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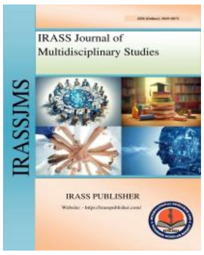

Evaluating the Potential of a Solitary Enzyme ‘α-amylase’ on Saccharification of Deteriorated Rice for Bio-Ethanol Production

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Abstract: Rice (Oryza sativa) is a vital staple food for over half of the global population, with Chhattisgarh, India, being a significant producer often referred to as the 'Rice Bowl' of the country. Despite its large-scale production, substantial quantities of rice become deteriorated and unfit for human consumption, resulting in considerable waste. This study investigates the conversion of deteriorated rice into bioethanol as a sustainable solution to address both environmental and economic challenges. The process involves starch liquefaction, enzymatic hydrolysis, fermentation, and distillation. This manuscript focuses on optimizing the use of α-amylase, a key enzyme for converting rice starch into fermentable sugars. Three varieties of deteriorated rice were evaluated, with DRS 2 showing the highest starch concentration of 73.61%. Optimal hydrolysis conditions were determined to be 8% α-amylase concentration, pH 6.5, and 90°C, which yielded 79.78% reducing sugars. Subsequent fermentation with Saccharomyces cerevisiae and a two-stage distillation process produced an ethanol yield of 22.8 grams per 100 grams of deteriorated rice. This study demonstrates that utilizing deteriorated rice for bioethanol production is a viable, cost-effective, and environmentally friendly alternative to fossil fuels. It offers a means to reduce waste, lower greenhouse gas emissions, and potentially boost local economies by providing a renewable energy source.

Keywords: Bioethanol, Single enzyme hydrolysis, Renewable energy, Waste reduction, Environment sustainability, Deteriorated rice feedstock, Saccharification, Starch Liquefaction.

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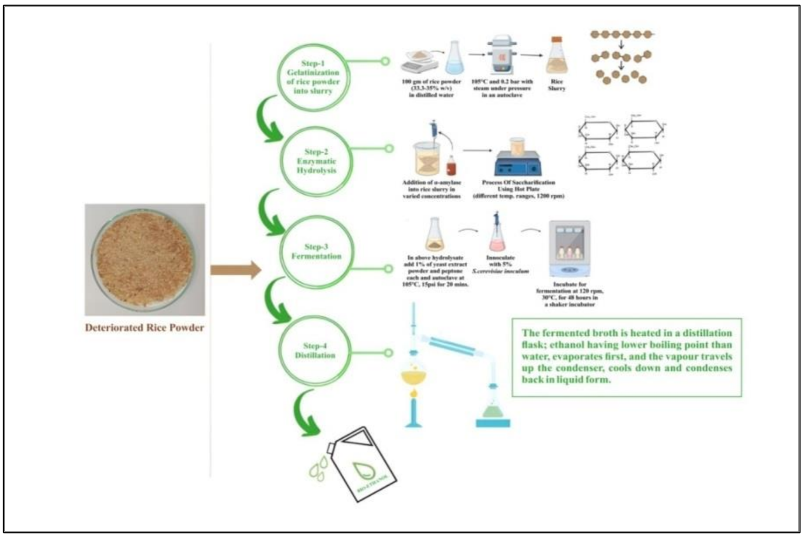
Graphical Abstract-



1. Introduction

Rice *(Oryza Sativa)* is known to be a staple food of more than half of the global population, among which 90% are Asians[1]. Chhattisgarh is a significant rice-producing state in India, often referred to as the 'Rice Bowl' of the country. The state has inevitably maintained the rice production levels. Recently collected data from Knoema World Data Atlas shows that after substantial fluctuations due to various causes. In 2020, 6.5 million tons of rice was effectively produced in Chhattisgarh[2].While exact figures for annual rice consumption in the state are not readily available, significant quantities of rice grain are wasted due to multiple reasons. A large amount of rice grains are inadequately kept open under tarpaulin sheets making the grains vulnerable to moisture and fungal infections resulting in spoilage and degradation in quality hence unfit for human consumption[3].According to the Food Corporation of India (FCI) major reason behind the degradation in rice grain is due to non-appropriate storage environment which then leads to the discarding of unusable portions and increasing waste adversely affecting the environment[4], [5]. Other factors causing the devaluation of rice are overuse and misuse of chemical fertilizers and pesticides harming the crop and affecting its nutrient value[5].An increase in temperatures causes sterility in rice plants decreasing both yield and quality, changing weather and improper harvesting i.e. too late or early harvesting severely decreases the grain quality.

