet potato were procured from the local market. All the chemicals used in this study were analytical grade and procured from Acros, BDH, Fluka, E-Merck, and Sigma Aldrich.

2.2 Culture maintenance and inoculum development

A novel fungal strain, *A. oryzae* IIB-6, was isolated by the serial dilution method as described previously [16]. The strain was grown for three days on PDA (4%, pH 4.8) to get a profuse growth in test tubes, slanting medium, and stored in the refrigerator (P342; Griffin) at 4 °C. It was refreshed after 15 consecutive days. Inoculum containing viable spore density was prepared as described previously [17].

2.3 Production of crude enzyme

A. oryzae IIB-6 was grown on solid medium (moist wheat bran) for crude enzyme production. Solid-state fermentation was conducted in 250 ml Erlenmeyer flasks. Wheat bran (7.5 g) with 80 % moisture content was taken in an individual flask. Moisture level was maintained using (Eq. 1) by salt solution containing $FeSO_4.7H_2O$ 0.016, $MgSO_4.7H_2O$ 0.8, $(NH_4)_2SO_4$ 3.5, KH_2PO_4 0.48, K_2HPO_4 1.12 mg/gds.

$$Moisture\ contents\ (\%) = \frac{wt.of\ moist\ wheat\ bran - wt.of\ dry\ wheat\ bran}{wt.of\ dry\ wheat\ bran} \times 100 \ldots\ldots\ldots\ldots (1)$$

The pH of the salt solution was set to 5.0. Then the solution was added to each flask individually, and the contents of the flasks were mixed thoroughly to spread the moisture evenly so that each particle of the bran got moistened. The flasks were cotton-plugged and steam sterilized at 121 °C, 15-lbs/in² pressures using an autoclave (Model: KT-40L, ALP Co, Ltd 3-3-10, Midorigaoka, Hamara-shi, Tokyo, Japan) for 15 min. The sterilized medium was kept cool at RT. Spore suspension (10 %) was used as inoculum (1.2×10^7 CFU.ml^{-1}) and transferred aseptically. The flasks were shaken vigorously to ensure the spread of fungal spores throughout the flask's contents. The inoculated medium was incubated (Model: MIR-153, SANYO, Japan) at 30 °C for 72 h. After the completion of 72 h, the crude enzyme was extracted as described previously [17].

2.4 Partial purification of the enzyme

Ammonium sulphate crystals were added to the crude enzyme to 50-70 % saturation with constant stirring at 0 °C. The sample was incubated overnight at 4 °C. The precipitated GGH was recovered by centrifugation (25,900×*g*) at 4 °C, in 15 min. The pellets were re-suspended in sodium acetate buffer (50 mM, pH 5.0), and dialyzed as described previously [18].

2.5 Analytical methods

All the analytical reaction's products were quantified by a UV/VIS double beam scanning spectrophotometer (Cecil CE 100-series, Aquarius Inc., London, UK). Total protein contents were monitored at 595 nm, taking BSA as a standard [19]. The enzyme was assayed by Caldwell et al. [20], taking D-glucose as the standard. Soluble starch solution (5 %, w/v, Sigma S9765) was used as substrate. All the reaction mixtures were prepared in sodium acetate buffer (0.05 M, pH 5) by taking GGH and substrate in 1:1 ratio for experimental reaction, replacing the GGH and substrate with buffer and D-glucose in standard reaction, replacing the GGH and starch with buffer in enzyme blank, and substrate blank reactions, respectively to deduce possible reducing sugar contents in GGH and starch. All the reactions were performed at 60 °C for 60 min with constant stirring at 100 rpm. The reactions were quenched by adding the DNS reagent in a 1:1 ratio to the reaction mixture, and absorbance was taken at 546 nm [21]. The amount of reducing sugar liberated was quantified by using Eq. 2.

$$\frac{A_{std.}}{C_{std.}} \times \frac{A_{Ex}}{C_{Ex}} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

Where, A_{Std} = absorbance of standard, C_{Std} = concentration of standard, A_{Ex} = absorbance of experimental, C_{Ex} = concentration of experimental. Total starch concentration in all raw starches was determined by using an Iodine reagent (5 mM I_2 and 5 mM KI) [22]. The formation of blue coloration was monitored at 580 nm. The soluble starch (Sigma S9765) solution was used as a standard. The AM contents of all the raw starches were determined by the blue color developed against a standard AM (Sigma, A7043) [23]. The AMP content was calculated by subtracting the amylose content from 100 % against a standard (Sigma, A7780).

2.6 Extraction of raw starches

2.6.1 Raw starch extractions from cereals: Raw starches from corn (*Zea mays* L.), rice (*Oryza sativa* L.), and wheat (*Triticum aestivum* L.) were extracted by using the method of Udachan et al. [24] (Fig. 2). The grains were washed several times by adding fresh water every time and rubbed thoroughly to remove fungi, rotten spots, skin, soil, and dirt. For alkaline steeping, 100 g of grains were soaked in 200 ml of NaOH (0.25 % w/v) and incubated at 4 °C for 24 h. After that, the grains were washed again thoroughly with sterilized deionized water to remove the caustic soda completely. The grains were added to a kitchen blender with an equal volume of sterilized deionized water, and ground the grains for 10-30 min to make a homogenous slurry. A 200-mesh screen was used to filter the slurry. The residue material on the sieve was collected, and rinsing, grinding, and filtering were repeated three times on the residues generated every time. The final residues were discarded. The filtrate was allowed to settle for 60 min. The homogenized slurry was separated into three-phase fractions: (i) Heavy phase of starch, at the bottom, (ii) Middle phase of gluten, and (iii) Upper light phase of pentosanes. A wooden spatula was used to remove the upper light phase and the middle phase. Excess water was added to the heavy phase, and the settled material was resuspended. It was centrifuged at 4 °C, 6000 rpm for 10 min. In the strong gravitational field of the centrifuge, starch settles quickly, while fibers and pulp residues float in water. Water with floating materials was removed, and more water was added to the settled material, re-suspended, and centrifuged again. These steps were repeated several times until the top starch layer was whitened in color. An appropriate amount of this whitened material was added to petri dishes and incubated for 24 h at 60 °C till to it dried completely.

