BIOETHANOL PRODUCTION FROM RICE WATER WASTE: A LOW COST MOTOR FUEL

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Summary

Studies on bacterial amylase, especially, in the developing countries have concentrated mainly on *Bacillus* species, probably, because of the simple nature and nutritional requirements of these organisms. At present, bacterial amylases are vastly produced and used under extreme conditions of pH and temperature. The effect of temperature and pH on the activity of crude amylase of *Bacillus licheniformis* was examined and maximum activity was found at 60°c at pH 7.5. The enzyme showed an increase in activity with the addition of $CaCl_2$. Bioethanol produced using different methods of saccharification were analyzed and observed that acid treatment followed by enzyme mediated saccharification method was the most efficient one with highest bioethanol yield.

Keywords: amylase, *Bacillus licheniformis,* $CaCl_2$, saccharification

Introduction

Motor grade ethanol is the fastest growing market for ethanol production worldwide [1]. At present, the largest bioethanol producer at commercial scale is U.S. (895,000 barrels/day) using corn starch as substrate. However, corn is a major food crop in India meeting 89% of food demands in the country, and therefore may not be a suitable long-term solution. Starch in corn kernels is the current substrate for making bioethanol which is a costly part of the plant. This will increase the food prices in U.S and also in the developing countries in the nextcoming years if the current methodology of bioethanol production is continued [2]. As starch is ubiquitous and is an easily accessible source of energy [3], an alternative starch substrate that can be used is rice water waste; the left over liquid after cooking rice. It consists of vitamins such as niacin, riboflavin and thiamine, minerals such as calcium, magnesium and iron along with very high content of starch. Regarding the suitability of bioethanol production, starch content of rice water waste is the most important criteria, which depends on the quality of rice. Rice water waste obtained from low quality rice is a better substrate for bioethanol production than that obtained from high quality rice. Amylases have central role in bio-conversion of starch to bioethanol [4]. Rice water waste, in the form of liquefied gel, can be easily attacked by amylases, which has an advantage over corn, which is found in nature as insoluble, non dispersible crystalline granules resistant to enzymatic breakdown [4].

Keeping in view of above parameters, the present study was designed for cost-effective fermentation process of bioethanol production. It was conducted at lab scale starting with isolation and screening of bacteria, optimization of fermentation conditions and increasing the

thermostability of the enzyme. Further saccharification of rice water waste into fermentable sugars was studied using four different approaches of starch hydrolysis. Anaerobic break-down of hydrolyzed sugars into bioethanol employing immobilized yeast was studied and the results were compared. Rice water waste in the present study has been found to be easily broken down into fermentable sugars that can contribute to reduced cost of bio-ethanol.

Materials and methods

Isolation of Amylase Producing Bacteria:
Water samples were collected in sterile containers from Bangalore Sewage Water Board (BSWB). The pH of the water was measured, which was around 4.8. The collected water sample were suspended and serially diluted in sterile distilled water up to 10^{-4}, 0.1ml of each dilution were placed on sterile starch agar plates and was spread uniformly over the agar surface and incubated at 37°c for 18-24 hours [5]. The medium consisted of peptone 5.0g, yeast extract 1.5g, soluble starch 2.0g, Na_2HPO_4 4.0g, KH_2PO_4 2.0g, NaCl 0.5g, $MgSO_4$ 0.24 g, $CaCl_2$ 0.01g, agar 15g in distilled water 1000ml and pH was adjusted to 6.0 [6,7].

Screening and Selection of Amylase producing Bacteria:
The plates were stained with Gram′s iodine (0.01 M I2-KI solution), and scored for a clear halo surrounding the colony. The zones of hydrolysis were measured with scale. The organisms that formed the largest zone of hydrolysis was selected for the present study [8,9].

Identification of the strain:
Gram staining was performed to know whether the selected organism was Gram positive or negative. Morphology was observed under light microscope. The results obtained from various morphological and biochemical analysis was used to identify the organism *Bacillus licheniformis* [10].