Rice mostly contains carbohydrates mainly starch (~75-80%)consisting of amylose & amylopectin, moisture about 12%, and ~7% of proteins[6].This composition of rice gets altered in the deteriorated grains making it unfit for human consumption. It is disposed as waste in landfills causing harm to the environment, a major cause of greenhouse gas emission. Scientists all around the globe are working towards the valorisation of waste into bio energy to preserve the environment and turn waste biomass into useful resources. Waste grain can be effectively used as a rich substrate for the production of bio-fuels proving to be an excellent substitute for fossil fuels[7]. Utilizing waste grain to produce biofuels like bioethanol has both financial and environmental benefits, as bioethanol has the potential to significantly strengthen the local economy.

Ethanol can be made from any substrate containing polysaccharides such as starches and sugars[8], Rice is a rich source of starch and is produced abundantly in Chhattisgarh, therefore this study especially focuses on the production of Bio-ethanol from deteriorated rice which is stated as unfit for consumption by living beings and is discarded as waste. The bioconversion of rice into ethanol is also known as first generation Bio-ethanol production which consists of major steps like liquefaction of starch, breakdown of starch into fermentable sugars by enzymatic hydrolysis, fermentation of sugars into ethanol, and then separation of ethanol from the production medium by the process of distillation[9] Each of these steps plays an important role in obtaining the maximum ethanol yield [10], [11]. Among all the steps of conversion of rice into ethanol, conversion of starch into simple sugars by hydrolysis is a challenging one as it determines the final product's yield. This process usually involves the use of a cocktail of enzymes which are usually expensive[11], [12], [13] however, the present research work intends to assess the potential of a single enzyme, α-amylase for the efficient breakdown of starch. The current study aims to develop a streamlined and cost-effective production process for conversion of starchy grain into ethanol.

α-Amylase is the most well-known type of endo-amylase, which primarily breaks the α-1,4 glycosidic bonds found in amylose and amylopectin of starch, converting it into oligo-dextrins and helps to liquefy starch during the production of bioethanol[14]. Because of recent technological developments, various strains of genetically modified microorganisms are being used to produce thermostable forms of amylases, which can be more useful for an efficient conversion of starch into simpler sugars. Therefore, in this study varied concentrations of α-amylase were utilized for optimizing the production conditions to analyse the efficacy of α-amylase to use it as a solitary enzyme for bioethanol production specifically from rice.

2. Materials & Methods

2.1 Microorganism

A pure culture of *S. cerevisiae* ATCC 9763 was maintained on YPD agar slants containing 1% yeast extract, 2% peptone and dextrose each and 15% of agar for short term storage. The subculture of the yeast species was performed monthly and yeast glycerol stocks having the same media composition with 30% (v/v) of sterile glycerol were stored at 4ºC for long-term storage of pure culture.

2.2 Rice Samples Collection & Preparation

Three varieties of deteriorated rice samples (DRS) were collected from different areas of Chhattisgarh, India which were unfit for human consumption due to rotting and other quality issues. The selection of such rice samples ensures the utilization of discarded rice and repurposes it into a resource material. The collected samples were coarsely ground to the size of approximately 2.5mm, stored in a dry and cool place, and given tags as DRS1, DRS2, and DRS3.

2.3 α-amylase

Crude α-amylase (ECOENZYME-AAHT) was obtained from the Infinita Biotech Pvt. Ltd. Vadodara, Gujarat, India

2.4 Method

Selection of Suitable Sample

The selection of suitable sample of rice among the three varieties obtained, for efficient production of bio-ethanol was done by analyzing the presence of the highest starch content by estimating the total reducing sugar post acidic and enzymatic hydrolysis of all three samples by DNS method.