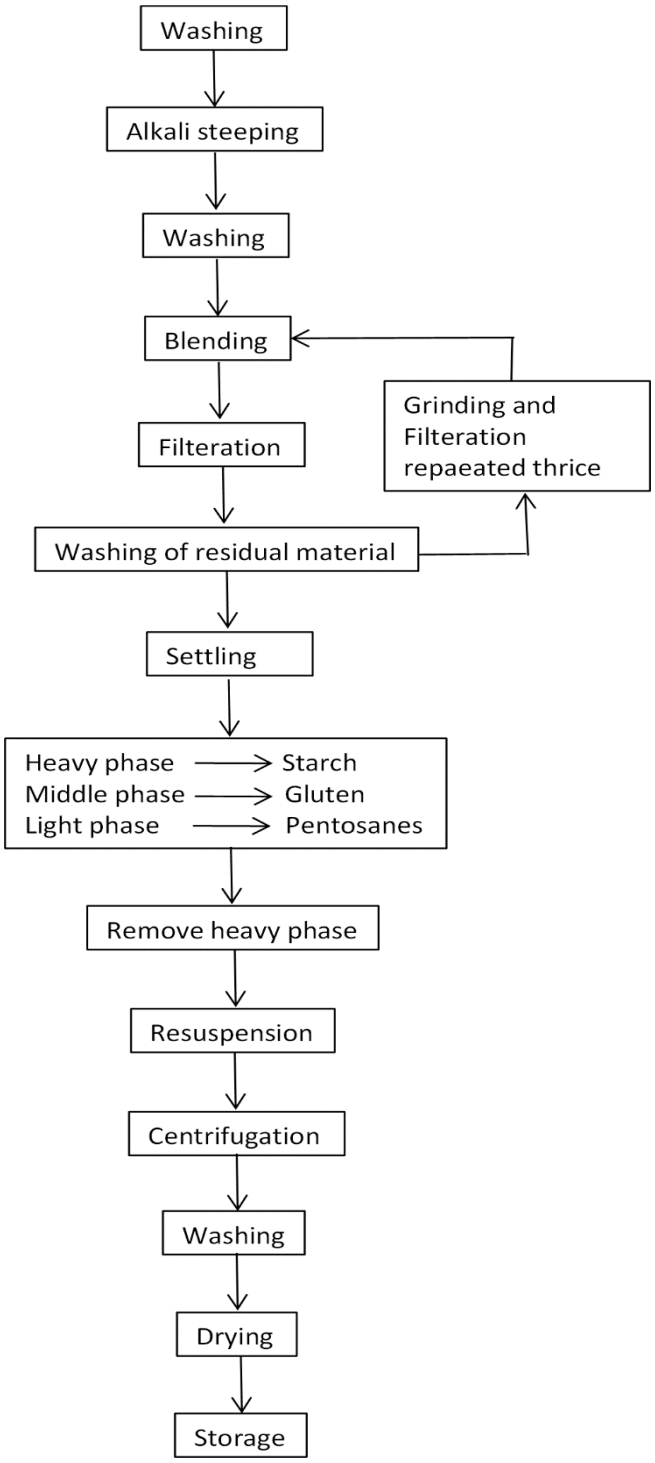

Fig. 2. Extraction of raw starch from cereals.

2.6.2 Raw starch extraction from tubers: Clean potatoes (*Solanum nigrum* L.) and sweet potatoes (*Ipomoea batatas* (L.) Lam) were washed with tap water and finally distilled water to remove dirt (Fig. 3). The thin outer skin was removed by peeling. The tubers were washed again and sliced into 2 - 3 cm cubes. Blending was done for 3 - 4 min in deionized water (1:1 w/w) to make a homogenous slurry. Cell debris and the translucent suspension were removed by passing through fine muslin cloth. The collected filtrate was subjected to washing filtration steps three times through fine muslin. The final white filtrate was allowed to settle down for 60 min. The upper water layer was decanted, and the settled starch was transferred to petri dishes. The petri plates were revolved clock and anti-clockwise to spread the starch evenly and then incubated at 60 °C in a hot air oven for 24 h, till to dried completely [25].

The dried starches were finely ground to a fine powder using a pestle and mortar, packaged in sealed polypropylene bags, and stored at room temperature until needed.

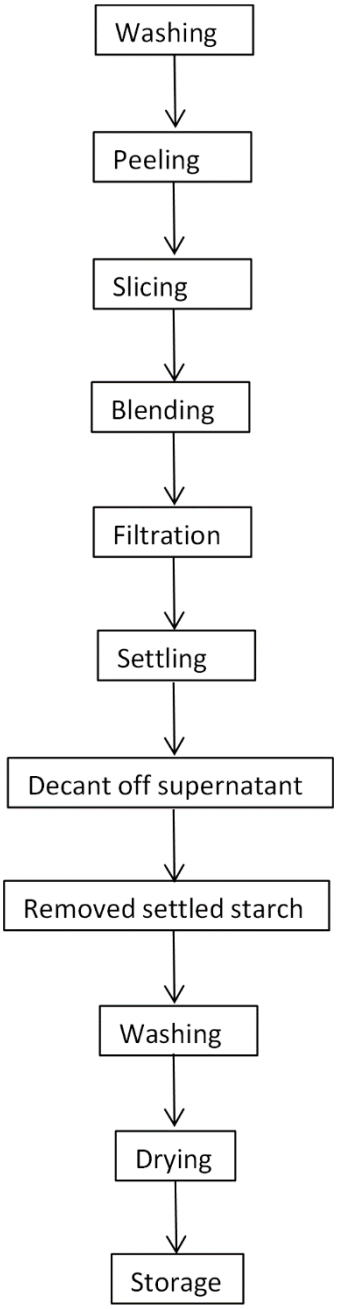

Fig. 3. Extraction of raw starch from tubers or roots

2.7 Raw starch saccharification

The saccharification of raw starches by partially purified RSD-GGH was studied using raw starches extracted from corn, wheat, rice, potato, and sweet potato. The results were compared with commercial soluble starch (Sigma S9765, potato-derived modified starch) used as a control.

