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Research Article

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Production of Industrial Applicable Enzymes from Rhizopus Oryzae through Response Surface Methodology

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Abstract

The agricultural wastes are used for production of vital enzymes like cellulase, protease and lipase through Solid State Fermentation (SSF), which reveal their industrial potential aspect. This study was done to find out the ability of Rhizopus oryzae to produce lipase, protease and cellulase through SSF. Wheat bran showed maximum enzyme activities as related to others agricultural wastes. The enzymes showed maximum activities (21.4±0.04) (4.6±0.02 IU/mL) and (1.1 ± 0.01 IU/mL) for cellulase, lipase and protease respectively at temperature 25°C and incubation period 72 hours. Response surface methodology (RSM) used to optimize conditions likes temperature, pH, moisture contents, inoculum size and substrate concentration. RSM revealed maximum enzyme activities using 100% moisture contents, temperature 25°C, pH 5, substrate concentrations 12g and inoculum size of 6 mL. The method of ammonia sulphate precipitation used for the purification and crystallization of enzymes like lipase, protease and cellulase. The precipitation of cellulase, lipase and protease performed with 80%, 70 % and 90% ammonium sulphate concentrations to increase yield. The characterization of lipase, protease, cellulase revealed that it worked best below acidic conditions (pH 5) and at moderate temperature (25°C). The results revealed that the mold oryzae had potential to produce industrially important enzymes at acidic ph.

Keywords: Rhizopus Oryzae, Response Surface Methodology, Cellulase, Lipase, Protease

1. Introduction

The SSF has many advantages over SmF especially in high productivity and low cost. Due to many process problems, SmF can be limited to some extend in enzyme production [1]. In SSF, substrate morphology can be improved, so yield increases [2,3]. SSF is also valuable for production of many antibiotics such as penicillin, which is produced from mixed culture [4]. R. oryzae has showed excellent potential for soybean meal as a substrate to produce protease enzyme. R. oryzae is basically a fungal strain that usually grow on fruit and vegetables [5]. The enzymes are proteinaceous agents which are playing important role in various industries in these days. These are eco-friendly and non-toxic to the human body [6]. Cellulases, Lipases and proteases are using in many industries like paper, starch production, textiles, animal feed, detergent, leather and food processing [7]. Proteolytic enzymes are also known as proteases. These enzymes have the capability of hydrolyzing peptide bonds in protein molecules. Proteolytic enzymes are everywhere and found almost in all organisms, and these enzymes are especially important for cell differentiation and for cell growth [8]. The enzyme protease is vital industrial enzyme and used in detergent, pharmaceutical and leather industries [9]. The valuable sources of proteolytic enzymes are microbes and they can be used for the manipulation and production of new enzymes for industrial applications because of their fast growth rate [10]. These proteolytic enzymes find numerous applications in pharmaceutical industry, food, and detergent industries. Their importance in these fields is due to their key role in biological processes [11]. Proteases or proteolytic enzymes are usually found in all living organisms, like viruses, animals and in humans. Proteolytic enzymes play an important role in life cycle of many pathogens [12]. Proteolytic enzymes play an important role in many research applications include nucleic acid purification, digestion of unwanted proteins, cell culturing, diagnostic of many diseases and in proteomics proteolytic digestion of proteins. In nucleic acid purification, proteases play their important role by digestion of unwanted proteins. Protease enzymes are widely used in waste management, pharmaceutical, food industry like in cheese making, baking, in

hydrolysis of proteins and in leather industries [12]. Lipases are involved in the metabolism and mobilization of lipid within the cells as well as in the transfer of lipids from one organism to another. Lipase belongs to group of hydrolases that acts on the triacylglycerols and break down into a fatty acids and glycerol [13]. Lipases can be produced from fungi, plants, and bacteria. Mostly used microorganisms to produce lipase are Aspergillus Niger, Penicillium and Rhizopus species [14]. The lipases are playing a crucial role in the field of research and food, detergent, pharmaceutical and chemical industries [15]. Lipases are widely used in paper manufacture, the synthesis of fine chemicals the processing of oils and fats production of cosmetics, and pharmaceuticals and detergents and degreasing formulations, food processing [16]. The main parts of polysaccharide are lignin, cellulose, and hemicellulose. The main component found in the earth crust is cellulose and it is organic in nature. This cellulose is found in the cell wall of the plant and percentage of cellulose is different in every cell of the plants. The cellulase is frequently used in the production of paper processing, textiles and production of ethanol. This enzyme is non-toxic to human body and its works under specific temperature and pH [17]. In order to check the efficiency of R. oryzae to produce lipase, amylase and proteases for industrial applications and characterization of multiple parameters for enzymes production have become warranted. The present study was designed to evaluate the industrial applicability of lipase, cellulase and protease yield from R. oryzae.

2. Material and Methods

2.1. Substrates Collection

Agro-industrial wastes (Wheat straw& bran) were used as substrates in this study. These substrates were collected from local areas of Gujrat, Pakistan. After collecting, these substrates were subjected to dry under sunlight then stored in an airtight jar to prevent from moisture and further studies [18].

2.2. Microorganism

The strain of R. oryzae collected, isolated from rotten fruit, and cultured in Department of Biochemistry and Biotechnology, University of Gujrat, Pakistan. The culture was maintained on agar slants and stored at 4°C for later use [18].

2.3. Substrate Screening

Twelve grams of both substrates wheat straw and bran were taken in triplicate using volumetric flasks. Substrates were moistened with 6ml Vogel's medium and then kept for 3 days for maximum enzyme yield. Wheat bran showed maximum expression for enzymes, so for further experiment wheat bran was selected as a substrate.

2.4. Enzyme Extraction

After the stipulated fermentation period, 50 mL cool distilled water were added in fermented flask and stirred at 120 rpm for 30 min for the extraction of extracellular enzymes. Muslin cloth used for filtration while, extracted filtrates were centrifuged at 15000 rpm for 10 min, at 4°C to take out the crude enzyme. Pallet containing fungal biomass debris discarded, and the cell free supernatant that contained desired crude enzyme obtained [18].

2.5. Assay of Protease Activity

Protease activity of both purified and crude enzyme were measured using casein as a substrate by method describe by Malik & Shinde [19]. