Asian Journal of Mycology 6(2): 1–25 (2023) ISSN 2651-1339

www.asianjournalofmycology.org **Article**

Doi 10.5943/ajom/6/2/1

Analyses of challenges faced during the production of amylolytic enzymes and bio-ethanol using left-over cooked-rice, and rice unfit for human consumption

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Kumar R, Babu P, Vinoth V, Vignesh K, Rani S, Renganathan S, Sarma V 2023 – Analyses of challenges faced during the production of amylolytic enzymes and bio-ethanol using left-over cooked-rice, and rice unfit for human consumption. Asian Journal of Mycology 6(2), 1–25, Doi 10.5943/ajom/6/2/1

Abstract

Rice-based enzyme and alcohol production bio-processes using fungi have been explored for years, but they have suffered from the long-pending predicament of presenting their challenges. Currently, major agricultural countries have been resorting to the usage of surplus first-generation biomass sources in bio-refineries. The present lab-scale work proposes, analyzes, and presents the scale-up challenges of such processes while using a novel mixture of left-over cooked rice and market-rejected rice grains. Fractionated rice grains were used as the substrates for enzyme production. Alpha-amylase (365 IU/mL) was produced by employing *Aspergillus oryzae* 113 ATCC [9102], while glucoamylase (96 IU/mL) was produced by employing *Aspergillus niger* A1143 NRRL3 [DSM2466] in separate 144-h-long bio-processes. A mixture of left-over cooked rice and rice grains was sequentially saccharified using the produced enzymes, showing a cumulative 51.66% saccharification. Fermentation of the saccharified hydrolysate using *Saccharomyces cerevisiae* yielded 59.2 g/L bio-ethanol (starch-conversion: 63.3%) with 190-proof purity (95% v/v). The impact of the enzyme/bio-ethanol production on the coefficients of fungal biomass yield ($Y_{x/s}$, $Y_{x/O2}$) and maintenance (m_{sx}, m_{O2x}), the rates of specific growth (μ) and substrate uptake (SUR) were analyzed individually. A fundamental techno-economic analysis of the entire process has been presented.

Keywords – Hydrolysate – Fungal biomass yield – Saccharification – Yield and maintenance coefficients – Specific growth rate – Techno-economic analysis.

Introduction

The worldwide fossil fuel demand had risen to 103 mb/d (million barrels per day) by 2021 (Ramamoorthy et al. 2022a). By 2022, the Indian government had proposed to market ~20% (v/v) ethanol-blended fuels (Ramamoorthy et al. 2022a). Reducing oil imports could greatly influence the exchange rates between various currencies (Vochozka et al. 2020). Currently, the 2G bio-refineries (second-generation/lignocellulose-based bio-refineries) have been catering to the worldwide ethanol requirement (Ramamoorthy et al. 2022b). In an earlier hypothetical data-based analysis performed

Submitted 20 August 2022, Accepted 6 March 2023, Published 8 August 2023
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by our research group (Ramamoorthy et al. 2022a), considering a 100% saccharification (Ramamoorthy et al. 2018) and a 100% theoretical fermentation yield (Ramamoorthy et al. 2020b) from an average of 35% (w/w) cellulose-containing (Ramamoorthy et al. 2020a) 146 billion tons of lignocellulosic biomass sources (Ramamoorthy et al. 2022a), it could be surmised that a total of 12.2 million barrels per year (mb/y) of ethanol could be produced. For a worldwide 20% (v/v) ethanol-fossil fuel blending, the supposed bio-ethanol production of 12.2 mb/y would be manifolds lesser than its single-day requirement of 20.6 million barrels (Ramamoorthy et al. 2022b). The first-generation bio-refineries, though faced criticism owing to the fuel-food debate (Muscat et al. 2020) they created, are now being re-considered by several nations to combat the burgeoning fuel crisis (25 % subsidy for investors for ethanol production 2021). In 2021, approximately 78,000 tons of rice from the Food Corporation of India (FCI) were diverted towards ethanol production (The Hindu 2021).

In India, a majority of produce from marginal community farmers is market-rejected (Singh 2020). In the southern parts of India, during the devastating floods of November 2021, sewage mixed with flood water entered household grain storage spaces (Jane 2021). The sewage-immersed rice is beyond the standards of human consumption. With regard to recycling, it fails to meet the "Hazard Analysis and Critical Control Point" set by poultry/cattle farms following the guidelines included in the manual "Good practices for the feed sector" published by the Food and Agriculture Organization of United Nations (FAO and IFIF 2020), implementing the Codex Alimentarius Code of Practice on Good Animal Feeding. On an average, in a country's semi-urban/rural household (Liu et al. 2020), restaurants/cafeteria, around 2 – 2.5 Kg of cooked rice is left-over per day (Liu et al. 2020); and are dumped in trash.

Alpha-amylase (E.C.3.2.1.1) (apparent molecular weight – 58.4 kDa) (Ramamoorthy et al. 2022c), an endo-acting, calcium-dependent metallo-enzyme, hydrolyses the interior of α-1,4-glycosidic bonding in starch and results in the release of maltose and glucose (Sethi et al. 2016). The terminal region and the α-1,6-glycosidic linkages cannot be hydrolyzed by this enzyme (Sundarram et al. 2014). Alpha-amylase production has been attempted from edible oil cakes (Balakrishnan et al. 2021), soybean husk, flour mill wastes (Melnichuk et al. 2020), and rice bran (Paul et al. 2020). The exo-acting microbial glucoamylase (EC 3.2.1.3) (apparent molecular weight – 68.3 kDa) (Ramamoorthy et al. 2022c) hydrolyses partially-digested starch to glucose. The enzyme is capable of hydrolyzing both the α-1,4-glycosidic linkages and α-1,6-glycosidic linkages (Karim & Tasnim 2018). Glucoamylase production has been attempted using rice (Mikai et al. 2015), rice *koji* (Takefuji et al. 2016), food waste (Kiran et al. 2014), agricultural wastes (Singh et al. 2019), and wheat bran (Adefisoye et al. 2018). Though our previous works recorded a 1.5-fold increase in enzyme yields, while using solid-state fermentation (SSF) (Sambavi et al. 2019), its scale-up is tedious. Hence, submerged fermentations (SMF) are largely-preferred (Ramamoorthy et al. 2019a).

For centuries, researchers have used *A. oryzae* for the production of a plethora of enzymes (Arnau et al. 2020) apart from proteases and amylases (Kjærbølling et al. 2020). Certain species of *Aspergillus*, such as *A. niger* (Cen et al. 2020), *A. awamori*, and *A. Japonicas*, have been applied in glucoamylase production (Arnau et al. 2020). *Aspergillus* spp. has been reported to display maximum extracellular protein secretion (Fani et al. 2020), and it has been given a GRAS (Generally Recognized as Safe) status (Fani et al. 2020).

In the present study, an integrated and sequential bio-process, employing a novel mixture of left-over cooked rice and rice grains has been performed. Detailed analyses pertaining to the process constraints, fungal growth, and bio-reactor operational kinetics, during the production of α-amylase, glucoamylase, and bio-ethanol, have been presented. Apart from providing valuable process-linked analytical data for scale-up (Nosrati-Ghods et al. 2022), the work may offer a futuristic, self-capitalized, entrepreneurial proposition to community farmers.

Materials & Methods

Conventionally, "rice grain/whole rice grain" refers to rice along with its bran and husk (Bodie et al. 2019). But in this study, to differentiate between the two forms of rice (cooked and uncooked),

"rice grain/whole rice grain" has been used to describe uncooked rice. Sucrose, a non-reducing sugar and one of the major components of the substrate mixture, has been listed in the proximate analysis sections along with the quantities of various reducing sugars. But for the convenience of accommodating the total quantities of various sugars in a single tabular representation, the non-reducing sugar component has also been listed in the tables.

Collection of Rice

Market-rejected rice grains, sewage water-immersed rice, and cooked-rice were collected from small, surrounding rural districts of Puducherry, Tanjore, Tindivanam, and Chennai, from the state of Tamilnadu, India. While the rice grains were sun-dried for 4-h and packed into autoclavable bags (500 g each), the left-over cooked rice was collected in battery-operated refrigerated boxes and brought to the laboratory.

Pre-processing and preparation of the rice-based substrate for bio-ethanol production

The cooked and uncooked rice samples were subjected to a 15 min water-jet rinsing, heat-based drying (50 ^{o}C for 6-h in a hot air oven, Kencore systems, 250 L, 1.5 kW), and autoclaving as separate units (Lab-scale Autoclave, Biotechniks, 15 L, 2kW) at 15 psi, 121°C, for 1-h. The individual batches were cooled, mixed in a 1:1 ratio (on a weight basis: left-over cooked rice: rice grain) and powder-milled (Natraj enterprises, 10 kg/h, 746 W) for 1-h. The powdered mixture was subjected to a stage of intermittent beat-sieving process (U-Tech Coarse - ASTM Mesh 35, US sizing, 500 μm). The process flow has been pictorially depicted in Fig. 1a and Fig. 1b.