The reaction mixtures consisted of 2 g of various raw starches, each was prepared in wide test tubes with a cork containing 10 ml of 0.05 M sodium acetate buffer (pH 5.0) and 1 ml of partially purified RSD-GGH with a final concentration of 20 % starch. The reactions were carried out at 60 °C, 100 rpm for 3 h, and the samples were centrifuged at 10,000 rpm for 10 min. The reducing sugar (mg/ml) released was determined by the standard assay method. The extent of raw starch saccharification (R_S) was calculated by using Eq. 3.

$$R_s(\%) = \left(\frac{A_1}{A_0}\right) \times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3)$$

Where, A_1 = the amount of reducing sugar as glucose in 1 ml of supernatant after the saccharification reaction, and A_0 = the amount of raw starch before the reaction [26].

2.9 Kinetics of starch saccharification

The kinetics of starch saccharification catalyzed by partially purified RSD-GGH followed the model Eq. 4.

$$V = \frac{V_{max}[S]_0}{K_m + [S]_0} \quad (4)$$

The Michaelis-Menten kinetic constants, i.e., K_m and V_{max}, were calculated from the Lineweaver-Burk plot [27]. The 1/V versus 1/[S] was plotted with different starch concentrations, 2.5-20 % (w/v), which were saccharified by the fixed amount of GGH at pH 5.0 separately. All the samples were assayed by the standard analytical method in triplicate (n=3).

2.10 Molecular Modeling

The tertiary structures of GGH were predicted by homology modeling using the M4T server version 3.0, [28–30], and created by Py-MOL De-Lano Scientific LLC, South San Francisco, California, USA [31].

2.11 Statistical analysis

Treatment effects were compared by the protected LSD method (Costat cs6204 W.exe). The significant differences among the replicates (n=3) are presented as Duncan´s multiple range test in the form of a *p*-value ($p \leq 0.05$) [32].

3. RESULTS AND DISCUSSIONS

3.1 Raw starch contents measurement

The starch content measurement included only the major components AM and AMP in different raw starches (Table 1), which were statistically significantly varied ($p \leq 0.05\%$) among different starches, i.e., AM = LSD ~0.753 and AMP = LSD ~1.202. Among all the raw starches studied, raw rice starch has one of the lowest AM contents. It has AM = 16.56 % and was found as the most susceptible substrate to hydrolysis. Zhu et al. [33] also reported 16.1 % AM in rice starch, and they categorized this fraction as low AM rice starch. Raw wheat, corn, and potato starches have 20.53, 25.13, and 51.63 % AM contents, respectively. The well-consistent values of AM contents for wheat and corn were reported as 28 and 23 %, respectively. However, the AM content of 21 % was reported for potato starch [34]. The potato starch with 51.65 % AM contents was found to be the most resistant starch to hydrolysis as compared to all the other starches studied. The AM and AMP contents of raw sweet potato starch were 30.56 and 69.44 %, respectively. Similarly, the values for the same were reported as 28.69 and 71.31 %, respectively, [35], which agree well with our findings. In contrast to our study, it was reported that AM contents were found in greater proportion in starches from cereals than those isolated from tubers or roots [36].

Table 1. Measurement of the major contents of different raw starches.

Raw starch source	Amylose (%)	Amylopectin (%)	Amylose/Amylopectin
Rice	$16.56^{d}\pm1$	$83.44^{c}\pm1$	0.198
Wheat	$20.53^{e}\pm0.9$	$79.47^{d}\pm0.9$	0.258
Corn	$25.13^{c}\pm0.3$	$74.87^{c}\pm0.3$	0.335
Sweet Potato	$30.56^{b}\pm0.7$	$69.44^{b}\pm0.7$	0.44
Potato	$51.63^{a}\pm0.63$	$48.37^{a}\pm0.63$	1.067
LSD	~0.753	~1.202	-

Means in the same column with different letters are significantly different (≥3 One-Way ANOVA, $p \leq 0.05\%$). ± indicates the standard deviation (±S.D) of parallel replicates (n = 3).

The AM/AMP for different raw starches was calculated (Table 1). The AM/AMP ratio in raw starches and their structural variability strongly depend on their botanical origin [34]. Hence, the number of binding sites on substrate molecules for RSD-GGH hydrolytic reaction is dependent on their botanical origin. The AM and AMP ratios provide distinctive characteristics specific to each type of starch, and it is critical to obtain gels after gelatinization with good mechanical properties because they affect their solubility and degradability. As substrate diversity was investigated, it was demonstrated that AMP concentrations can vary by up to a factor of 5 within a given botanical source, either cereals or tubers, and by more than a factor of 20 when comparisons were made among different sources, i.e., grains and tubers [37]. Additionally, Absar et al. [38] reported that the raw starches' median granule size and AM content differed greatly. It was proposed that the variations in AM contents across different starches could be caused by a variety of reasons, including genetics, environmental factors, agricultural practices, etc. Even after the extraction and purification process, the minute amounts of protein and lipid molecules are retained in every starch preparation, because these molecules integrate into the cover of the native starch's granules so that they form complexes with the starch's components. These minor constituents of proteins and lipids impart specific characteristics to each type of starch [39]. Starches with rich protein constituents are generally highly prone to undesirable browning colorations due to Maillard-type's reactions, which occur at high temperature (100-105 °C) between the ε-amino group of lysine and glucose, during gelatinization of the starch [40].