Amylase assay:
The enzyme, α–Amylase (EC 3.2.1.1) is a bacterial thermo stable endo-amylase, which rapidly hydrolyze α-1,4-glycosidic bonds at random points in amylose and amylopectin of starch to reduce its viscosity but, cannot hydrolyze α-1,6-glycosidic bonds in amylopectin and thus, by pass these branch points [11,12]. The products of the reaction are glucose, dextrin and small amounts of maltose [11]. It has been reported that this enzyme requires calcium ions for its maximum activity and stability [13].

Production of Crude amylase:
The production of crude amylase was carried out in the same medium used for isolation without adding agar. A colony of culture from agar plate was inoculated into 50ml glass conical flask containing 10ml of production medium and incubated with 120rpm at 30°c for 18 hours [14,15]. This culture was then inoculated into 250ml flask containing 100ml of the same medium and incubated at 30 °c for 18 hours. 10ml of the medium was withdrawn and was centrifuged at 10,000 rpm for 15 mins at 4°c [16,7]. Cell-free supernatant was taken and was used as the source of crude amylase enzyme.

Measurement of Amylase activity:
Amylase activity was measured by DNS method [17]. Amount of reducing sugars liberated from 1.1% soluble starch solution solubilised in 50mM phosphate buffer, pH 7.2 (33) mixed with 0.5ml of enzyme was determined [8]. This mixture was incubated for 10 mins at 30°C for the enzyme substrate reaction to occur and the reaction was stopped by adding DNS solution. The treated samples were boiled for 10 mins, cooled in water for colour stabilization, and the optical density (OD) was measured at 540nm using spectrophotometer against a suitable blank. The amylase activity was determined using a calibration curve for glucose (standard glucose 1000µg/ml). One unit of enzyme activity was defined as the amount of enzyme that released 1 µmol of glucose per minute [18].

Effect of Temperature and pH on the Amylase Activity:
The influence of temperature and pH on the catalytic activity of amylases was determined by measuring the enzyme activity at temperatures ranging from 30°c to 80°c and varying pH values ranging from 6.0, 6.5, 7.0, 7.5 and 8.0 using phosphate buffer under the standard assay conditions as described above.

Effect of $CaCl_2$ on Amylase Activity and Stability:
The influence of $CaCl_2$ on catalytic activity and stability of amylase was determined by adding $CaCl_2$ (3.4ppm) at temperature ranging from 30°C to 90°C and the enzyme activity was measured under standard assay conditions as described above. $CaCl_2$ helps in maintaining the stability of the amylase [3].

Bioethanol production from saccharification and fermentation:
Bioethanol production involves two steps, firstly starch hydrolysis in an aerobic condition and fermentation by yeast in an anaerobic condition [17]. Starch hydrolysis can be achieved by acid hydrolysis or enzymatic hydrolysis [19].Yeast is a eukaryotic organism and can ferment saccharified starch into bioethanol and carbon dioxide [11].

Preparation of rice water:
A total of 1kg of low quality rice was obtained, mixed with 3L of water and cooked for 20mins. The excess water left out was collected, sterilization and used as substrate for bioethanol production.

Inoculum preparation:
Bacterial culture was inoculated onto same medium used for isolation. Colony of culture from agar plate was inoculated into 10ml of production medium and incubated with 120rpm at 30°c for 18 hours. This inoculation was extended to 100ml, 1000ml of the medium and incubated at 30°c for 18 hours.

Acid hydrolysis of rice water waste:
In acid hydrolysis, α-1,4-glycosidic linkage and α-1,6-α-glycosidic linkage are cleaved at about the same rate. The rate of hydrolysis of starch was increased by raising the temperature [19]. Acid hydrolysis of rice water waste was carried out by adding concentrated HCl to 500ml of fermentation medium. This was kept in water bath at 50°c for 90 mins [20,21]. The media was cooled and neutralized with KOH and 0.2% $Ca(OH)_2$ to increase the solubility and digestibility of starch media [22].

Enzymatic hydrolysis of rice water waste:
Enzymatic hydrolysis of rice water waste was carried out by adding crude amylase extracted from the 24 hour culture by centrifugation or by adding directly bacterial inoculum of along with 50mM phosphate buffer (pH 7.5) and 3.4ppm of $CaCl_2$ to 500ml of fermentation medium. The reaction mixture was incubated on a water bath rotary shaker at 60°C and120rpm.