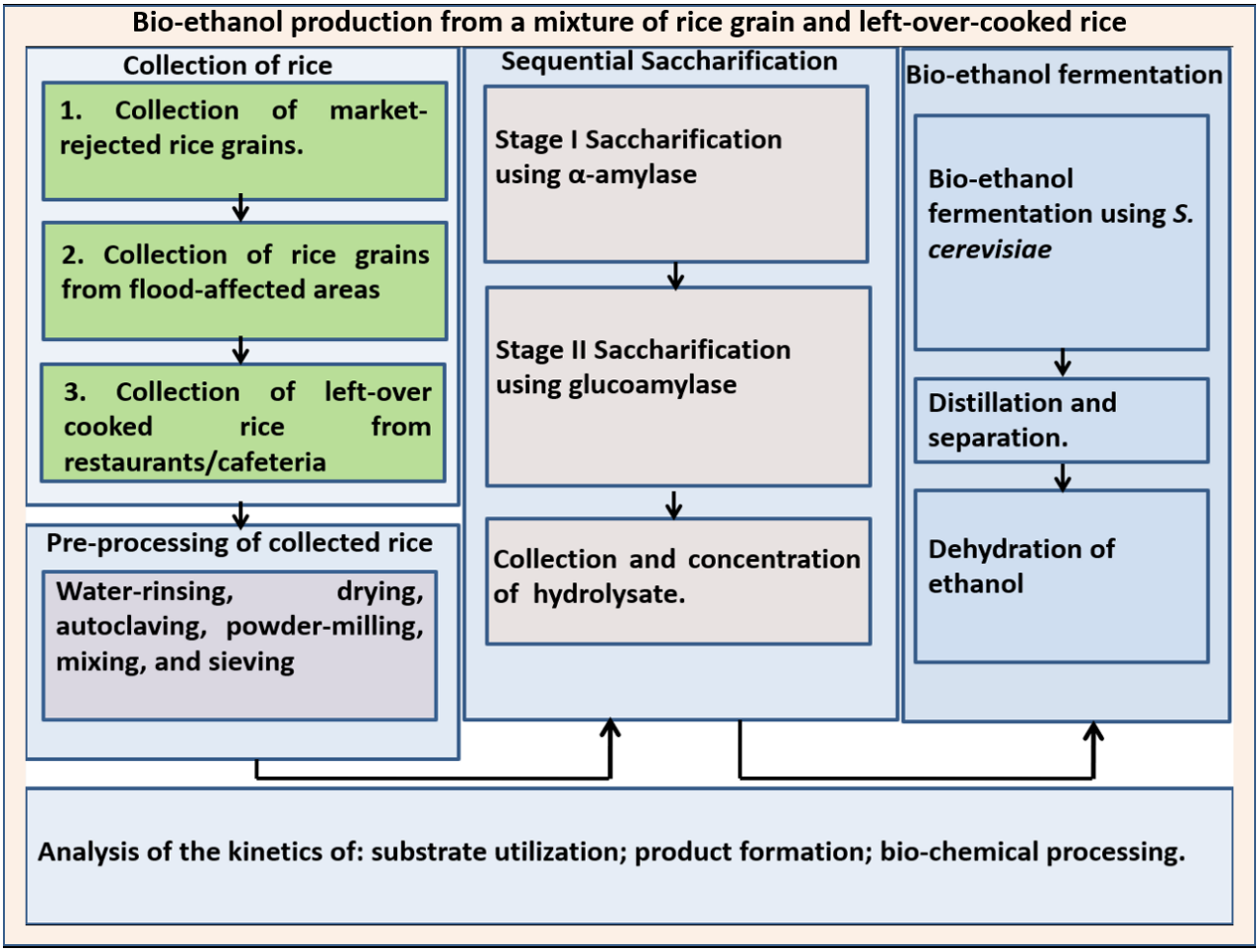

Fig. **1a** – Workflow for the production of bio-ethanol from a mixture of the collected rice grain and left-over cooked rice.

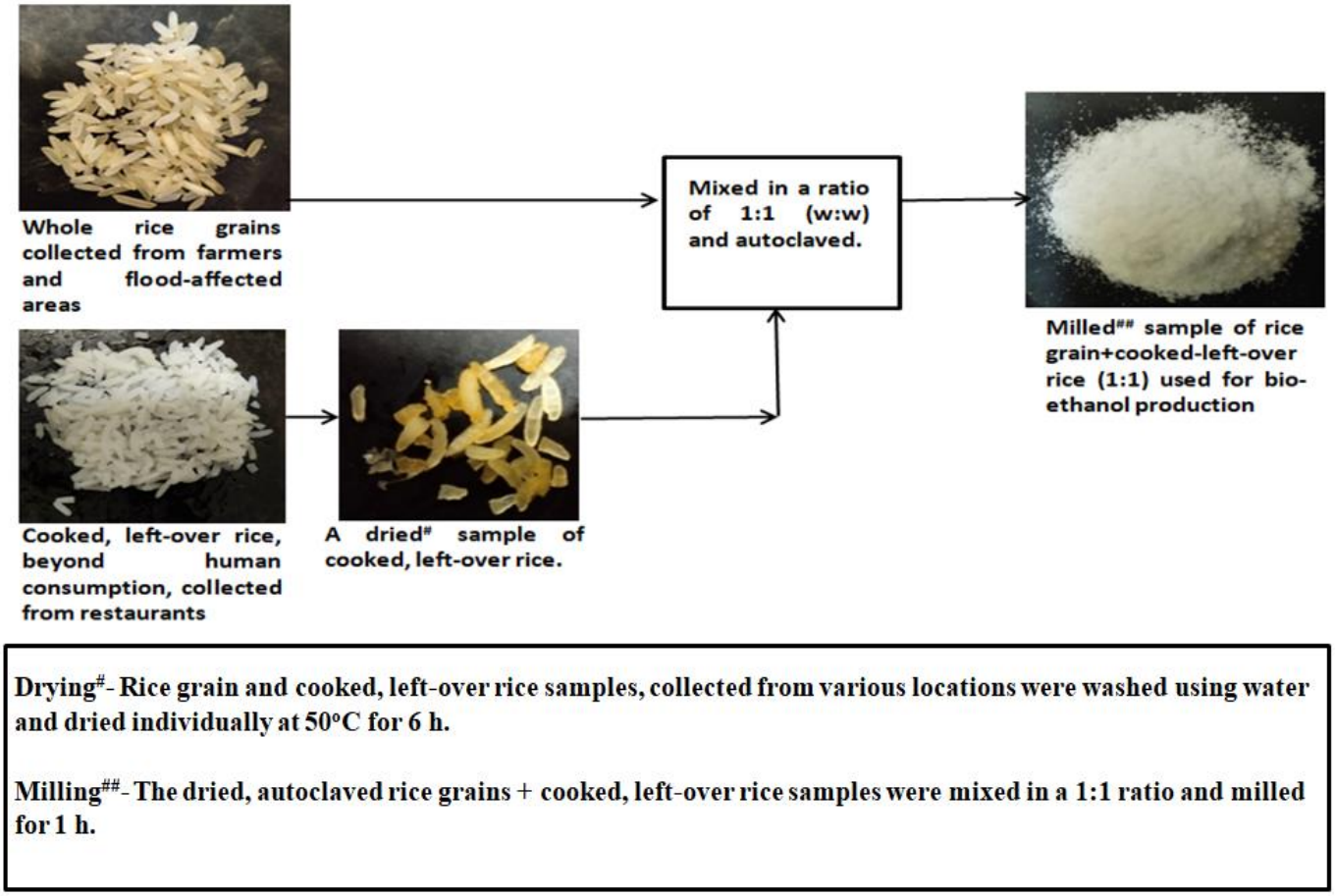

Fig. **1b** – Pre-processing and preparation of the rice grain and cooked, left-over rice as a substrate mixture for the production of bio-ethanol.

Proximate analysis for the estimation of total reducing sugars (**TRS**) **in the substrate mixture**

In a capped, hard-glass tube, 300 mg of the sample mixture was treated with 3 mL of 72% (v/v) sulphuric acid and incubated at 30 ^{o}C for 60 min in a water bath (Bioinnova Tec 5 L). The acidity was reduced to 4% by adding 84 mL of water. The proximate analysis of liquor was subjected to semi-preparative thin-layer chromatography, followed by Miller's DNSA method of sugar estimation.

Pre-processing and preparation of the rice-grain-based substrate for the production of amylolytic enzymes

To the rice grain sample collected, in an open glass beaker (Borosil 5 L capacity, laboratory glassware), two volumes (w/v) of distilled water were added, and it was heated over a hot plate (Labcare, 100 to 1000 mL, max temp 100 ^{o}C, 550 W) at 70 ^{o}C for 20 min. Post heating, the sample was separated into two fractions. The gel-like supernatant (shown in Fig. 2b) and the bottom fraction containing rice were dried at 50 ^{o}C for 6-h and 4-h, respectively, in a hot-air oven. Due to moisture loss, the dried top fraction appeared like a film (shown in Fig. 2b) (Lumdubwong 2019). The dried fractions were individually powder-milled for 30 min (shown in Fig. 2b), autoclaved at 15 psi, 121 °C for 1-h (as separate units), and subjected to proximate TRS content analysis. Sucrose, a hetero-di-saccharide (composed of glucose and fructose monomers), is quantified using the double-equivalent of the quantified fructose units. However, for the ease of comprehension, the quantified sugars are represented as equivalents of glucose and sucrose.

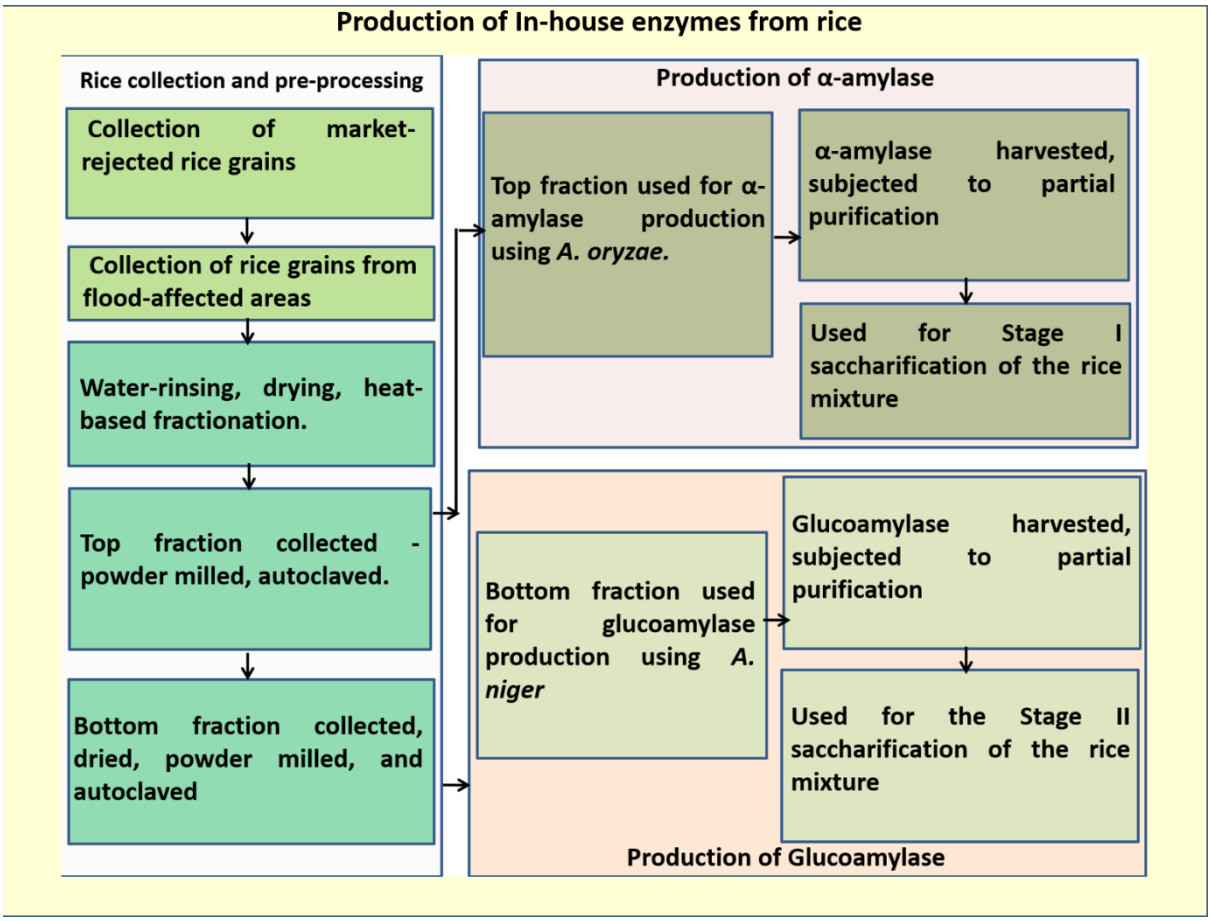

Fig. **2a** – Workflow for the production of amylolytic enzymes.