3.2 Raw starch saccharification

Among the cereal raw starches that were tested, rice starch was found to be more vulnerable to the saccharification process than wheat and corn (Fig. 4). The reducing sugar released as glucose from raw rice starch was 0.53 mg.ml^{-1}, which is 29.3 % less as compared to soluble starch. Both tuber raw starches were found utmost resistant to saccharification. Raw potato starch released only 0.2 mg.ml^{-1} glucose in the reaction mixture under the same given conditions as were for other replicates.

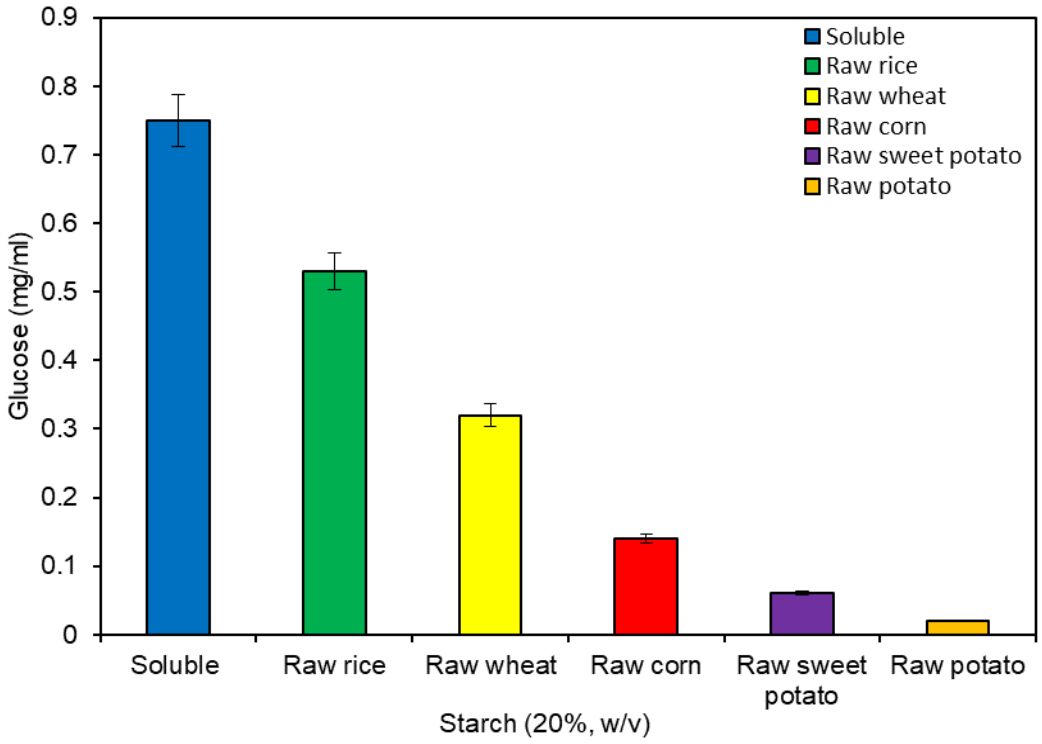

Fig. 4. Starch saccharification by partially purified RSD-GGH from *A. oryzae* IIB-6. Y-error bars show the standard deviation (±S.D) of parallel replicates (n = 3). Each mean value differs significantly at $p \leq 0.05\%$.

In starch hydrolysis, the action pattern of the specific amylase and the botanical origin of starch govern the degree of hydrolysis [41]. The extent of saccharification (R_S) of soluble and raw starches was calculated under the same reaction conditions, i.e., pH, temperature, concentrations of enzymes and substrates, and ionic strength (Table 2). The R_S value for soluble starch (potato-based modified starch (Sigma S9765)) was 3.75 %. Among the raw cereal starches, raw rice starch was saccharified to the highest extent with R_S = 2.65 % which is 1.4-fold less than soluble starch. The R_S values for wheat and corn starches were 1.6 and 0.7 %, respectively, which may be due to that wheat and corn starches have 1.2397and 1.5175-fold higher AM contents than rice starch, whereas the AMP contents of raw wheat (79.47 %) and corn (74.87 %) starch are much lower than raw rice starch (83.44 %).

Hence, the number of linkage sites, i.e., α-1, 4 glycosidic linkage in AM and α-1, 6 glycosidic linkage in AMP available for RSD-GGH hydrolysis, is reliant on the botanical origin of the starch used. Furthermore, the rate of hydrolysis of α-1, 6 glycosidic linkage is much higher as compared to α-1, 4 glycosidic linkage. The extent of saccharification for tuber starches, i.e., sweet potatoes and potatoes, was calculated as 0.3 and 0.1 % (Table 2), as these starches have much higher AM contents (as described above) but have the lowest AMP, 69.44 and 48.37 %, respectively.

Table 2. Saccharification of starch by RSD-GGH from *A. oryzae* IIB-6

Starch	**Enzyme activity ($U.ml^{-1}.min^{-1}$)[a]**	**Extent of saccharification R_S (%)[b]**	**Dextrose equivalent *DE* (%)[c]**
Soluble (potato-based modified starch)	69.45	3.75	0.0375
Raw Rice	49.07	2.65	0.0265
Raw Wheat	29.63	1.6	0.016
Raw Corn	12.96	0.7	0.007
Raw Sweet Potato	5.56	0.3	0.003
Raw Potato	1.852	0.1	0.001

[a]One unit of activity was the amount of enzyme that liberates 1 µmol of glucose at 60 °C, pH 5, per min.
[b]R_S (%) = (A_1/A_0) × 100.
[c]The dextrose equivalent (*DE*) is the measure of "degree of hydrolysis" and is defined as "the direct reducing sugar content (RSC) expressed as glucose (%) on a dry basis (maltodextrins DE < 20; syrups DE ≥ 20).

