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Production of Amylase by Potent Isolate Bacteria and Fungi in Submerged Fermentation

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Abstract: In the present study, isolation of potent amylase producing bacteria was screened using soil sample by serial dilution and inoculated onto starch agar plates to observe for clearance zone by addition of iodine solution. The isolates were subjected to various biochemical tests. The potent bacterial isolate I_4 was co-cultured with fungi *Trichoderma reesi* and as monoculture to assess the biochemical changes in production of α-amylase, reducing sugars, protein and ethanol at different incubation period and effect of carbon sources *viz*., sucrose and fructose using wheat bran as substrate in submerged fermentation.The production of α-amylase by potent isolate I_4 bacteria and fungi *Trichodermareesi* grown separately and co-cultured in liquid medium using wheat bran in whichamylase enzyme exhibited the maximum production after 24 hrs of incubation (4.5 , 6.9 and 10.1 IU/ml liquid) respectively. Higher amylase production was observed in Fructose (7.0 IU/mland 6.2 IU/ml) as carbon source for both bacteria and fungal as mono cultures and co-culture in wheat bran (9.2 IU/ml) at 48 hrs of incubation period. The maximum protein was 26 mg/ml by co cultured indicating the amylase productionon 72 hrs of fermentation using wheat bran in submerged fermentation and a slowly declined on 96hrs of incubation (20 mg/ ml) as compared with addition of sucrose inoculated with co culture organism produces 18.6 mg/ ml of protein during 48 hrs of incubation.There was an increase in production of ethanol (5mg/litre to 14.2 mg/litre) during 96 hrs of fermentation by co cultures inoculated in wheat bran submerged fermentation. In sucrose supplemented, ethanol amounted to 16.6 mg/litre in 48 hrs of incubation respectively.

Key words: Wheat Bran · Isolate Bacteria I_4 · *Trichodermareesi* · Submerged Fermentation

INTRODUCTION

Amylases constitute one of the important groups of enzymes that are used in a wide range of starch industries and account for nearly 25% of the total sale of enzymes. Although there are many microbial sources available for producing amylases, only a few such as *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus amyloliquifaciens* are recognised as commercial industry [1].

The utilization of wheat bran as solid substrate in submerged fermentation had a great advantage in its low cost could lead to large scale production of this could lead to large scale production of this enzyme for industrial use in starch liquefaction. There are a few reports in the literature on the use of fungi in pure or mixed cultures for bioconversion of starchy substrates into ethanol. For instance, the hydrolytic enzymes and *A. awamori*for ethanol production from cassava starch and obtained 10%

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ethanol in five d at 30°C [2]. Ethanol production from cassava starch by co-cultures of selected such as *Aspergillusoryzae* and *Rhizopusoryzae* under submerged fermentation [3]. Keeping these views, the present study was on bioconversion of wheat bran waste into more useful product amylase using potent isolated bacteria and fungi in submerged fermentation at different time intervals and supplemented with two different carbon sources.

MATERIALS AND METHODS

Wheat bran was procured from local market of Shengottai, Tenkasi (Dt.,) Tamil Nadu and used as substrate for amylase production in submerged fermentation.

Isolation of Amylase Producers: The collected soil sample was diluted by serial dilution technique. The diluted samples (0.1ml) at 10^{-5} to 10^{-7} were spread by spread plate technique on the starch agar plates. After incubation at 37°C for 24 hours, the plates were added with a pinch of iodine crystals. The colonies forming clear halo zones of hydrolysis were selected [4].

Characterization of the Isolates: The isolates were subjected to Gram's staining procedure and various biochemical tests *viz.*, indole test, methyl red test, catalase test, gelatine hydrolysis test and starch hydrolysis test based on the standard protocols described [4].

Fermentation Process: Submerged fermentation (SmF) was carried out by following the methodology defined by Varalakshmi *et al.* [5] with sight modifications using wheat bran as substrate respectively.

Cultures of *Trichoderma* were maintained by stock culture in Potato Dextrose Agar (PDA) slants. They were grown at 32°C ± 2°C for 24 hours and stored at 4°C for regular sub-culturing, 100ml of inoculum was prepared for each culture using czapek-dox broth in 250ml flasks. The inoculums was kept in shaker (200pm) at 37°C for 24hours, before it was used for the fermentation process.

Submerged fermentation was carried out in 250ml Erlenmeyer flasks containing 100ml of fermentation medium. The composition of the medium contained the following g/l of distilled water KH_2PO_4 - 0.2, $(NH_4)_2SO_4$ - 0.14, Urea - 0.03, $MgSO_4$ - 0.03, $CaCl_2$ - 0.03, $FeSO_4$ - 0.5, $MnSO_4$ - 0.16, wheat bran - 100gram. The medium was sterilized by autoclaving at 121°C for 15min. Each flask was inoculated with 1 ml of potent isolate bacteria (10^{-9} cells / ml) and fungal inoculum in separate flasks and also as mixed cultures. The cultures were incubated on a rotate shaker (120 rpm) at 32°C for different intervals (24 hours, 48 hours, 72 hours, 96 hours) [5].

Effect of Supplement Sources: Various carbon sources (Sucrose, Fructose) at a concentration of 1% w/v were supplemented as individual components to the fermentation medium containing wheat bran as substrate. The medium was autoclaved at 121?C for 15 to 20 mins and cooled. To that inoculate the 1ml of potent bacterial isolate (10-9cells / ml) and fungal culture to the carbon supplemented flasks as individually. The flasks were incubated at 30°C for 3 days in on rotary shaker (120 rpm). The broth was centrifuged and the enzyme assay was carried out.

The fermented broth inoculated with potent bacterial isolate and fungal culture in individual and mixed after different time intervals (24 hours, 48 hours, 72 hours, 96 hours) and carbon supplements (sucrose, fructose) after the 48hours of incubation were filtered with filter paper to remove mycelium / cell debris. The clear supernatants were analysed for change in pH, production of protein content, reducing sugars, amylase activity and ethanol content. The pH of culture broth was measured using a pH meter (Elico model) to investigate the effect for the growth of two strains [6].

Enzyme Assay: The amylase activity was assayed by measuring the reducing sugar liberated in the reaction mixture by the standard methodology [6].

Protein Assay: The protein proportioning was made according to Lowry *et al.* [7], the enzyme extract, add 5ml of 10% Tri Chloro acetic acid (TCA) and shake it well. Then centrifuge the solution at 2000 rpm for 5mins. Discard the supernatant, to the pellet add 5ml of 0.1N NaOH solution and shaked vigorously. To this one ml of protein extract, add 4 ml of Alkaline copper tartrate reagent, shake it well and kept it in dark for 5mins. Then add 0.5ml of Folin phenol ciocaltaechu reagent and blue colour was developed. The O.D was measured with spectrophotometer with 660 nm against the blank without protein extract.

Bio Ethanol: Fermented samples were regularly collected for quantitative estimation of ethanol by Potassium di chromate method [8].

About, 0.5ml of absolute alcohol was taken in different test tubes and the volume was made up to 5ml by adding distilled water in each test tube. 0.3ml of test

samples were taken and the volume was made up to 5ml by adding distilled water in test tube. One ml of Potassium dichromate reagent was added in each test tube. Then 2ml of NaOH solution was added in each test tube. The test tubes were incubated at 50?C for 30 minutes. The absorbance was measured at 600 nm using a Spectrophotometer.

Preparation of Reagents

Acid Dichromate Solution: 125ml of water was added to a 500ml conical flask. Then 325ml of concentrated sulphuric acid was carefully added. The flask was cooled under cold water tap and 34 grams of Potassium dichromate was added. Dilute 500ml with distilled water. 2M Sodium hydroxide solution: Add 40grams and NaOH in 100ml of distilled water.

Determination of Total Soluble Carbohydrates: Total soluble carbohydrates were determined by the method [9]. The reaction mixture contained 25ml of a 4:1 mixture of phenol and water 0.8ml of crude extract and 2ml of concentrated Sulphuric acid. Then mixed well and heated in a boiling water bath for 30 mins. The absorbance was determined at 480nm. Glucose served as the calibration standard for total carbohydrate determination.

Determination of Total Reducing Sugars: Total reducing sugars were determined by the method [6] the reaction mixture contained 0.5ml of crude extract and 0.5ml of Dinitro salicylic acid reagent (DNS). The tubes were heated in a boiling water bath for 10mins. After cooling to room temperature the absorbance was measured at 560nm. Glucose served as the calibration standard for total reducing sugar determination.

RESULTS AND DISCUSSION

The serially diluted soil sample was inoculated on to starch agar plates and observe for zone of clearance after the addition of iodine solution. Different bacterial isolates were tested for the amylase production by Starch hydrolysis test.

When starch agar medium was inoculated / streaked with bacterial organism and subsequently flooded with iodine solution, production of amylase was indicated by the zone of clearance around the microbial growth. On the basis of area of clearance only one potent isolate bacteria was selected for further studies on α-amylase production. Five bacterial strains showing zone of clearance were isolated designed as I_1, I_2, I_3, I_4 and I_5 were considered for further analysis (Table 1).

Characterization and Identification of the Isolates: The isolates were observed for microscopic and selected biochemical tests for characterization.

Microscopic Characterization: The results of gram staining reveals that the strains I_2, I_3 and I_4 were gram negative rods where as I_1 and I_5 are gram positive rods. The isolated fungi showed the morphological characters as greenish, uniformly dispersed colonies and fairly rapid growth on PDA medium and designated as *Trichodermreesi*.

Biochemical Characterization: The results of the response of the isolates I_1, I_2, I_3, I_4 and I_5 to the selected biochemical tests are given in Table 1. Based on the above results, the potent bacterial strain was provisionally selected for further studies. Similar results [10] using soil sample for isolating amylase producing soil bacteria and revealed the initial amylase production was confirmed by inoculating the isolates in Berg's broth. Production of α-amylase and saccharification content (total soluble carbohydrates and reducing sugars) by potent bacterial strain and fungi *Trichodermareesi* and their co cultures in submerged fermentation using wheat bran as substrate.

Table 2 showed the effect of incubation period on production of α-amylase by potent isolate bacteria and fungi *Trichodermareesi* are grown separately / individually in submerged flasks and also as a mixed /co cultures in liquid medium using wheat bran as substrate. Amylase activity exhibited the maximum production after 24 hrs (4.5, 6.9 and 10.1 IU/ml), respectively inoculated with potent bacterial strain and the maximum production of total soluble carbohydrates (8.07 mg/ ml) and reducing sugars (11 mg/ml) were detected after 96 hrs respectively as inoculated with bacterial strain (Table 3 and 4).

The maximum production of reducing sugars was in agreement with the maximum production enzymes. Similarly the maximum activity of Amylase was observed at 40 hrs incubation of which the wheat bran (WB) was proved as the best substrate source [11].

The maximum protein concentration 26 mg/ml by co cultured indicating the amylase production (Table 5). It has also been proved by protein concentration of enzyme extracts of wheat bran as substrates. Study on the evaluation of wheat bran as a substrate and analysing the effect of various fermentation parameters (Fermentation period and carbon sources) for the production of amylase activity by using monocultures of potent isolated bacteria and fungi (*Trichodermareesei*) and their co-cultures were carried out.

Table 1: Biochemical characters of the Amylase producing bacteria isolatedfrom soil

S.No	Biochemical tests	I_1	I_2	I_3	I_4	I_5
1.	Starch Hydrolysis	+	+	+	+	+
2.	Catalase test	+	+	+	+	+
3.	Indole production test	+	-	-	+	-
4.	Methyl red test	+	+	+	+	+
5.	Gelatin hydrolysis test	+	+	-	+	-
6.	Gram staining	Rod Positive	Rod Negative	Rod Negative	Rod Negative	Rod Positive

Table 2: Production of α-amylase (IU/ml) in by the selected microbes in submerged fermentation

Incubation period (hrs)	Bacteria	Fungi	Bacteria + Fungi
24	4.5	6.9	10.1
48	6.5	7.4	13.4
72	7.4	9.2	15.4
96	4.6	10.4	14.8

Table 3: Submerged fermentation of Wheat bran (10g/ 100ml) by potent isolate bacteria and mixed cultures for the production of total sugars (mg / ml)

Incubation period (hrs)	Bacteria	Fungi	Bacteria + Fungi
24	2.47	5.06	2.14
48	6.27	4.29	3.63
72	4.01	8.4	6.38
96	8.03	5.6	3.13

Table 4: Submerged fermentation of Wheat bran (10g/ 100ml) by potent isolate bacteria and mixed cultures for the production of Reducing Sugar (mg / ml)

Incubation period (hrs)	Bacteria	Fungi	Bacteria + Fungi
24	2	2.6	4.4
48	4.2	4.4	8.6
72	6.5	7.4	10.1
96	11	9.5	7.4

Table 5: Submerged fermentation of Wheat bran (10g/ 100ml) by potent isolate bacteria and mixed cultures for the production of Protein (mg/ ml)

Incubation period (hrs)	Bacteria	Fungi	Bacteria + Fungi
24	10	11	16
48	14	15	24
72	17	18	26
96	14	22	20

The incubation time was found to affect enzyme production (Fig. 1) as it was related to the growth of organism. There was a gradual increase in enzyme production through 24 hr and 48 hr and maximum 72 hrs. This may be because the cultures might be at stationary phase as Malhotra *et al.* [12] should that enzyme production was maximal when cells entered stationery phase. The lowest enzyme production was found at 24 hrs (10.1 IU/ml) and was 14.8 IU/ml at the 96 hrs. The decline in enzyme production with prolonged incubation may be due to loss of moisture, slower growth and lower enzyme production rates etc [13, 14]. The utilization of wheat bran as solid substrate in submerged fermentation had a great advantage in its low cost could lead to large scale production of this could lead to large scale production of this enzyme for industrial use in starch liquefaction.

Similar results are positively correlated [15] observed that different carbon sources (starch casein) supplemented with wheat bran enhanced maximum amylase production under solid state fermentation. *Bacillus* bacterial growth and polymer production were enhanced with the supplementation of hydrolysates of wheat bran / rice bran individually or in combination, based on weight of soluble substrates [16].

The production of amylase activity (9.2 IU/ml) was more in mixed cultures of isolated potent bacteria + *Trichodermareesi*, grown in carbon source as sucrose supplemented in wheat bran growing media at 48 hrs of fermentation / incubation period (Table 7). This was followed by amylase activity is less / minimum (4.9 IU/ml) in fructose supplemented wheat bran growing media fermented or inoculated with *Trichoderrmareesi* alone during 48 hrs of culture (Table 7). Whereas the production of amylase activity was more or less same as carbon source (fructose and sucrose) supplemented with wheat bran growing medium fermented by potent isolated bacteria alone in 48 hrs of incubation respectively (Table 7).

There was a gradual increase in the amylase activity from 24 hrs to 72 hrs (10.1IU/ml – 15.4 IU/ml) were observed in co cultures (Bacteria + fungi) and bacteria alone as mono culture in wheat bran growing media / liquid submerged fermentation and as slightly declined as 96 hrs of incubation period (14.8 IU/ml and 4.6 IU/ml) respectively. Whereas in fungal inoculated wheat bran submerged fermentation shows that there was an steady increase in the production of α-amylase activity (6.9 IU/ml and 10.4 IU/ml) during 96 hrs of incubation period.

The results depicted that the nature and amount to carbon source in culture media is important for the growth and production of extracellular amylase in bacteria [16] and amylase production in solid-state fermentation with wheat bran and rice husk as substrates [17].

Table 6: Submerged fermentation of Wheat bran (10g/ 100ml) by potent isolate bacteria and mixed cultures for the production of ethanol / litre

Incubation period (hrs)	Bacteria	Fungi	Bacteria + Fungi
24	4.76	2.9	5.0
48	5.9	4.3	7.3
72	6.6	4.9	9.9
96	4.6	5.3	13.2

Table 7: Effect of carbon sources on wheat bran in submerged fermentation

		Sucrose			Fructose		
S.No	Parameters	Bacteria	Fungi	Bacteria + Fungi	Bacteria	Fungi	Bacteria + Fungi
1.	pH	5	6	6-7	5	6-7	5-6
2.	α - Amylase	6.5	5.6	9.2	7.0	4.9	6.2
3.	Total sugars	7.3	3.5	3.8	7.4	3.4	7.5
4.	Reducing sugars	2.5	2.63	3.1	3.33	2.9	3.05
5.	Protein	11.8	8.96	18.6	11.5	10.2	8.64
6.	Alcohol	5.9	8.6	16.6	4.0	5.9	6.6

Biosynthesis of amylase was performed on agro industrial wastes and byproducts such as starch materials to solve pollution problems and obtain a low cost medium [18]. Rice husk, wheat bran, potato starchy and sugarcane bagasse were used as a low cost carbon substrate for amylase activity by *B. subtilis* and *Pleurotus djamor* [19, 20].

Ethanol: Table 6 depicted the ethanol production by submerged fermentation. There was an increase in the production of ethanol (5mg/litre to 14.2 mg/litre) during 96 hrs of fermentation by co cultures inoculated in wheat bran submerged fermentation. Whereas isolated potent bacteria fermented the wheat bran as a substrate and produced the maximum amount of alcohol (6.6 mg/litre) on 72 hrs of incubation and later steadily declined on 96 hrs of fermentation (4.6 mg/litre) respectively.

There was low production of ethanol produced by fungus (*Trichodermareesi*) during the 96 hrs of incubation fermented by using the wheat bran as a substrate. In carbon sources supplanted (Sucrose) in wheat bran submerged with fermentation the co cultures fermented the wheat bran and produced ethanol amounted to 16.6 g/litre in 48 hrs of incubation respectively. As compared to fructose supplemented wheat bran medium in (6.6 g/litre). The alcohol production was very low in isolated potent bacteria and fungi as monocultures inoculated in fructose and sucrose supplanted wheat bran medium (Table 7).

Protein: The maximum protein was produced in co cultures (26 mg / ml) on 72 hrs of fermentation using wheat bran as a substrate in submerged fermentation and a slowly declined on 96hrs of incubation (20 mg / ml) respectively. There was on steady increase in the production of protein by fungus fermentation (11-22 mg / ml) during 96 hrs of fermentation. The isolated potent bacteria produced protein content (17 mg / ml) on 72 hrs of incubation and sleepily declined on 96 hrs respectively (Table 5).

The carbon source supplemented with sucrose in wheat bran medium the co culture organisms produces 18.6 mg / ml of protein during 48 hrs of incubation. Whereas Fructose and Sucrose supplemented as carbon sources for the grown of isolated potent bacteria and 9 g/litre proteins was produced during 48 hrs of incubation respectively (Table 7).

Influence of Carbon Source on Amylase Production: Amylase production under the influence of carbon sources is indicated in Table7. Higher amylase production was observed in Fructose (7.0 IU/ml) as carbon source for both bacteria and fungal as mono cultures and co-culture inoculated in wheat bran (6.2 IU/ml) and (9.2 IU/ml) when compared to control at 48 hrs of incubation period respectively.

Amylases constitute one of the important groups of enzymes that are used in a wide range of starch industries and account for nearly 25% of the total sale of enzymes. Although there are many microbial sources available for producing amylases, only a few such as *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus amyloliquifaciens*are recognised as commercial industry. Amylases are also used in textile, detergent, paper and distilling industries [1].

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