Acid Hydrolysis of Rice

The rice powder (5gm) was incubated in a water bath with 10ml of 1M HCl for 20 minutes and then centrifuged at 4000rpm for 10 minutes. The supernatant was collected in a 100ml volumetric flask pooled with distilled water and made up to the final volume. 1ml of diluted sample was then made to react with DNS reagent under specific conditions described by G.L. Miller[15] and total reducing sugars were estimated considering glucose as standard using Systronics UV-VIS Double Beam Spectrophotometer 2201.The amount of starch present in the rice samples was quantified as the product of total reducing sugars and a conversion factor of 0.9[16]

Enzymatic Hydrolysis with α-amylase-

A 100 gm of rice powder with 1:3 w/v concentration with water was first gelatinized to prepare a slurry under hydrothermal conditions of 105°C and 0.2 bar with steam under pressure in an autoclave for up to 60 minutes and then the rice slurry was loaded with a definite concentration of α-amylase (ECOENZYME-AAHT) according to the conditions formulated by Infinita Biotech Pvt. Ltd. (Table 1) for the process of Saccharification i.e. breakdown of soluble starch obtained by the Gelatinization process into simpler sugars using a Hot Plate. The enzymatic hydrolysis of starch was optimized for time, pH, temperature, and enzyme concentrations. In this study, A single enzyme, α-amylase, was employed in varying concentrations, ranging from 1% to 10% v/w. Using a single enzyme α-amylase in place of a conventional enzyme cocktail was a trial to assess its potential for efficient saccharification and to make this process cost-effective. For an enzyme to complete its activity, an optimum temperature is required and the obtained α-amylase was claimed to be thermo stable for which the temperature range from 80-120°C was checked in varied pH ranges of 4 to 7. All the trials were performed in triplicates.

Table 1 Formulated conditions for hydrolysis of starch utilizing ECOENZYME-AA-HT

Trade name of Enzyme	**Conditions for starch hydrolysis**					**Functions**
	Enzyme	**Miscibility**	**Working pH range**	**Temperature (°C)**	**Dose (%v/w)**	
ECOENZYME AA-HT	α-amylase	Miscible in water	4.5-6.5	80-110°C	25-30 DS%	Liquefaction and saccharification in fermentation

Inoculum Preparation and Fermentation-

After successful screening of a suitable sample and optimization of saccharification process parameters for fermentation of rice into bio-ethanol, a single colony of *S.cerevisiae* was plated into a YPD agar plate from the agar slants, and was incubated at 30ºC for 24 hrs. The next day a single colony of the yeast culture was selected and inoculated under aseptic conditions in 100ml of sterile YPD broth medium in a 250mL Erlenmeyer flask and then incubated at 30ºC overnight in a shaker incubator at 120 rpm, which was finally used as inoculum for fermentation. Prior to fermentation, 1% (w/v) peptone and Yeast extract each were added as nutrients to the hydrolysate for yeast cells to perform a maximum conversion of sugar into ethanol, which was then sterilized at 105°C for 20 minutes and then aseptically inoculated with 5% (v/v) of fresh broth culture and incubated at 30°C, 120 rpm for 48 hours for conversion of total sugars into ethanol.

Distillation-

The fermented broth was centrifuged to separate the yeast cells. Collected supernatant was undertaken into a two-stage distillation process to separate produced ethanol from other liquid components. The first distillation of the broth was performed at 80°C and then the Second distillation was done with the first distillate at 70-75°C to improve purity and enhance the overall quality of the final product ethanol. This two-stage distillation process plays an important role in separating the other by-products like butanol and oils from the liquid and improving the concentration of ethanol in the second stage of distillation enhancing the purity of the product.

2.5 Statistical analysis

All the data obtained by the experiments in this study were statistically analyzed using Origin Pro 2022 Software through the test of significance performed by analysis of variance (ANOVA). All the evaluation procedures in the study were performed in triplicates and were analyzed according to Tukey's test and compared at the 5% level of significant difference.