Further, the tuber and root starches, i.e., potato starch, and tropica starch produce highly viscous gel after gelatinization under same conditions (pH, Temperature, concentration) as compared to cereal starches which are owing to the difference in swelling power of their granules present in each type of starch which is further due to the difference in their chemical composition such as AM/AMP ratio, and lipids and phosphorus contents [38]. The lipids present in the starch are not only responsible for the rancidity of starch during storage but also prevent the binding of water molecules with starch granules. For this, inorganic ions such as Na^+, K^+, Mg^{2+}, $Ca^{2+,}$ and P^{5+} came from the process water. Therefore, the presence of lipids has a negative effect on the functional properties of the starch, such as clarity, solubility, and water-absorbing capacity [38,42,43].

Furthermore, the hydrolysis rate of raw cereal starches is very high when digested by a single purified GGH; they hydrolyze completely and rapidly than those from tubers or roots. This may be owing to differences in their granular sizes. Sizes increase from rice < 20 µm, corn (<25 µm), wheat (< 30 µm), potato (< 110 µm). In addition to this, there have been seen on wet granules or the surfaces of dehydrated granules have been seen by scanning electron microscopy. These depressions are clustered equatorially as seen on wheat starch granules or randomly distributed over surfaces of the granules as observed on corn starch surface; however, they are not seen on granules of potato starch. These depressions may be architecturally

susceptible sites for enzyme absorption. Physical adsorption of enzymes on starch granules is another important factor. Adsorption of *A. oryzae*'s GGH is inversely correlated with raw starch hydrolysis [44].

3.3 Kinetics of raw starches' saccharification

The selection of the best substrate (starch) from a set of candidates requires comparing the kinetic capabilities of the RSD-GGH towards different raw starches. The kinetic constants (K_m and V_{max}) of RSD-GGH were determined from double reciprocal rate and concentration plots (Fig. 5). The enzyme showed highly variable affinity for different substrates. The K_m (mg.ml^{-1}) values of the enzyme were 14.851, 36.875, 40.671, 57.192, 113.23, and 453. 01 for soluble (potato modified starch (Sigma S9765)), rice, wheat, corn, sweet potato, and potato starches, respectively (Table 3). The initial rate of starch hydrolysis is affected by the specific surface area of the starch granules, as there is a direct relationship between surface area and starch volume. Thus, contact between starch and RSD-GGH decreases as the size of the granule increases [45]. When comparing the ratio of the initial hydrolysis rate of insoluble to soluble starch, the reported rate data represent all possibilities. The same botanical starch digested more quickly when it was in the form of solubilized soluble starch than native granules by a factor of up to 40 [44]. This value is consistent with the present study, the digestion of corn-derived commercial soluble starch is faster than raw corn starch by a factor of 43.

The kinetic parameters are significantly impacted by substrate variations resulting from not only botanical and chemical diversity but also by physical diversity. For example, branching frequency: relative ratio of AM glycosidic linkage (linear polymer of α-1, 4) and AMP glycosidic linkage (polymer having α-1, 6 at the branched point). The activity (k_{cat}/K_m) towards the α-1, 6 linkage is only 0.2 % of that for the α-1, 4 linkages. Numerous investigations have demonstrated a negative correlation between starch digestion and the AM/AMP ratio [46–56]. In soluble starch, the AM complex form is different [57]. Unlike native raw potato starch with a double helix, AM and AMP have a single helix in modified potato starch, making it soluble (Sigma S9765) [58].

This is the probable reason that in the present study, RSD-GGH showed the highest affinity for soluble starch than raw rice starch, but it has much less V_{max} value than raw rice starch, that have AMP = 83.44 %. It is owing to the fact that the rate of saccharification is highest for AMP contents [13]. The AM contents were not very different for raw corn and potato starch, but there is a huge difference in their K_m value. This is probably because potato starch contains trace amounts of covalently bonded phosphate groups in its constituents, with an average of 1 in 200–500 glucose residues being phosphorylated and amylolytic enzymes are incapable of bypassing the phosphorylated glucosyl residue indicated that phosphatase, which is active toward glucose-6-phosphate, would be necessary along with amylase to fully saccharify starches that include phosphate esterified with some glucose units, like potatoes [38].

Table 3. Kinetics of GGH for different starch saccharification.

Starch source	Mechaelis Menten constants	
	K_m (mg.ml^{-1})	V_{max} (mg.ml^{-1}.min^{-1})
Soluble potato-based (*Solanum nigrum* L.)	14.851	34.488
Rice (*Oryza sativa* L.)	36.875	1250.2
Wheat (*Tritium aestivum* L.)	40.671	833.34
Corn (*Zea mays* L.)	57.192	476.19
Sweet Potato (*Ipomoea batatas* (L.) Lam))	453.01	454.02
Potato (*Solanum nigrum* L.)	113.23	370.37

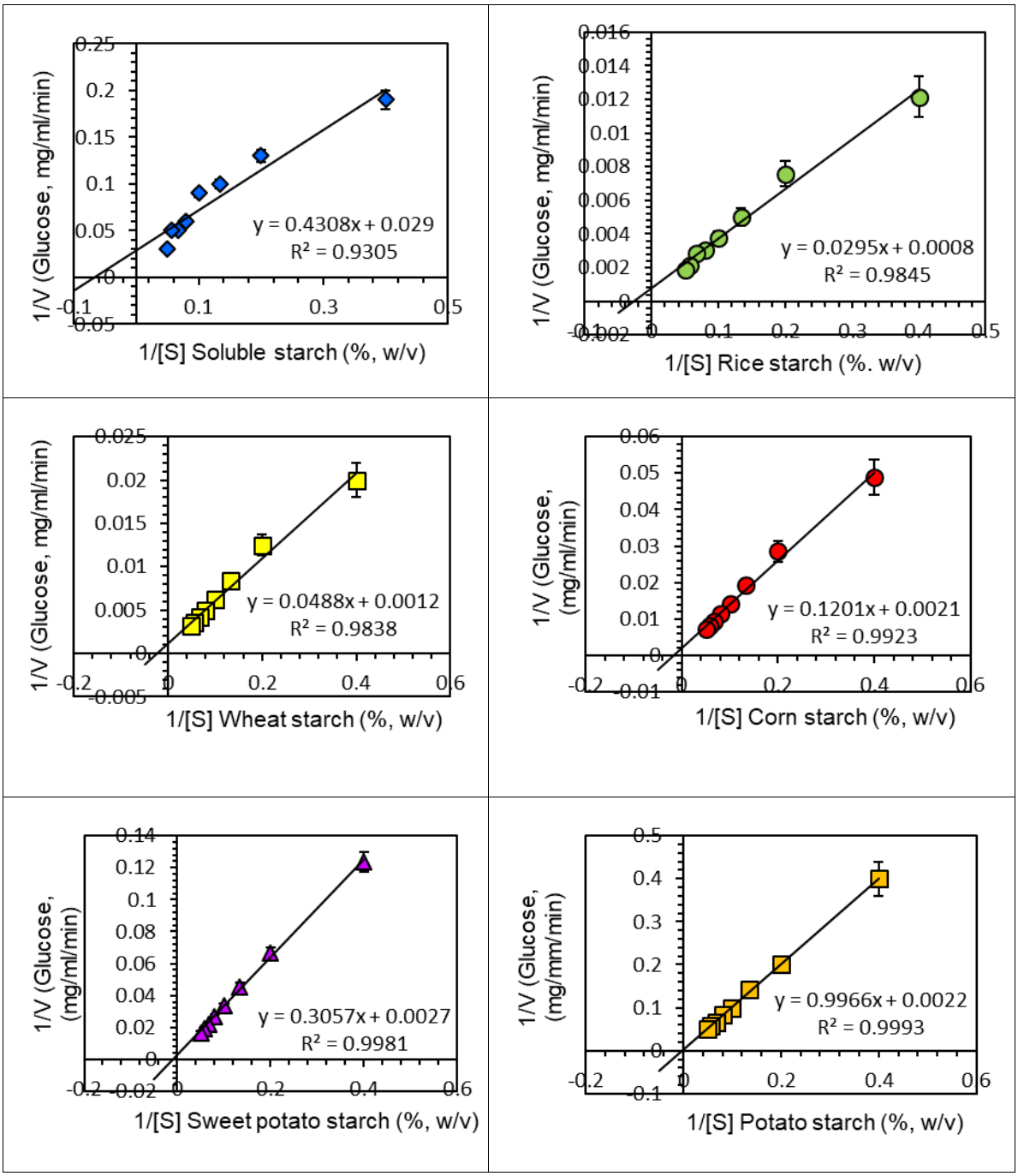

Fig. 5. Lineweaver-Burk plots for the determination of kinetic constants for soluble starch, raw rice starch, raw wheat starch, raw corn starch, raw sweet potato starch, raw potato starch, saccharification by partially purified GGH from *A. oryzae* IIB-6 at 60 °C, pH 5. Where the intercept on the *y*-axis corresponds to $1/V_{max}$ and the intercept on the *x*-axis to $-1/K_m$. Y-error bars show the standard deviation (±S.D) of parallel replicates (n = 3). Each mean value differs significantly at $p \leq 0.05$ %.

3.4 Molecular basis for raw starch digestion

The structure of the eukaryotic GGH from the fungus *A. awamori* was determined [59]. The amino acid sequences of the GGHs from *Aspergillus* species, including *A. niger*, *A. awamori*, *A. awamori* var. *kawachii*, and *A. shirousami*, exhibit a high degree of similarity (94–100%). The so-called $(\alpha/\alpha)_6$-barrel fold is formed by the helical catalytic domain (CD) of GGH, which is made up of 12 α-helices. It is made up of an inner core of six α-helices that are mutually parallel to one another and a peripheral set of six α-helices that are parallel to one another but roughly anti-parallel to the inner core [10].

Aspergillus GGH exists in two major molecular types, namely GGH I and GGH II. From residues 1 to 512, the amino acid sequence of GGH II is identical to that of GGH I. Only GGH I can bind to and hydrolyze granular raw starch, although both GGH I and GGH II hydrolyze soluble substrates. *Aspergillus* sp.'s GGH I comprises the three functional units that are (i) a CD, (ii) a strongly O-glycosylated domain (OD), and (iii) a SBD. The CD comprises 1-440 residues (Fig. 6), and it has a strong sequence homology with *Rhizopus oryzae*. The second domain, OD of GGH I (Fig. 7), consists of residues 441-512 (green) (Fig. 7B), is highly O-glycosylated and rich in Ser and Thr residues. This domain seems to be a predominantly extended conformation and to be involved in stabilizing the overall 3D structure of GGH I. One of the roles of the OD is to maintain the CD and the SBD apart from each other at a fixed distance. The carbohydrate moieties, mainly mannose residues, constituting the OD are thought to be involved in the hydrolysis of raw starch. The SBD (Fig. 7), comprises 513-616 residues (magenta) (Fig. 7A), which are involved in the absorption of RSD-GGH I to the starch and hydrolyze starch granules [10].

The CD and the SBD are different regions of the GGH molecule. The hydrolyzing capacity of GGH I to granular raw starch is linked to its ability to absorb and hydrolyze the starch granules. After digestion with subtilisin, the GGH I lost its absorption ability and it became unable to hydrolyze the granular (raw) starch; however, the resulting glycopeptide (Gp-1) retained its ability to absorb to granular starch, but it lost its digestibility. This Gp-1, which is part of the GGH I, is named the "raw-starch affinity site" (Fig. 8). This site is located between Ala-471 and Val-514. The Gp-1 is 45 residues long and is highly homologous to the OD region of GGHs from *A. awamori*, *A. niger*, and *R. oryzae*. In *Aspergillus* GGH, the SBD is located at the C-terminus, and it is constituted upon Trp-590 and Trp-615 [10].