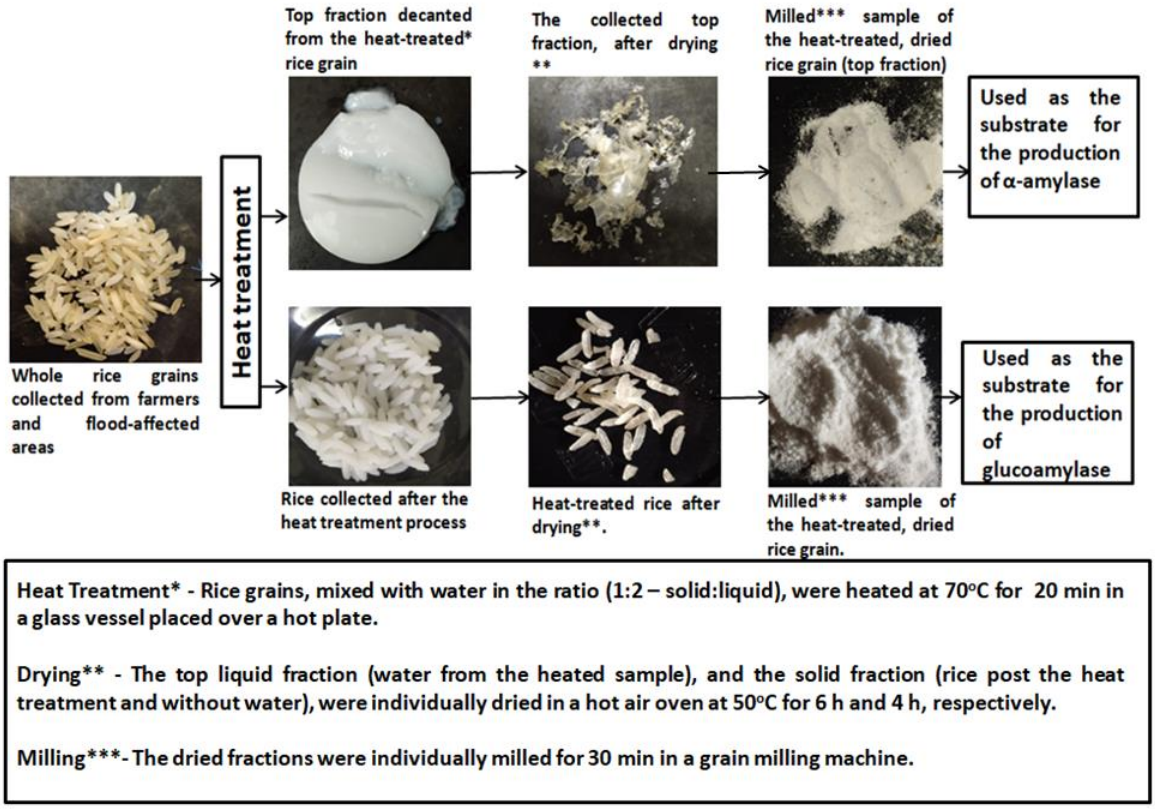

Fig. **2b** – Pre-processing and preparation of the rice grains as substrates for the production of α-amylase and glucoamylase.

Production of amylolytic enzymes

Preparation of starter cultures

Aspergillus oryzae 113 ATCC [9102] and *Aspergillus niger* A1143 NRRL 3 [DSM 2466] from the fungal consortium in our laboratory were used for the production of α-amylase and glucoamylase, respectively. The reagents used in the work were procured from Hi-Media Laboratory. Two batches of 60 mL pre-primary inocula were prepared in potato dextrose broth (microbiological grade) in 250 mL Erlen Meyer flasks. Spore suspensions of 4 x 10^{10} of *A. oryzae* and 3 x 10^{10} of *A. niger* were used to inoculate the pre-primary culture flasks, which were incubated at 35 ^{o}C for three days at 85 RPM shaking (Biosystems shaker 5 L, 0.5 kW). The 200 mL of primary inoculum for the phase of bio-reactor cultivation was prepared in a chemically-defined media. Approximately, 20% (v/v) pre-primary cultures were used to inoculate the primary inocula (200 mL), which were subjected to similar incubation regimes. A primary culture should facilitate a decent proportion (at least 60-70 % of the growth in an undefined media) of fungal mycelial biomass accumulation (Chen et al. 2022).

Production medium for the enzyme-production phase

The defined media comprised (in % w/w) of 0.01% calcium chloride (microbiological grade), 0.1% tryptone (Type I - Casitose), 0.14% magnesium sulphate heptahydrate (Analytical grade), 0.2% ammonium nitrate (Analytical grade), 0.25% tri-sodium citrate (microbiological grade), and 0.5% di-potassium hydrogen phosphate (Analytical grade). To function as carbon sources, from the heat-fractionated rice grain samples, 1.5 % (w/v) of the top-fraction was used for α-amylase production while 3.5% (w/v) of the bottom-fraction was used for glucoamylase production. Prior to autoclaving, the media's pH was adjusted to 4.5.

Operational conditions maintained during the enzyme-production phases

Alpha-amylase and glucoamylase were produced in separate 144-h-long submerged fermentation (SMF) batches. A 1-L working volume (production media + inoculum) was used in the bio-reactors (Scigenics, 3 L, India). To maintain the media pH between 4–4.5, 2N ammonium hydroxide and 2N hydrochloric acid were used as the alkali and acid inlet lines, respectively. Polypropylene glycol (4–6 ppm) was used as the antifoam. The pH probe was calibrated between the ranges of 3 and 7 prior to autoclave. After sterilization, the dissolved oxygen (DO) probe was polarized (for 100 % saturation) for 8–10 h. The inocula and carbon sources were exactly similar to the percentages used for primary cultures. The temperature was set to 37 ^{o}C; the aeration rate was manually-varied between to suit the oxygen requirement and incessant foaming. The culture broth was periodically analyzed for variation in viscosities (Viscometer - Acutek LM 60, max 20,00,000 cP, 20 W) and sugar accumulation.

Estimation of enzyme activities

Citrate buffer (1M, pH - 3.5) was prepared by dissolving citric acid monohydrate (Analytical grade) in 750 mL of distilled water. DNS reagent was prepared by mixing 10.6 g 3,5- di-nitro-salicylic acid, 1420 mL distilled water, 19.8 g sodium hydroxide, and 306 g sodium potassium tartrate. In addition, 7.6 mL phenol (melted at 50^{o}C) and 8.4 g of sodium meta-bisulfite were added to the mixture.

The 1 IU (international unit) refers to the quantity of enzyme which releases 1 μ.mol per min of glucose at the stipulated reaction conditions. Rice-based starch (50 mg, Microbiological grade - Bioinnovations) was used as the substrate for the assay of α-amylase, and 50 mg of soluble starch (GR for analysis –Merck) was used as the substrate for the assay of glucoamylase. The 0.5 mL of the enzyme (appropriately diluted using citrate buffer) was added to a test tube containing 50 mg of the respective substrate and incubated at the enzyme-specific temperature for 60 min. For α-amylase, the incubation temperature was 73 ^{o}C, while for glucoamylase, the incubation temperature was 64 ^{o}C. Later, 3 mL of the DNS reagent was added and the sample was boiled for 3 min in a water bath. The 20 mL of distilled water was added to the test samples and the absorbance of the solution was

measured at 540 nm using a spectrophotometer (UV spectrophotometer - Perkin Elmer). For the estimation of the enzyme activity of α-amylase, the release of maltose was considered; for glucoamylase's activity estimation, the release of glucose was considered.

Estimation of the quantities of fungal biomass

The weight of fungal biomass during the enzyme-production phase was estimated as follows: 10 mL of the production medium was collected and centrifuged at 9000 RPM for 20 min at 20 ^{o}C. The pellets were collected, dried at 45 ^{o}C for 24 h, and weighed. This includes the weight of the insoluble substrate as well.

The weight of the insoluble substrate was estimated as follows: 10 mL of the medium was collected and centrifuged at 9000 RPM for 20 min at 20 ^{o}C. The pellets were treated with 15 mL of an acid mixture solution containing in a 1:10 ratio (on a volume basis) nitric acid and acetic acid (80% v/v). The acid-treated mixture was boiled for 30 min in a water bath, centrifuged at 5000 RPM for 15 min, and the supernatant was discarded. The acid-treated pellets were dried overnight at 40 ^{o}C and weighed; this accounts for the weight of the insoluble substrate (in g). This value was subtracted from the sample weight of the mycelia+substrate to calculate the weight of the mycelia alone.

Partial-purification of the produced enzymes

The culture broth was centrifuged at 15,000 RPM for 20 min at 4 ^{o}C. The obtained supernatant was subjected to cross-flow filtration (Sartorius VivaflowR, 100 KDa cut-off, 0.2 W). A 12-fold concentration was performed, after which the enzyme was collected and stored at 4 ^{o}C.

Sequential saccharification using the produced amylolytic enzymes

Stage I: saccharification using α-amylase

An enzyme loading of 10 IU/g of α-amylase (10 IU per gram of the 1:1 rice mixture), 1:10 (w:v ratio) of citrate buffer, 0.25% (v/v) Tween 80, and 0.01% (w/v) of sodium azide were added to the saccharification reactor (CSTR – Scigenics 3L with programmable temperature ramping) operated at 73 ^{o}C for 6 h. The obtained hydrolysate was centrifuged at 6000 RPM for 15 minutes, and the supernatant was subjected to stage II of saccharification.

Stage II: saccharification using glucoamylase

The reaction conditions, buffer, and other reagent concentrations were the same as those used for the stage I of the saccharification process. A glucoamylase load of 6 IU/g was used (6 IU per gram of the rice mixture used for stage I saccharification). Centrifugation was performed, and the supernatant (hydrolysate) was collected and stored at 4 ^{o}C. The percentage of saccharification was calculated using equation 1, and the saccharification yield was calculated using equation 2.

Equation 1:

$$\text{Efficiency of saccharification} = \left\{\frac{(TRS\ in\ the\ saccharified\ hydrolysate\ (g)\ x\ 0.9)}{(Weight\ of\ the\ rice\ mixture\ (g))}\right\}\ x\ 100$$

0.9 is the factor for the conversion of polysaccharide to monosaccharides.