3. Results and Discussion

Selection of Rice Sample-

The most appropriate rice variety was selected after determining the initial starch content in the substrate, a necessary step for assessing the effective hydrolysis capacity of α-amylase[17]. Among the three varieties, the sample tagged as DRS 2 contained the highest i.e., 73.61% of starch (fig. 1). The amount of starch in the sample was almost the same as the results of Goel & Duan (2012), who studied the production of bioethanol from Indian broken rice containing 68.23% starch[18].Another study by K. Suresh et al. (1999) focused on producing ethanol through SSF using a mixture of sound and damaged rice grains containing 52-72.2% starch, supporting our findings[19]. Ketut et. al (2016) worked on production of bioethanol from liquid waste of rice flour and found 79% of starch content in rice while 30% in rice flour waste water supporting our results[20]. The previous work done by Frie et. al (2003) on the invitro starch digestibility and glycemic index of different varieties of rice and found 74.12% of starch content in whole grain rice strongly backing up our results [21]. Schneider et. al (2018)while studying the production of bioethanol from broken rice grains found 80±1.2% of starch content[22].

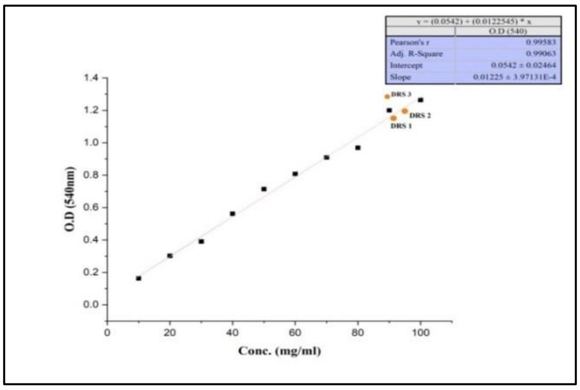


Figure 1 Glucose standard curve for reducing sugars

Optimization of Hydrolysis Conditions-

There are essentially three stages involved in the hydrolysis of starch. The first step in the process is called starch gelatinization, which is the hydrothermal breakdown of the hydrogen bonds between starch granules. After comes the liquefaction stage which is catalyzed by amylase enzyme that produces oligosaccharides which are further broken down into simpler sugars like glucose and maltose by the saccharification step. The proficiency of hydrolysis of starch is based on its quality. Starch containing high amount of amylopectin, makes the process of enzymatic hydrolysis easier and more efficient[23].The present study focuses on evaluating the potential of α-amylase in efficient conversion of starch into glucose, varied concentrations of the enzyme (1-10% v/w) were assessed. The maximum yield of 75.8% of reducing sugars was recorded at 8% v/w enzyme loading at 80℃, pH-6 for 2 h (fig. 2). In order to obtain high sugar concentrations in the hydrolysate it is important to optimize the reaction conditions. Therefore, variations in certain physiochemical parameters such as pH, reaction time of hydrolysis, temperature of hydrolysis were further studied.

A variation in concentration of hydrogen ion highly affects the sensitivity of an enzyme, thus care of pH is must to control during the hydrolysis process. Variations in pH influence the efficiency of an enzyme to perform maximum activity. In the current study, pH- 6.5 was recorded as optimum for the maximum conversion of starch into sugar yielding 79.04% of reducing sugar (fig. 3) The results of the current study are in correspondence with the work done by Schneider et. al (2018) who found pH-6 to be optimum for hydrolysis of broken rice using α-amylase for production of bioethanol [22]. Aggrawal et.al (2001) worked on process optimization for formation of sugar from starch and found hydrolysis activity of α-amylase to be optimum at pH-6.5-7, supporting our findings[16] Ma'As et. al (2020) while working on production of bioethanol from brewer's rice using α-amylase and glucoamylase found out pH 7.0±0.5 to be most effective for hydrolysis of starch with α-amylase, validating our results [24]An overview study done by Castro et. al (2011) on production and use of α-amylases in bioethanol production processes implied that bacterial amylases used for production of bioethanol from starchy feedstock are highly active at pH-6 for the process of starch hydrolysis, which aligns with our current findings[8]