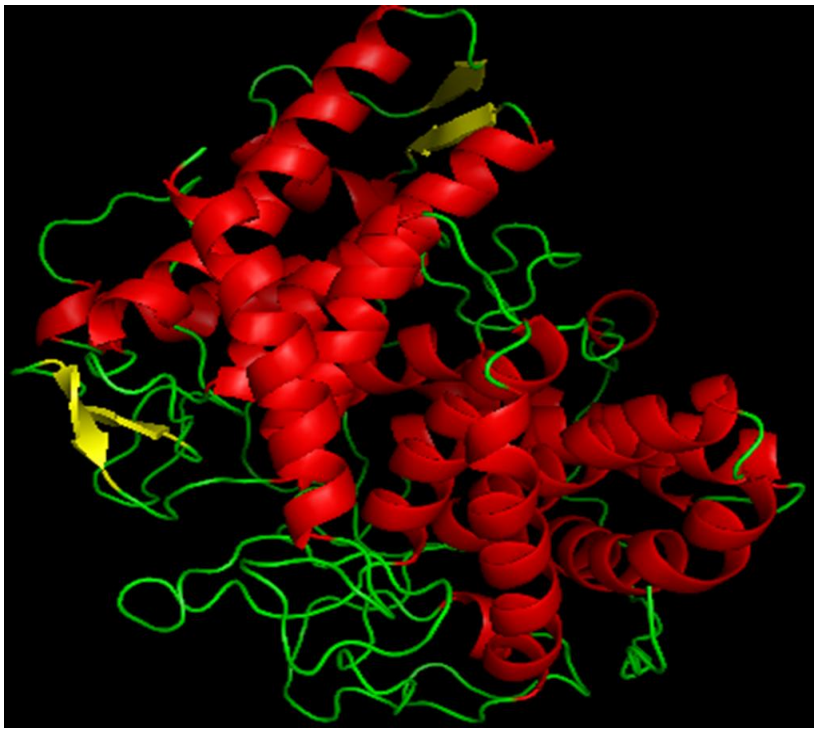

Fig. 6. CD of GGH I from *A. oryzae* IIB-6. Helix (Red), Sheet (Yellow), Loop (Green).

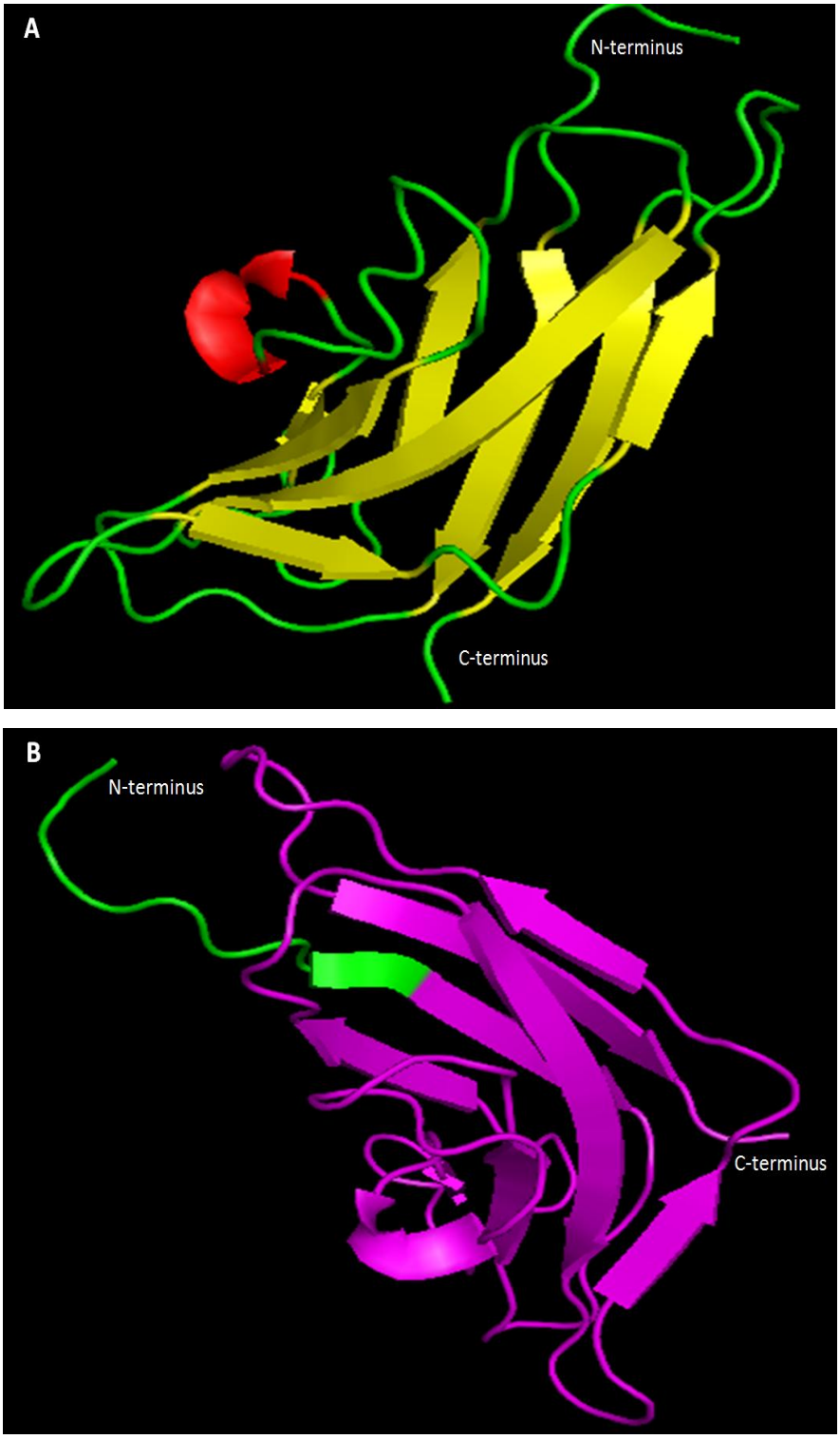

Fig. 7. 3D structure of SBD of GGH I from *A. oryzae* IIB-6. (A) Helix (Red), Sheet (Yellow), Loop (Green) (B) 0-glycosylated domain (Green); starch binding domain (magenta).