Ethanol production at lab scale from rice water waste was studied following four different approaches of starch hydrolysis into fermentable sugars and their further breakdown into ethanol by *Saccharomyces cerevisiae* (baker's yeast) under anaerobic condition.

Enzyme mediated saccharification:
In this method, only enzymatic hydrolysis of the substrate was done and then the medium was incubated on a rotary shaker at 60°c for 18 hours.
Acid treatment + Enzyme mediated saccharification:
In this method, acid hydrolysis followed by enzymatic hydrolysis of the substrate was done and then the medium was incubated on a rotary shaker at 60°c for 18 hours.
Acid treatment +Bacteria mediated saccharification:
In this method, acid hydrolysis of the substrate was done and then bacterial inoculums (24hours culture) was added and the medium was incubated on a rotary shaker at 30°c for 18 hours.
Acid treatment + Enzyme + Bacteria mediated saccharification:
In this method, acid hydrolysis followed by the substrate was done and then enzyme and bacterial inoculum were added together and the medium was incubated on a rotary shaker at 30°c for 18 hours.

Calculation of theoretical yield:
To calculate the efficiency of the fermentation, the theoretical ethanol yield from starch is calculated [4]. From Gay-Lussac's equation the 1.11g glucose would theoretically yield 0.567g ethanol. Using the density of ethanol, we calculated the yield of 100% ethanol.
$C_6H_{12}O_6 \rightarrow 2C_6H_5OH + 2CO_2$

Medium for *Saccharomyces cerevisiae* culture:
Saccharomyces cerevisiae cells were grown aerobically on YPD (Yeast extract Peptone and Dextrose) medium at 30°c with continuous shaking at 250rpm for 48hours.

Medium of bioethanol production:
The medium used for bioethanol production was refined in the present study and it consisted of 500ml hydrolyzed rice water along with trace amount of $(NH_4)_2PO_4$ [7] and $ZnCl_2$ [23]. The reason behind adding these nutrients was to improve yeast growth for better bioethanol yield because yeast is the catalyst for bioethanol production and it produces bioethanol almost 33 fold faster during its growth phase than lag phase and stationary phase [24]. The pH of the medium was adjusted to 3.5 [25]. Potassium disulphite, an antiseptic, was added to the medium to prevent contamination with harmful microorganisms [5]. The medium was introduced in four different 1L capacity flasks.

Bioethanol production:
The flasks were then inoculated with immobilized cells of *Saccharomyces cerevisiae* entrapped in calcium alginate gel [26] to overcome the product inhibition [27] and for better substrate utilization [28]. The flasks were then incubated at 32°c under static anaerobic conditions. A CO_2 outlet was bent and inserted into the hole of rubber cork stopper and was immersed into a glass tube filled with $Ca(OH)_2$ solution. As fermentation proceeds CO_2 released reacted with $Ca(OH)_2$ solution and formed $CaCO_3$ precipitate [29]. This was done to remove the heat formed during fermentation so as to keep the fermentation temperature constant and also to keep observing the fermentation process taking place. $Ca(OH)_2$ also prevents O_2 to enter into the fermentation flask and this will prevent oxidation of bioethanol into acetic acid [29]. Bioethanol formed was estimated after 12, 24, 36 and 48hours of fermentation.

Bioethanol Assay by Potassium Dichromate ($K_2Cr_2O_7$) Method:
Alcohol standard was prepared by dissolving absolute ethanol in water to get 10mg/ml concentration. Different aliquots of standard ethanol (0.1-1 ml) were taken and volume was made up to 5ml with distilled water. Then 1ml of Potassium dichromate reagent (0.1g/ml) was added. All the test tubes were kept in ice water and 4 ml of conc. H_2SO_4 was added to each tube gently through the walls. The OD was measured at 660 nm and standard graph was plotted to obtain the concentrations of unknown samples [30].

Results and discussion

Sl. No.	Test	Result
1	Gram Staining	Gram Positive Rods
2	Triple Sugar Iron	Positive
3	Indole Test	Positive
4	Methyl Red Test	Positive
5	VP Test	Negative
6	Citrate Test	Positive
7	Starch Hydrolysis Test	Positive
8	Gelatin	Positive
9	Nitrate Reduction Test	Positive
10	H_2S Production Test	Positive
11	Oxidase Test	Positive
12	Catalase Test	Negative
13	NaCl	Positive

Table 1: Morphological and biochemical analysis done to identify the organism

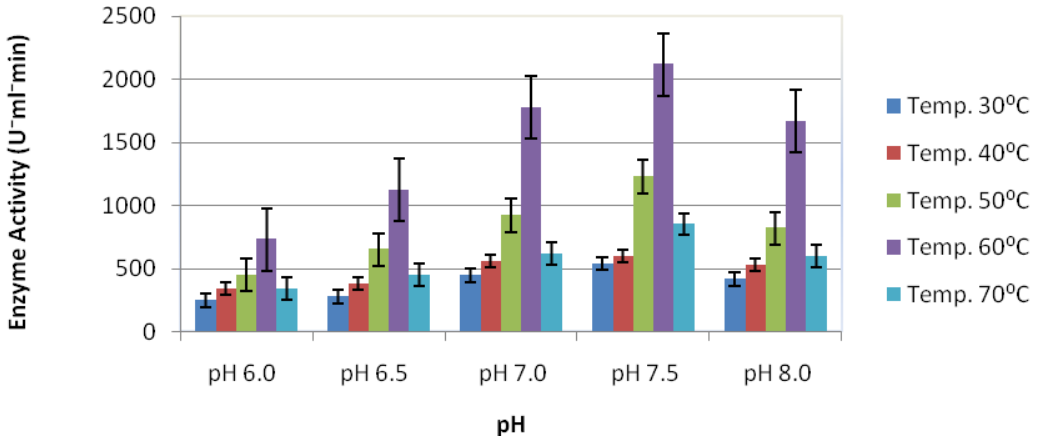

Figure 1: Effect of temperature and pH on amylase activity

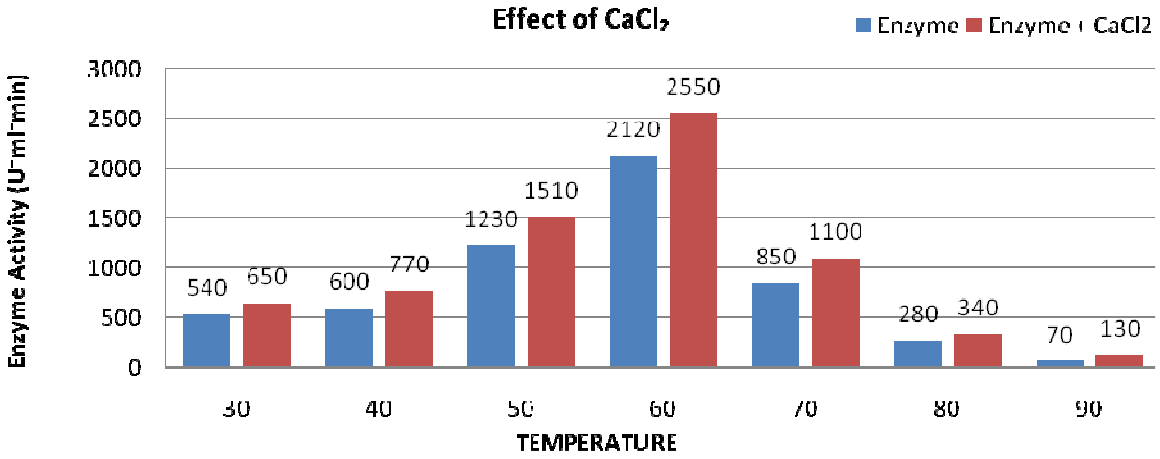

Figure 2: Effect of $CaCl_2$ on amylase activity

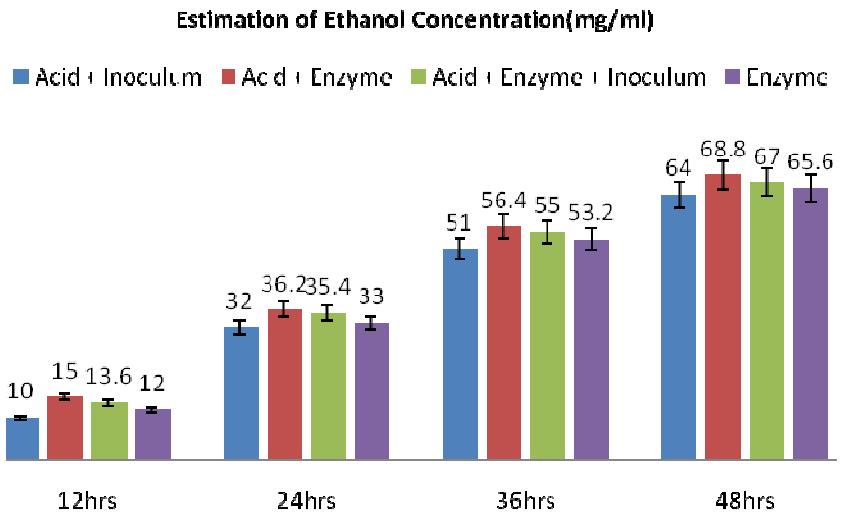

Figure 3: Bioethanol concentration obtained after 12hrs, 24hrs, 36hrs and 48hrs of fermentation with four different approaches.

The results of amylase activity obtained in this study showed that there is an appreciable high production, activity and stability of the enzyme optimized at higher temperatures and at slightly alkaline pH. The enzyme showed a good activity between temperature 50°c to 60°c and pH 6.5 to 7.5 with maximum activity at 60°c at pH 7.5. The results are shown in **figure 1.** The enzyme showed an increase in activity with the addition of $CaCl_2$, and maximum activity was obtained at 60°c and pH 7.5 as shown in **figure 2**. The theoretical yield of bioethanol obtained after saccharification were 98.54mg/ml, 95.12mg/ml, 96.42mg/ml, 93.28mg/ml for Acid + Enzyme mediated saccharification, Enzyme mediated saccharification, Acid + Enzyme + Bacteria mediated saccharification, Acid + Bacteria mediated saccharification respectively. The graph representing this statistics are presented in **figure 3**. It has been revealed in past literatures that optimum temperature and optimum pH range are the two most important physiological parameters for enzyme production by microbes [4,31]. It is also concluded from the present study that the enzyme activity and stability was significantly enhanced at higher temperature by adding $CaCl_2$ at a very low concentration. This suggests that *Bacillus licheniformis* can be a potential producer of extracellular thermostable amylase which could find applications in higher bioethanol yield.

The results of bioethanol production using different methods of saccharification were comparatively analyzed and it was observed that acid treatment followed by enzyme mediated saccharification method was the most efficient one with highest bioethanol yield (68.8mg/ml). Second highest ethanol yield was obtained with acid treatment followed by enzyme along with bacteria mediated saccharification method (67mg/ml). Third highest bioethanol yield was obtained with acid enzyme mediated saccharification method without any acid treatment (65.6mg/ml). The lowest bioethanol yield was obtained with acid treatment followed by bacteria mediated saccharification method (64mg/ml). Thus, we can conclude that it is least preferable to use bacterial inoculum during saccharification method. The reason behind this can be assumed that bacterial culture, added to the starch medium for saccharification, utilizes the hydrolyzed

products (reducing sugars) formed as a nutrient for their own growth, and this leads to lesser reducing sugars available for bioethanol production. Similar correlation was observed by R. SatishBabu et al. [31]. So, it is preferable to use the amylase extracted from the bacteria for the saccharification process of the starch media that was pretreated with acid.

Conclusion

A higher bioethanol yield obtained during this study is attributed to process refinement and the use of liquefied, easily hydrolyzed substrate – rice water waste. The results of this study indicated that rice water waste is a rich source of starch, which can be easily broken down into reducing sugars, and thus can be used to improve the process of bioethanol production. So, further studies are recommended on this substrate, as it is thrown as a waste. Therefore, in developing countries such as India, it can be used as an alternative substrate for bioethanol production.

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