Equation 2:

$$\text{Yield of saccharification} = \left\{\frac{(Actual\ yield\ of\ reducing\ sugars\ (g)\ )}{(Theoretical\ yield\ of\ reducing\ sugars\ (g))}\right\}\ x\ 100$$

Fermentation for the production of bio-ethanol

Saccharomyces cerevisiae 3-VVS FB, with an ethanol tolerance of ~15% (v/v), was employed in the fermentation process. A concentrated form of the sequentially saccharified hydrolysate containing 15% (w/v) glucose was used. The media components include 0.5% (w/v) yeast extract and 0.3% (w/v) peptone. The 48-h-long fermentation was performed at a neutral pH, at 28 ^{o}C, using 10% (w/v) of the pelleted *Saccharomyces cerevisiae* (from a large volume primary culture). The reactor was started at 100% DO, and the aeration lines were cut to allow the formation of a micro-aerophilic environment after 24 h. The media was harvested after 48 h, centrifuged at 10,000 RPM for 20 min at 4 ^{o}C. The supernatant was subjected to distillation. Equation 3 was used to quantify the bio-conversion of starch to ethanol. Equation 4 was used to calculate the percentage yield of ethanol. Samples collected during fermentation and after distillation were analyzed using ^{1}H NMR spectroscopy (400 MHz Fourier-transform Nuclear Magnetic Resonance Spectroscopy, Bruker BioSpin, Avance – II, USA). The ^{1}H NMR spectroscopy library was used to assess the presence of various constituents of each of the sample mixtures. Prior to performing an ^{1}H NMR, the samples were concentrated to attain the ^{1}H NMR's threshold limit of analyte detection. The qualitative NMR spectroscopy was performed to ascertain the presence of various components of the analyte.

Equation 3:

$$\text{Starch bio-conversion} = \left\{\frac{(Bio-ethanol\ produced)}{0.51\ x\ (fraction\ of\ starch\ x\ weight\ of\ rice)}\right\}\ x\ 100$$

Equation 4:

$$\text{Yield of bio-ethanol} = \left\{\frac{(Bio-ethanol\ produced\ )}{(Amount\ of\ glucose\ consumed)}\right\}\ x\ 100$$

Results and Discussion

α-Amylase production, its impact on *Aspergillus oryzae's* growth kinetics and bio-process kinetics

The present section discusses the trend curves representing the impact of the subtrate concentration on the enzyme activity and the accumulation of fungal biomass within the reactor, as shown in Figure 3a. The 200 mL primary inoculum showed an α-amylase activity of 160 IU/mL, which was reduced to 32 IU/mL (due to dilution) during inoculation in the bio-reactor. As both the primary and the bio-reactor cultures used a similar carbon source, there was a significant reduction in the lag/adaptation phase of the fungus (Godbey 2014) (shown in Fig. 3a from 0 h – 24 h). The α-amylase-producing genes are signaled based on the carbon source that the mycelia contacts (Ramamoorthy et al. 2019b). AmyR, a $Zn(II)_2Cys_6$-type transcriptional factor, regulates the expression of amylase when it binds to the $CGGN_8CGG/CGGN_8AGG$ sequence of the promoter region and initiates the expression of the α-amylase genes (*amyA*, *amyB*, and *amyC*) (Tanaka et al. 2021). While Fig. 3b shows the combinatorial impact of the viscosity, fungal biomass accumulation, and agitation on the dissolved oxygen concentration of the broth, the Fig 3c shows the impact of the concentrations of glucose and maltose, and the broth viscosity on the enzyme activity. A decrease in dissolved oxygen concengration on the 6th day with a corresponding increase in the fungal biomas, glucose concentration and viscosity are found. The presence of 8.2 g/L of mature, enzyme-producing mycelia in the primary culture also diminishes the adaptation phase of the fungus. In an SMF, mature mycelia are reported to form within 20 h (Ma et al. 2013).

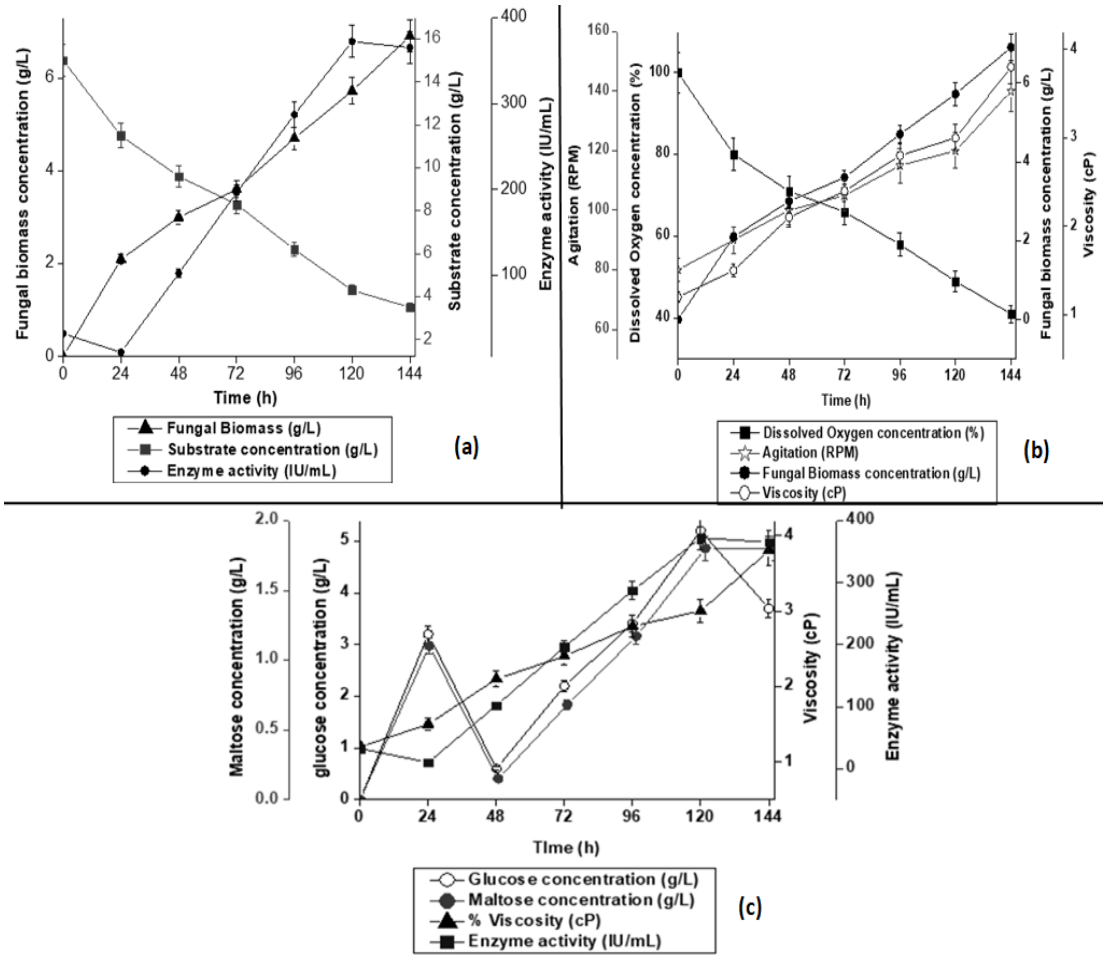

Fig. **3** – The variations of significant bio-process kinetics during the production of α-amylase using *Aspergillus oryzae* 113 ATCC [9102].

During 0 h–24 h, a 23% fall (15 g/L - 11.5 g/L) in the substrate concentration, 68% reduction (32 IU/mL to 10 IU/mL) in the enzyme activity (Fig. 3a), a reduction in dissolved oxygen concentration (Fig. 3b) and an increased glucose concentration of 3.2 g/L (Fig. 3c) were observed. This may have resulted from the α-amylase-catalyzed saccharification of the fractionated rice grain substrate mixture (Ramamoorthy et al. 2019a). Additionally, during this period, the fungus displayed a specific growth rate of the fungus at the rate of 0.08 g/h, a 1521-fold increase in its fungal biomass (1.4 mg/L to 2.1 g/L) (Fig. 3a), and $Y_{x/O2}$ of 0.49 g biomass/g O_2, implying the occurrence of an accelerated growth phase and rapid utilization of a portion of the glucose (Vrabl et al. 2019, Mäkelä et al. 2018). The fungus consumes the sugars from the broth in a sequential pattern, starting from the easily digestible-glucose, followed by switching over to the less-preferred sugars (fructose, maltose, and sucrose in this case) (Adnan et al. 2017). Despite the presence of another monosaccharide fructose, the fungus has been reported to show a significant affinity for glucose uptake because it (glucose) facilitates an improved nuclear-accumulation of the transcriptional regulator AmyR to favour α-amylase secretion (Murakoshi et al. 2012).

The Fig. 4a shows the variations in the fluid properties of the broth wherein an increase in viscosity corresponds to a decrease in the dissolved oxygen concentration, while Fig. 4b shows the impact of glucose accumulation in the broth on the specific growth rate, substrate uptake rate, and the resultant specific activity of the enzyme. A diminished specific α-amylase activity of 0.45 IU/mg (Fig. 4b) (ratio of the proportion of enzyme to the proportion of fungal biomass) was recorded at the end of 24 h. This probably was because of the absence of a nutrient limitation-induced stress to initiate the expression of the enzyme-producing genes; (Sánchez et al. 2010). Under nutrient-limiting

conditions (absence of glucose), a higher amount of cytoplasmic cAMP has been reported. The cAMP activates transcription as it binds to the promoter region of the DNA through the CRP (Sánchez et al. 2010), forming a CRP-cAMP complex. The presence of excess glucose may cause preferential glucose consumption followed by a possible carbon catabolite repression (CCR), wherein the glycosyl hydrolase genes required for the synthesis of the amylolytic enzymes are repressed and a transcription factor CreA/Cre1, a C_2 H_2-type transcription factor mediates CCR (Tanaka et al. 2018). Glucose gets phosphorylated as it is transported into the microbe's interior and hampers transcriptional activation of the required genes by causing deactivation of adenylate cyclase, reduction of the cAMPs, and a reduced formation of the CRP-cAMP complex, which is a critical step required to commence the transcription (Sánchez et al. 2010).