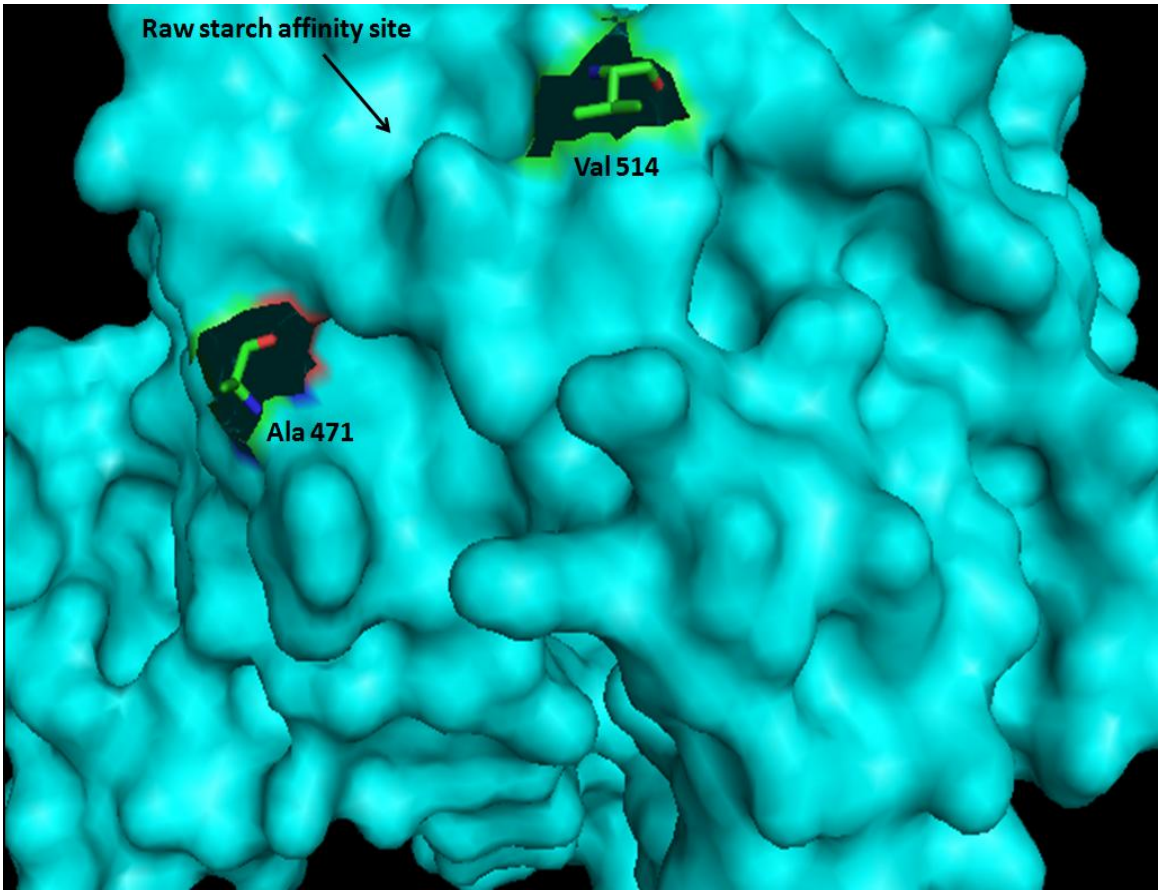

Fig. 8. Raw starch affinity site of GGH I from *A. oryzae* IIB-6.

4. CONCLUSIONS AND RECOMMENDATIONS

It is concluded that the AM and AMP ratios and botanical origin of the raw starches greatly affected the kinetics of the hydrolysis reaction. The direct saccharification process of raw starches has promising industrial applications for bioconversion to biofuel and other value-added products such as glucose, maltose, and high fructose syrup production. Since rice, corn, wheat, sweet potato, and potato are the cost-effective sources of starch, raw starch saccharifying enzymes that are capable of hydrolyzing starches from all these sources efficiently are economically attractive and have great potential in biotechnological applications in biofuel, food, and pharmaceutical industries. Hence, the kinetics of RSD-GGH from *A. oryzae* IIB-6 for hydrolyzing different raw starches have proposed this enzyme as a promising industrial catalyst.

ABBREVIATIONS AND SYMBOLS

RSDE = Raw starch-digesting enzymes
GGH = 1, 4-α-D-glucan glucohydrolase
GGNH = 1, 4-α-D-glucan glucanohydrolase
GRAS = generally regarded as safe
IIB = institute of industrial biotechnology
LSD = least significant difference
E.C. = enzyme commission number
R_s = extent of hydrolysis
DE = degree of hydrolysis
K_m = affinity of the enzymes with their substrate
V_{max} = maximum velocity of the enzyme
PDA = potato dextrose agar
CFU = colony-forming units

RT = room temperature
DNS = 3, 5-dinitrosalicylic acid
BSA = bovine serum albumin
CD = catalytic domain
SBD = starch binding domain
AM = amylose
AMP = amylopectin

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NOVELTY STATEMENT

It is the first report for the comparative kinetic study of novel RSD-GGH for the direct saccharification of raw starches extracted from different botanical sources.

AUTHOR'S CONTRIBUTION

Dr. Bilqees Fatima: conception of the study, performed the bench work, data analysis, wrote an original draft, reviewed, edited, and approved the final draft. **Prof. Dr. Muhammad Mohsin Javed:** conceived and supervised the study, reviewed, edited, and approved the final draft.

CONFLICT OF INTEREST

The authors have no relevant financial or non-financial interests to disclose.

ETHICAL APPROVAL

Not applicable.

SUPPLEMENTARY INFORMATION

Not applicable

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