A range of temperatures from 80℃-120℃ for efficient saccharification of starch by α-amylase were checked at optimum pH-6.5. The most suitable temperature noted in this study for maximum reducing sugar yield of 74.2% was at 90℃ whereas on increasing the temperature to 120℃, the activity of α-amylase severely decreased yielding 51% of reducing sugar (fig. 4). This experimental data justifies the formulated conditions of α-amylase by Infinita Biotech, providing a great energetic advantage unlike energy demanding steps in traditional processes of high heat gelatinization of starch. The current finding is in accordance with the work done by Kwiatkowski et. al (2005)on bioethanol process modeling by corn dry-grind process using thermostable α-amylase for hydrolysis of starch at 80-90℃[25]. Ma'As et. al (2020) while working on production of bioethanol from brewer's rice noted 80℃ to be optimum temperature for hydrolysis of starch with α-amylase [24]. Chuky et. al (2016) also noted 85℃ to be the optimum temperature for hydrolysis of starch in to reducing sugars [23]. The present study is justified by a review done by Assaf et. al (2024) which states that the optimal temperature ranges for starch hydrolysis are between 80 and 120°C depending on the maximum activity of α-amylase[26].The activity of α-amylase for maximum hydrolysis of starch under optimized pH and temperature was noted within the time intervals of 30 mins each starting from 0.5 hour to 3 hours. As a result, the maximum reducing sugar yield of 79.78% was recorded after 2 hours at 90℃ (fig. 5). Ketut et. al (2016) worked on bioethanol production from liquid waste of rice flour and noted 1 hour to be the optimum hydrolysis time [20].

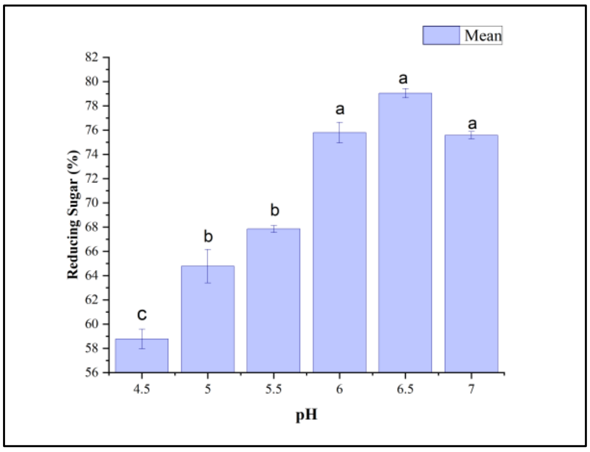


Figure 3 Optimization of hydrolysis pH

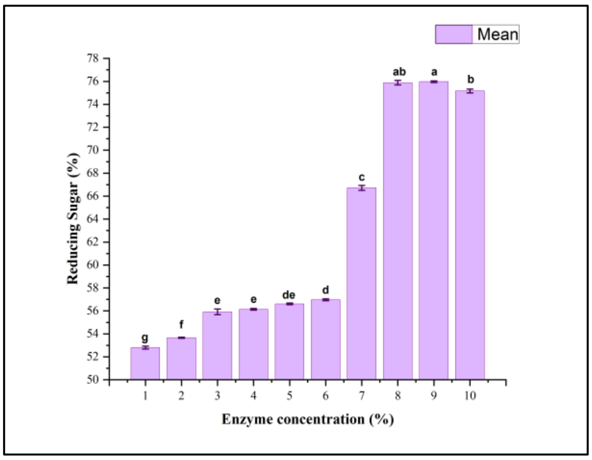


Figure 2 Variations in Enzyme Concentration

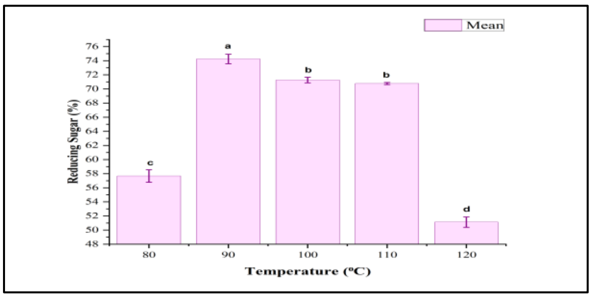


Figure 4 Variations in Temperature

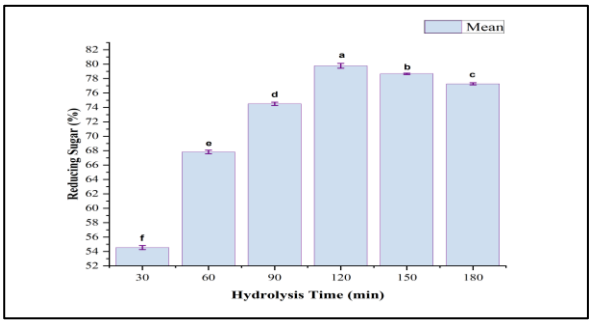


Figure 5 Variations in Hydrolysis Time

Means of each reducing sugar concentration within a bar followed by the same superscript letter do not differ significantly at 5% level by Tukey's Test.

Fermentation and Distillation

The final yield of ethanol after 48 hours of fermentation with 5%v/v of yeast inoculum was found to be 22.8grams of ethanol per 100 grams of deteriorated rice. 228g/L of ethanol was obtained by utilizing 736g/L of starch present in 1 kg of deteriorated rice The previous study of the scientists Gohel & Duan (2012) supports our work as it states that 100gm of starch should theoretically produce maximum 57.6 gm of ethanol[18]. Prior to this research, Chu-ky et al. (2015) noted an ethanol yield of 64.3g/l obtained from 150g/l of soluble starch in certain optimum conditions as 17% v/v of ethanol[23].

4. Conclusion

The continuous development of world economy is moving its attention towards the renewable resources of energy due to their finite supply, green house emission and global warming. All these drawbacks have drawn the attention of all the scientists working throughout the world towards the alternative, renewable and sustainable sources of energy known as biofuels specifically Bioethanol. Present study was undertaken with the aim of optimizing a process for the efficient saccharification of starch from deteriorated rice following a non- conventional approach by using only one enzyme α-amylase. Results add to the diversification in usage of cocktail enzymes, revealing the potential of a single enzyme α-amylase efficiently converting 79% of starch into fermentable sugars. By employing the above-described optimized parameters, a total 79.78% of reducing sugar yield from 736g/l of starch was obtained. Further after fermentation 228g/l of Bioethanol was produced, which is approximately 40%v/v of ethanol content. The findings also imply the sustainable use of waste rice as a raw material for Bioethanol production as even after being deteriorated 73% of starch content was recorded. In this way, the waste rice can be employed efficiently to produce biofuels and total waste generated can be reduced. Although processes to produce bioethanol from starchy feedstock have been used at large scale for decades, there is a continuous search for technological improvements leading to increases in yield as well as reductions in the costs associated to enzyme usage and to the final bioethanol production. A very balanced and intelligent combination of hydrolysis and fermentation process must be selected for maximum efficacy of the process. With the advent of synthetic hydrolyzing enzymes, other sophisticated technologies and their efficient combination, the process of bioethanol production employing deteriorated rice will prove to be a feasible technology in very near future. Thus, present study reveals the potential of converting those rotten grains into ethanol with added benefits of low capital investment/process, saving the operational cost, increasing fermentation efficiency by optimizing various production parameters and development of an environment friendly procedure.

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Author Contribution Statement

- **Sumit Sarkar:** Supervision, Resources and Funding.
- **Preeti Kaur:** Supervision, Sample analysis, Writing - Review & Editing.
- **Prachi Vaswani:** Methodology, Execution & Writing - Original Draft.
- **Purna Chandra Sahu:** Support in laboratory work.
- **Santosh Kumar Maitry:** Supervision & Review.

Disclosure Statement

The authors declare there is no conflict of interests.

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