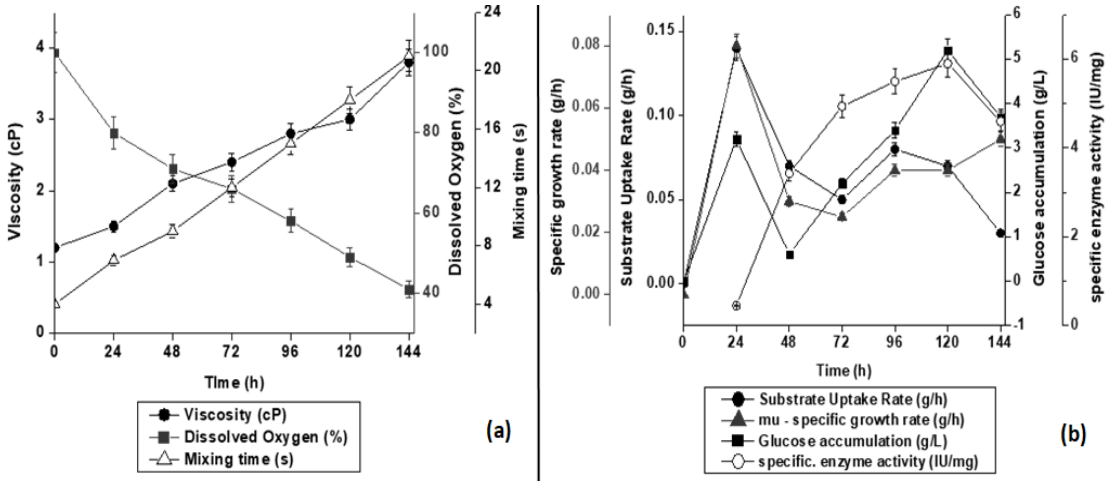

Fig. **4** – The variations of process kinetics during the production of α-amylase using *Aspergillus oryzae* 113 ATCC [9102].

During 24 h–72 h, the fungus displayed a marginal 1.7-fold increase in its biomass concentration (2.1 g/L to 3.6 g/L) (Fig. 3a) and a SUR of 0.05 g/h–0.07 g/h (Fig. 4b), as it may have been undergoing a stage of nutritional adaptation (from glucose to the rice grain-based substrate), thereby exhibiting a moderate diaxuie (Fig. 4b) (Teter et al. 2014). The rate of depletion of the DO concentration in the broth was slower (Fig 4a) (Yong et al. 2018). Furthermore, during this period, the pH of the production media fell to a low point in the range of 3.2– 3.5 pH, which further indicates that there was nutritional stress (non-availability of glucose), and the fungus may have started consuming the nitrogen source from the production media (Ramamoorthy et al. 2019a). In the time interval between 24–48 h, the glucose concentration reduced from 3.2 g/L to 0.6 g/L (Fig. 3c), and glucose, at this point of time, may have served as an inducer (Murakoshi et al. 2012) to accelerate the α-amylase production machinery of *A. oryzae*. Yuriko and his research group reported that glucose served as a good inducer in *Aspergillus* spp. at around concentrations of 0.05% (w/v) (Murakoshi et al. 2012). When glucose uptake occurs, AmyR, the transcriptional regulator, gets transferred to the nuclear region from the cytoplasm (Suzuki et al. 2015). As a result of the possible induction, there was a 19.6-fold increase in α-amylase activity (10 IU/mL to 196 IU/mL); additionally, a 1.6-fold increase in viscosity of the culture broth (1.5 cP to 2.4 cP) (Fig. 3c) was recorded at the end of 72 h, due to the accumulation of the extracellular enzyme (Blunt et al. 2019), and 11-fold increase in the specific enzyme activity (0.49 IU/mg to 4.94 IU/mg). This supports the fact that there could have existed an accelerated extracellular secretion of α-amylase during this 24 h–72 h period (Scargiali et al. 2013).

In the operational period between 72 h–120 h, there was a simultaneous increase in the fungal biomass concentration (3.2 g/L to 5.72 g/L) and the enzyme activity (196 IU/mL to 372 IU/mL) (Fig. 3a). Due to the possible simultaneous production of α-amylase and partial saccharification (by the α-

amylase) of the substrate mixture to release glucose (5.2 g/L) and maltose (1.8 g/L), there was a 1.25-fold increase in viscosity of the broth (2.4 cP to 3 cP) (Fig. 3c).

From 120 h–144 h of the bio-process, the fungal biomass reached a maximum concentration of 6.9 g/L (specific growth rate μ: 0.05 g/h), the glucose concentration reduced by 1.5-fold (5.2 g/L to 3.7 g/L), and the substrate uptake rate (SUR) reduced to 0.03 g/h (Fig. 4b). This indicates that enzyme production had stalled due to enhanced preferential glucose consumption and a possible CCR (Sánchez et al. 2010) initiated by the accumulated glucose (5.2 g/L) (Murakoshi et al. 2012). Consequentially, the α-amylase activity in the broth fell from 372 IU/mL to 365 IU/mL (Fig. 3c) as a portion of the produced enzyme may have catalyzed hydrolysis of the substrate mixture. Yuriko and his co-workers recorded that a 2% (w/v) of glucose concentration caused retardation of α-amylase synthesis in a species of *Aspergillus* (Murakoshi et al. 2012). A residual substrate quantity of 3.5 g/L was left unconsumed in the culture broth (Fig. 3a). Had the fermentation been prolonged (beyond 144 h), the unconsumed substrate (3.5 g/L), though may not have contributed to an increase in the enzyme activity, would have been partially hydrolyzed by the existing α-amylase in the broth, thereby resulting in a further decrease of the enzyme activity. In such enzyme-production processes, it has been reported that a considerable proportion of the enzymes (which are being produced) adsorb to the substrate (Ma et al. 2013) to catalyze enzymatic hydrolysis. This may result in the enzyme displaying a decreased enzyme activity (Ma et al. 2013), though its actual production could have been a few folds higher. Moreover, beyond 144 h, the media would not provide the culture with enough oxygen and nutrients as the high viscosity (3.8 cP) and the non-Newtonian fluid behavior of the culture broth (Cen et al. 2020) resulted in a prolonged mixing time of 21 s (Fig. 4a) (Sujan et al. 2018). A decreased K_La could also be attributed to an automated addition of 3 mL of the anti-foam solution to counteract severe foaming during this period. Though, by principle, anti-foam agents reduce excessive foaming, they are also detrimental to oxygen transfer (and hence K_La) as they break the number of air bubbles/foam and reduce the gaseous exchange, which the gas-entrapped bubbles may help in performing (Paciello et al. 2020). Furthermore, though the DO% and the rate of agitation (RPM) had been cascaded, an enormous concentration of the mature enzyme-producing mycelial biomass (close to 7 g/L) (Fig. 4a), growing on the internal components of the bio-reactor (Ramamoorthy et al. 2019b), may break as the impeller tip speed reaches the maximum speed (11.6 cm/s) to supply the broth with appropriate oxygen levels (Yong et al. 2018).

Saccharification of the amylose-rich substrate commences with the release of maltose, which is later hydrolyzed to glucose by maltase, also produced simultaneously along with α-amylase by *Aspergillus oryzae* (Visnapuu et al. 2019). The average m_{sx} was 0.02 g biomass/g substrate/h; the average m_{O2x} was 0.06 g biomass/g substrate/h, and the average $Y_{x/s}$ was 0.46 g biomass/g substrate.

At the end of 144 h, an α-amylase activity of 365 IU/mL, a specific enzyme activity of 4.6 IU/mg, and a partially-purified enzyme activity (12-fold partial purification) of 3650 IU/mL were recorded. In a 95-h solid state co-culture of *Aspergillus* spp. and *Rhizopus* spp. using a *koji* substrate, a 573 U/g α-amylase activity was reported by Takefuji et al. (2016). Using *Aspergillus oryzae* and a starchy substrate in an SMF, 37 IU/mL of α-amylase has been produced (Shah et al. 2014). In a 96 h SSF, Sethi et al. (2016) reported an α-amylase activity of 19.19 IU/g using pearl millet and *Aspergillus terreus* NCFT 4269.10 (Sethi et al. 2016). Mikai et al. (2015) has recorded a 4.68 IU/mL of α-amylase using *Aspergillus oryzae* and *Rhizopus* spp. in a 120 h SMF.

Production of glucoamylase and analysis of the bio-process constraints

The present section discusses the trend curves representing the impact of the substrate concentration on the enzyme activity and the accumulation of fungal biomass within the reactor, as shown in Fig. 5a. The primary culture contained around 8.6 g/L of mature mycelia and 95 IU/mL glucoamylase, which was subsequently reduced to 19.2 IU/mL during bio-reactor inoculation (due to dilution). There was a negligible adaptation phase (shown in Fig. 5a) due to nutritional similarity (Godbey 2014). A decline in the substrate concentration (35 g/L to 25.7 g/L) (Fig. 5a) and enzyme activity (19.2 IU/mL to 5.3 IU/mL) were observed between 0 h–24 h, followed by glucose

accumulation of 3.7 g/L as a result of the substrate's partial saccharification by the residual enzyme from the starter culture (Murakoshi et al. 2012). The above-stated event resulted in a μ of 0.09 g/h, a 1,466-fold raise in the mycelia biomass concentration (1.5 mg/L to 2.2 g/L) (Fig. 5a), and an enhanced $Y_{x/O2.}$

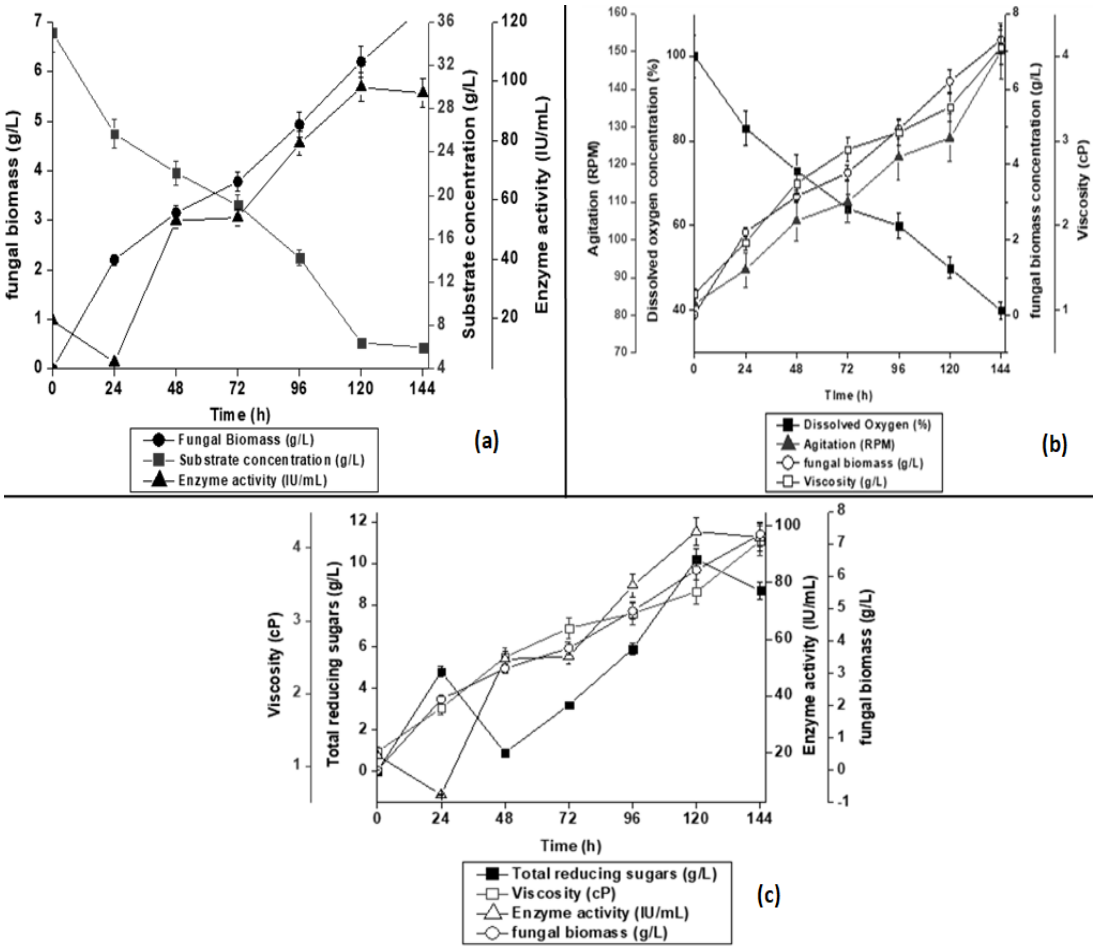

Fig. **5** – The variations of significant bio-process operational kinetics during the production of glucoamylase using *Aspergillus niger* A1143 NRRL3 [DSM 2466].

The Figure 5b shows the combinatorial impact of the viscosity, fungal biomass accumulation, and agitation on the dissolved oxygen concentration of the broth. An increase in fungal biomass and viscosity has shown a decrease in oxygen concentraton and the agitation is reducced. The Figure shows the impact of the concentrations of the total reducing sugars, fungal biomass, and broth viscosity on the enzyme activity. There was no fall in the enzyme activity on the 6th day.

The impact of glucose accumulation in the broth on the specific growth rate, substrate uptake rate, and the resultant specific activity of the enzyme is shown (Fig. 6a), while fig. 6b shows the variations in the fluid properties of the broth. A consequential higher growth rate increased oxygen consumption due to the presence of 3.7 g/L of glucose. Due to insufficient nutrient stress required to produce the target enzyme (Sánchez et al. 2010), a low specific enzyme activity of 0.23 IU/mg was recorded (Fig. 6a).

A 0.95-fold increase in the mycelial biomass (2.2 g/L to 3.2 g/L) (Fig. 5a) was observed between 24 h–72 h. The substrate uptake rate (SUR) was around 0.38 g/h–0.15 g/h (Fig. 6a) due to a possible nutritional adaptation phase (from glucose to the intended substrate) (Teter et al. 2014). A decreased specific growth rate (0.09 g/h to 0.03 g/h) was observed. Additionally, a 1.3-fold reduction in the pH was observed, which could be attributed to the consumption of nitrogen source before the fungus could start consuming the carbon source (Ramamoorthy et al. 2019a). During 48–72 h, there

was a marginal increase in enzyme activity (53 IU/mL to 54IU/mL), attributed to the presence of around 3.68 g/L of glucose, which was slightly higher than the required inducer concentration for *Aspergillus* spp. (Murakoshi et al. 2012).

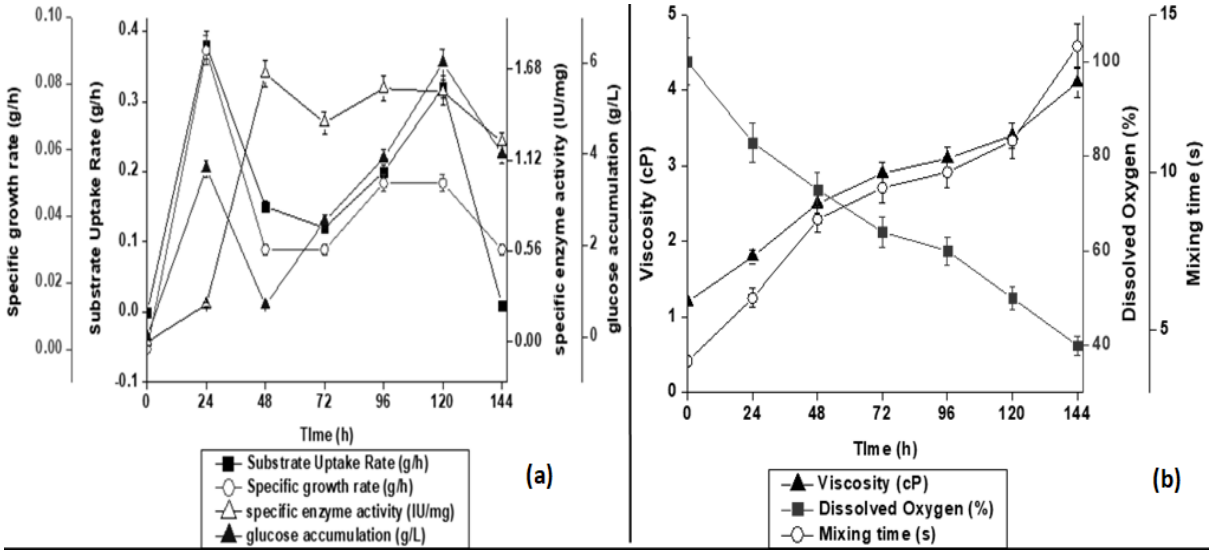

Fig. **6** – Variations of the bio**-**reactor**'**s operational kinetics during the production of glucoamylase using *Aspergillus niger* A1143 NRRL3 **[**DSM 2466**]**.

During the cultivation period between 72 h–120 h, there was a concurrent ascend of the glucoamylase activity (54 IU/mL to 98 IU/mL) and fungal biomass concentration (3.8 g/L to 6.2 g/L) (Fig. 5a). A partial hydrolysis of the substrate mixture could be attributed to the release of glucose (6 g/L), fructose (0.5 g/L), sucrose (2.5 g/L), and maltose (1.2 g/L). A 1.2-fold increase (2.9 cP – 3.4 cP) in the viscosity was observed (Fig. 6a).

From 120–144 h, the glucoamylase activity fell from 98 IU/mL to 96 IU/mL (Fig. 5c) as the glucose accumulation reached 6 g/L, resulting in catabolite repression (Sánchez et al. 2010). A 32-fold reduction in the substrate's uptake rate (SUR) (0.32 g/L to 0.01 g/L) was also observed (Fig. 5a), resulting in the non-consumption of 6 g/L of the substrate (Fig. 5a). At 144 h, the culture medium's viscosity rose to 4.1 cP, thereby lengthening the mixing time to 14 s (Fig. 6b). Due to high viscosity (Paciello et al. 2020) and a higher impeller tip speed of 12.5 cm/s (to facilitate DO% maintenance), a decreased K_La was observed.

Saccharification of the bottom fraction of the heat-treated rice grain would result in the release of sucrose, glucose, maltose, and fructose. Maltose and sucrose were saccharified by maltases and invertases, respectively, which are also secreted in minor quantities by *Aspergillus niger* (Visnapuu et al. 2019). Since the accumulation and consumption of fructose was minuscule in comparison to glucose, it is not considered in the present discussion. The average maintenance coefficient based on substrate uptake (m_{sx}) was 0.012 g/g/h, the average maintenance co-efficient based on oxygen uptake (m_{O2x}) was 0.13 g/g/h, and the average biomass yield coefficient based on the consumption of the substrate ($Y_{x/s}$) was 0.3 g/g.

At the end of 144 h, a glucoamylase activity of 96 IU/mL and a specific enzyme activity of 1.23 IU/mg were recorded (Fig. 5a). After a 15-fold partial purification, the resultant enzyme activity was 950 IU/mL. In a submerged co-culture of *Aspergillus* spp. and *Rhizopus* spp., a glucoamylase activity of 675 IU/mL has been recorded by Mikai et al. (2015). Using waste food and *Aspergillus awamori*, around 108 IU/mL of glucoamylase production has been reported (Kiran et al. 2014). In a 95-h solid-state co-culture of *Aspergillus oryzae* and *Rhizopus oryzae* in steamed rice *koji*, glucoamylase production of around 180 IU/g has been reported (Takefuji et al. 2016).

The monosaccharides in the media have to be kept low to ensure a sustained production of enzymes. In one of our previous works (Charles et al. 2018), to enhance cellulase production by

counteracting catabolite repression, a 5% (w/v) of a strain of baker's yeast was co-cultured along with the enzyme-producing fungus. A 1.045-fold enhancement in enzyme production was observed.

Factors influencing the dual-stage sequential saccharification of the rice-based substrate mixture

The complementing enzymes, α-amylase and glucoamylase (Xian et al. 2015), were used sequentially to saccharify the substrate mixture in two stages. In the 6-h-long first stage, an α-amylase loading of 10 IU/g (of the substrate) was used, while for the 6-h-long stage II, a glucoamylase loading of 6 IU/g was used. At higher enzyme loading, though there was a marginal increase (0.3 % w/w) in the TRS release (Alias et al. 2021), further increase had no significance. A prolonged substrate exposure to α-amylase results in the formation of α-limit dextrins, which cannot be further hydrolyzed (Zhu 2015). Similarly, extended substrate exposure to glucoamylase tends to catalyze the formation of maltose and isomaltose from the produced glucose units, reducing glucose yield (Hii et al. 2012).

During enzymatic hydrolysis, a higher surface area of exposure facilitates complete/maximum enzyme-substrate interaction (Karimi & Taherzadeh 2016). Smaller particle sizes (as low as 5 μm to a few hundred μm) improve the rate of saccharification using α-amylase (Ramadoss et al. 2018). Additionally, due to pulverization, a homogenous granular-gelatinization of the partially-crystalline substrate occurs at temperatures regimes of 60 ^{o}C – 75 ^{o}C (Lumdubwong 2019), which is due to the breakdown of hydrogen bonds in starch (Renzetti et al. 2021).

Table 1 sequentially presents the total quantity of sugars in the substrate mixture, and their variations during each of the saccharification stages and the bio-ethanol production stage.

Table 1 The quantities of total reducing sugars (TRS) estimated during each stage of the bio-ethanol production process.

Total Reducing Sugars (TRS) quantified during each stage of the bio-ethanol production process					
		Proximate analysis*			
1.	Process	Reducing sugar content (% w/w)			
		Glucose (%)	Fructose (%)	Total Reducing Sugar (%)	
	Estimation of the TRS in a 1:1 mixture of rice grain and cooked, left-over rice.	63.3	6.6	70	
Saccharification of the rice-based substrate mixture using the produced amylolytic enzymes					
2.	Process	Reducing Sugar (% w/w) released during enzymatic saccharification			
		Maltose (%)	Sucrose (%)	Glucose (%)	Fructose (%)	TRS (%)
	Stage I- Saccharification using **α-amylase**	10	1.5	0.5	0.2	12.2
	Stage II – Saccharification using **glucoamylase**	1.2	2.1	48.1	6	57.4
	Total Reducing Sugars (**%** w/w) released after the 2 subsequent stages of saccharification**:** 57.4**%**					
Bio-ethanol fermentation of the hydrolysate obtained after 2 stages of enzymatic saccharification					

Table 1 Continued.

Total Reducing Sugars (TRS) quantified during each stage of the bio-ethanol production process						
3.	Process	Reducing Sugar content (% w/v) during the course of bio-ethanol production				
		Maltose (%)	Sucrose (%)	Glucose (%)	Fructose (%)	TRS (%)
	Bio-ethanol fermentation – (sugar content at the **start** of the fermentation)	0.4	0.65	14.9	2.7	18.6
	Bio-ethanol fermentation – (sugar content at the **end** of the fermentation)	0.22	0.41	2.6	0.2	3.43
Bio-ethanol (%w/v) produced during fermentation – 5.92%						

The proximate analysis was performed by hydrolyzing the 1:1 rice mixture using a 72% (v/v) sulphuric acid solution.

In the stage I of saccharification using α-amylase, a TRS release of 12.2% (w/w) was recorded. The saccharification hydrolysate showed (in w/w) 10% maltose, 0.5% glucose, 1.5% sucrose, and 0.2% fructose (Table 1). Apart from the action of the mechanical treatment (powder milling) or the temperature-based (73 ^{o}C) hydrolysis of the substrate (Kong et al. 2018), the release of other sugars could be attributed to the α-amylase-catalyzed partial hydrolysis of amylopectin (Vigneswaran et al. 2014). A saccharification percentage of 11% and a percentage yield of saccharification of 17.42% were recorded.

In stage II of saccharification, there is maximum glucose release as glucoamylase hydrolyzes the α-1,4 glycosidic linkages (starting from the non-reducing end of the polysaccharide) and the α-1,6 glycosidic linkages (Livesey 2014) in the saccharified hydrolysate obtained from stage I. The stage II hydrolysate resulted in the release (in w/w) of 1.2% maltose, 48.1% glucose, 6% fructose, and 2.1% sucrose (Table 1). A percentage of saccharification of 51.66% and a percentage yield of saccharification of 82% were observed after the completion of the two stages of saccharification. In a similar work, a 4% (w/v) glucose release has been reported using an in-house α-amylase and amyloglucosidase (Pervez et al. 2014). Figure 7 shows the ^{1}H NMR spectra recorded for the two stages of enzymatic saccharification.

The saccharification sample from stage I showed the presence of (in **%**w/w basis)**:** Maltose **-** 10; Sucrose **-** 1.5; Glucose **-** 0.5; Fructose **-** 0.2. The saccharification sample from stage II showed the presence of (in **%**w/w basis)**:** Maltose **-** 1.2; Sucrose **-** 2.1; Glucose **-** 48.1; Fructose **–** 6.

Bio-ethanol fermentation: The process challenges, operational and growth kinetics

A concentrated, saccharified hydrolysate containing 18.6% (w/v) TRS (of which 15% (w/v) was glucose) was fermented to 5.92% (w/v) (59.2 g/L) bio-ethanol (Fig 8a, b) using a 10% (w/v) *S. cerevisiae* culture in a 36–48 h-long micro-aerophilic bio-process (Table 1). The fermentation sample showed the presence of (in **%**w/v basis)**:** glucose **-** 2.6; Maltose **-** 0.2; Sucrose **-** 0.4; Fructose **-** 0.2; bio-Ethanol **-** 5.92. Fig. 8a shows a ^{1}H NMR spectrum of an on-going fermentation for ethanol production. Fig 8b shows a ^{1}H NMR sample of the bio-ethanol obtained after distillation from the culture broth.

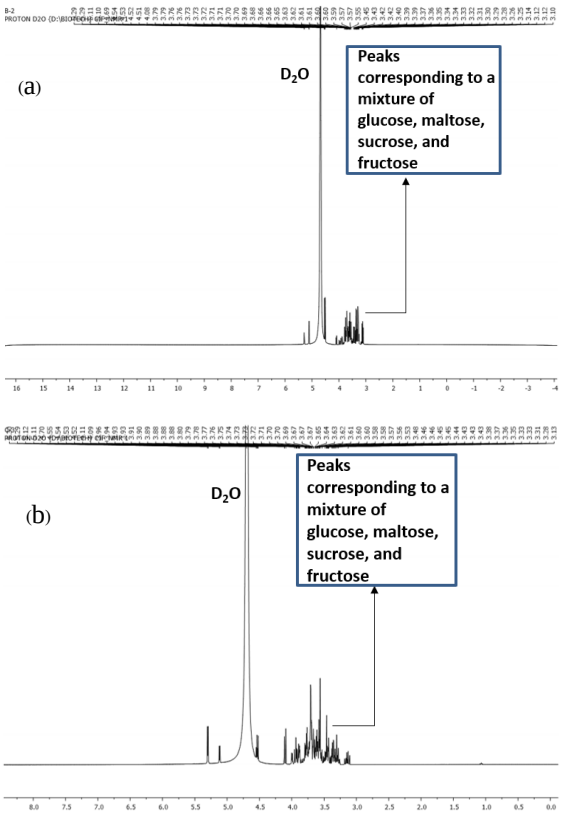

Fig. **7** – ^{1}H NMR spectra of the hydrolysates from. a Stage I and. b Stage II saccharification processes.

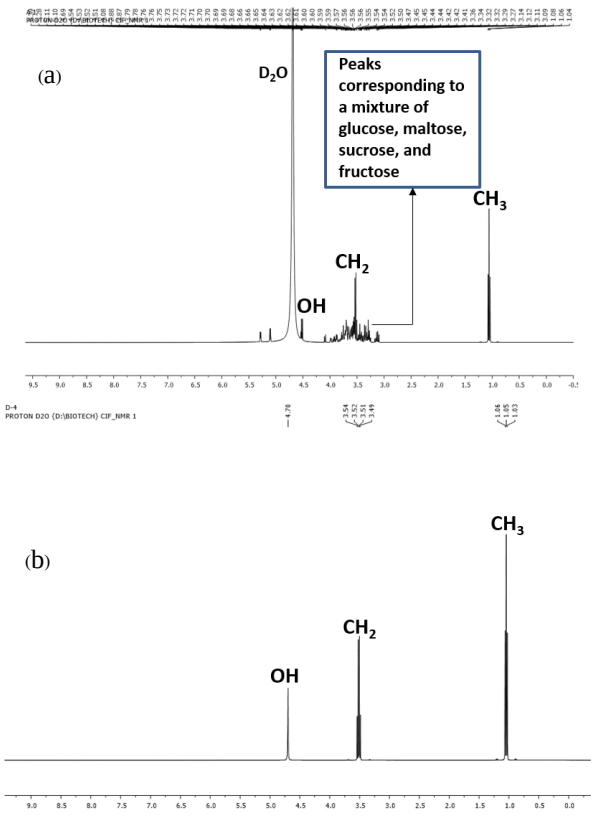

Fig. **8** – Analysis of the produced bio-ethanol using ^{1}H NMR spectroscopy. a at the end of the fermentation. b after distillation.

A higher concentration of sugars (25% (v/v)) may prolong the culture's lag phase and impart a higher osmotic stress to the cells (Silva et al. 2013). The process commenced with 100% dissolved oxygen (DO) due to accelerated biomass increase, reaching 0%, at around 36 h of the fermentation (Fig. 9). Fig. 9a shows the impacts of sugar concentration, vicosity, and fungal biomass concentration on the titers of ethanol. Fig. 9b shows the impacts of fungal biomass and substrate uptake rate on the production of ethanol while Fig. 9c shows the variation of the mixing time in response to the combined effects of the concentrations of the fungal biomass and total reducing sugar, and viscosity.

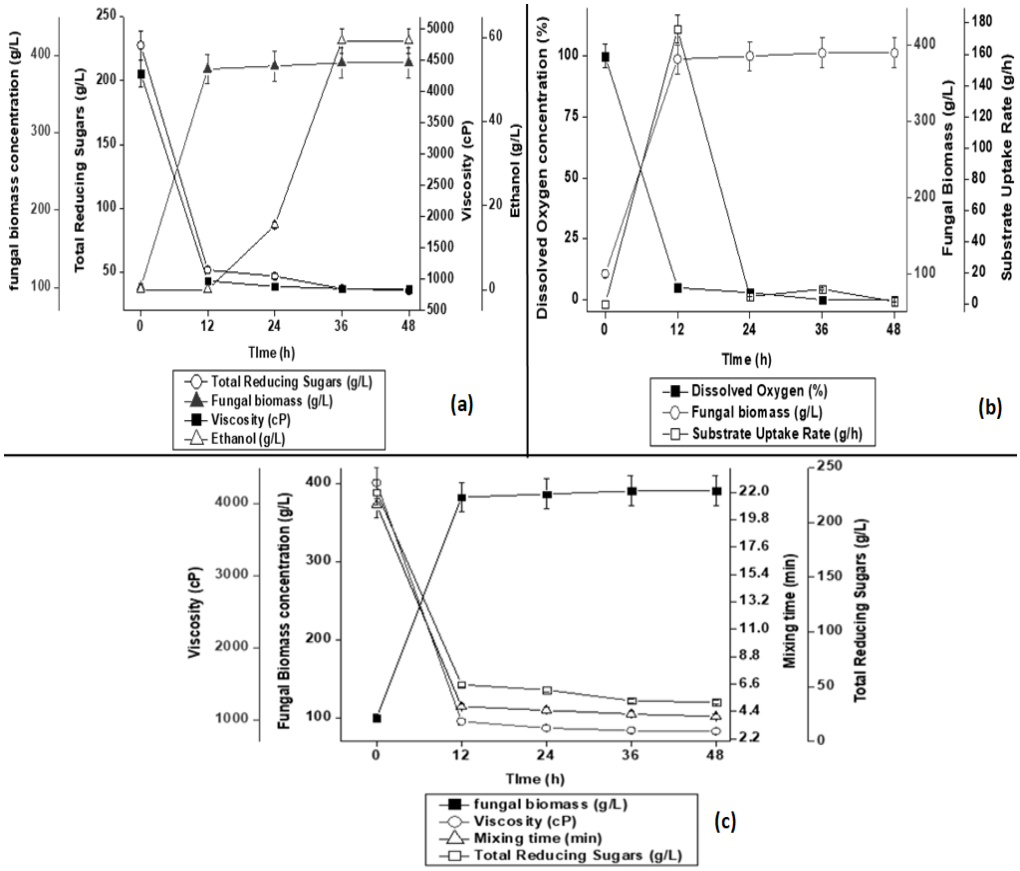

Fig. **9** – The variations in bioprocess kinetics during the production of bio-ethanol using *Saccharomyces cerevisiae*.

At the start of the fermentation, high volumes of inoculum and higher sugar concentrations resulted in a high broth viscosity of 4285 cP and a mixing time of 21 min (Fig. 9c). From 0–12 h, there was a 3.82-fold increase in the yeast biomass (100 g/L to 382 g/L), a 4.39-fold decrease in the TRS (227.5 g/L to 51.8 g/L), and a 4.4-fold reduction in the viscosity (4285 cPto 976 cP) of the media (Fig. 9a). Specifically, a 4.4-fold reduction in glucose concentration and an accelerated DO% decrease (Fig. 9b) implied that glucose had diffused into the flocs and the yeast cells by rapidly multiplying. In an oxygen-rich environment, the yeast cells are viable and multiplying, but the ethanol production phase is absent/inconspicuous (Slavov et al. 2014).

From 12–24 h, a 1-fold increase in the biomass (382 g/L to 386 g/L), a 1.1-fold reduction in the TRS (51.8 g/L to 46.9 g/L), a 1-fold reduction in the viscosity (976 cP to 885 cP), and ethanol accumulation of 15.3 g/L were observed (Fig. 9). The TRS from the fermentation media may have penetrated the yeast cells and could have begun undergoing fermentation to produce ethanol and CO_2, which would have later diffused into the broth (Walker et al. 2016). Additionally, the yeast's cell wall-localized invertases (Margetić et al. 2017) and maltases (Visnapuu et al. 2019) may have initiated the hydrolysis of sucrose and maltose, respectively. The resultant monosaccharides would be fermented to ethanol and diffused into media (Walker et al. 2016). Furthermore, the reaction rates are adversely impacted due to the diffusional and mass transfer limitations imposed by the highly

dense flocculated system (386 g/L) (Soares 2011). Yeast flocs build up by a bridging, facilitated by an ion (such as Mg^{2+}, Ca^{2+}/K^{+}), as the cell wall proteins and the cell walls of neighbouring cells interact (Soares 2011). The use of a polymer-based additive may decrease the rate of bridging (Bridle et al. 2014).

During 24 h–36 h, an ethanol concentration of 59.2 g/L, a 1.26-fold decrease in the TRS, an inconspicuous increase in the biomass along with reduced viscosity of 850 cP, and mixing time of 4.2 min were observed (Fig. 9a). The ethanol yield was 0.48 g ethanol/g glucose, the starch-ethanol conversion was 63.3%, and the yield percentage of ethanol was 48.1%.

From 36 h–48 h, a 0.6 g/L decrease in maltose and a 1 g/L decrease in sucrose were observed. Usually, in a mixed sugar-containing production media such as this, glucose is consumed first, followed by the consumption of maltose (Graham 2019). It could be interpreted as a mild inhibition/repression of the uptake of other sugars by glucose (Graham 2019). Prior to consumption of the second sugar, a stage of adaptation results in a diauxic growth pattern (Chu et al. 2016). However, in the present case, the glucose concentration had not reached complete exhaustion, which could signify the fact that the decrease in the quantities of sucrose and maltose could have been because of the uptake and hydrolysis by the cell wall-localized invertases (Margetić et al. 2017) and maltases (Visnapuu et al. 2019).

During 24 h–36 h, the DO% reached 0 (Fig. 9b). In such an anaerobic/micro-aerophilic environment, the accumulated ethanol permeates into yeast cells, denatures cellular proteins, hydrophobically inhibits glycolysis, and disintegrates the cell membrane's phospholipid lining (Ferraz et al. 2021). Additionally, the substrate and oxygen limitation may result in cell death (Pauline 2013). Endogenous and exogenous substrate uptake may be limited by diffusional limitations (Soares 2011). In a 36-h-long process, around 59.2 g/L bio-ethanol production has been recorded in this work. Using *Saccharomyces cerevisiae* and raw starch hydrolysate, around 57 g/L of ethanol production has been reported in a 36-h process (Xu et al. 2016). In a single-step fermentation employing *S. cerevisiase* and raw cassava starch hydrolysate, 81.8 g/L ethanol yield has been reported (Krajang et al. 2021).

Fundamental techno-economic analysis of the integrated-sequential production of α-amylase, glucoamylase, and bio-ethanol

Table 2 shows a fundamental techno-economic analysis of various stages recorded during the entire process of bio-ethanol production from rice-based substrates.

Table 2 A fundamental techno-economic analysis of the entire process for the production of α-amylase, glucoamylase, and bio-ethanol from rice grains and cooked, left-over rice.

Cost incurred during the production of α-amylase and glucoamylase							
S. No.	**Process**	**Operations**					**Cumulative cost**
1.	α-Amylase production	Pre-processing of the rice grains				Enzyme production	
		Heating*	Drying**	Milling***	Autoclaving	Bio-reactor operation	Partial purification of the enzyme#	$ 43.2
		$ 0.02	$ 1.3	$ 0.05	$ 0.35	$ 41.14	$ 0.08	
2.	Glucoamylase Production	Pre-processing of the rice grains				Enzyme production		
		Heating*	Drying**	Milling***	Autoclaving	Bio-reactor operation	Partial purification of the enzyme#	$ 42.9
		$ 0.02	$ 1.07	$ 0.05	$ 0.35	$ 41.14	$ 0.085	

Heating* – Rice grains were separated as fractions by subjecting them to temperature-regulated heating.
Drying** – The liquid fraction (supernatant from the heated sample), and the solid fraction (rice post the heat treatment and without water), were individually dried in a hot air oven at 50°C for 6 h and 4 h, respectively.
Milling***– The dried fractions were individually milled to a powder.

Table 2 Continued.

Pre-processing, enzymatic saccharification and production of bio-ethanol from the rice mixture					
S. No.	**Process**	**Operations**			**Cumulative cost**
1.	Pre-processing	Drying^{+}	Autoclaving	Milling^{++}	
		$ 1.28	$ 0.35	$ 0.10	$ 1.73
2.	Saccharification	Saccharification – I	Saccharification - II	Concentration of the saccharified hydrolysate	
		$ 1.71	$ 1.71	$ 0.74	$ 4.16
3.	Ethanol production	Fermentation		Distillation	
		$ 10.2		$ 0.5	$ 10.7

Drying+ – Rice grain and cooked, left-over rice samples, collected from various locations were washed using water and dried individually at 50oC for 6 h.
Milling++ – The dried, autoclaved rice grains + cooked, left-over rice samples were mixed in a 1:1 ratio and milled (to a powder) for 1 h.
Saccharification – I – α-Amylase, produced in-house, was used for the first stage of enzymatic saccharification of the rice mixture samples.
Saccharification – II – glucoamylase, produced in-house, was used for the second stage of enzymatic saccharification of the resultant hydrolysate obtained from Saccharification – I.

The Total cost incurred during the production of α-amylase and glucoamylase in two individual batch processes is $ 86.1. The total cost incurred in the production of bio-ethanol from the mixture of rice grain and left-over rice (1:1) is $ 16.6. The operational costs were recorded in Indian National Rupees (INR), and have been represented as United States Dollar (USD); corresponding date November 6, 2021.

During simultaneous pre-processing (as two batches), the costs of rice fractionation (Table 2) could be reduced by two-folds (from $3.44 to $ 1.74). Similarly, when drying, autoclaving, and powder milling are carried out together, a cumulative cost reduction of 3.3-fold could be anticipated. Of the produced α-amylase (3650 IU/mL) and glucoamylase (950 IU/mL), only 2% would be required for the saccharification process of 1 kg of the substrate. Considering the reduced enzyme loading rates (α-Amylase - 100 IU/kg; glucoamylase - 60 IU/kg) and a 50% recovery rate, around 1% of α-amylase and 3% of glucoamylase could be reused for 37 batches (1 kg each). Thus, a 1.03-fold reduction in the overall cost of enzyme and ethanol production could be surmised. On the whole, the valorization of waste into a valuable end-product with less expenditure of energy and more product yield (Maroušek et al. 2021) is a major salient feature of this "waste/rejected rice-ethanol" plant.

Conclusion

The study signifies that around 253.4 g/L of bio-ethanol could be consistently produced from a 1 kg mixture of rice grain and left-over cooked rice. The discussed analytical data elucidate the key process variables for scale-up. Variations of starch compositions among batches and lack of steadiness in the availability of surplus produce/waste rice are the shortcomings of the integrated bio-process. In the enzyme production phase, for lesser sugar accumulation, reduced viscosity, and to increase mass transfer, techniques such as a fed-batch or a repeated fed-batch, or a nutristat could be attempted. During sequential saccharification, a rigorously-standardized substrate's exposure time to α-amylase and glucoamylase could reduce the formation of the detrimental compounds, α-limit dextrins and isomaltose, respectively. Innovations, such as the employment of native/genetically-engineered fermenting/co-fermenting microbes with increased ethanol tolerance, timed-conversion rate-based pulse feeding of the substrate, the continuous recovery of ethanol during its accumulation, re-usability of inoculum and unutilized substrate from the reactor may contribute to boost the circular economy and bio-economy of the fermentation process. The study may interest alternate fuel researchers, bio-process engineers, and mycologists working on industrial enzymes by providing key

insights pertaining to growth kinetics of amylolytic fungi and ethanol-fermenting yeasts in real-time operations.

List of Abbreviations

μ (g/h): Specific growth rate of the fungus recorded during the bioprocess.
$Y_{x/s}$ - Biomass yield coefficient (g biomass/g substrate): Fungal biomass yield calculated on the basis of the consumption of substrate from the culture broth.
$Y_{x/O2}$ - Biomass yield coefficient (g biomass/g oxygen): Fungal biomass yield calculated on the basis of the consumption of the dissolved oxygen from the culture broth in the bio-reactor.
Enzyme activity (**IU/mL**): Enzyme activities recorded during the course of the bio-processes.
K_La (1/h): The mass transfer coefficient recorded in the bio-reactor during the enzyme production processes.
DO (%): The percentage of dissolved oxygen in the culture broth in the bio-reactor during the production of the enzymes.
SUR – Substrate Uptake Rate (g/h): Quantity of the substrate consumed per hour.

Acknowledgements

The authors would like to thank the Department of Biotechnology and the Department of Biochemistry and Molecular Biology, Pondicherry University, for providing the laboratory facilities. This work was funded by the Dr. D.S. Kothari post-doctoral fellowship scheme, and the first author is thankful to the agency for the support provided.

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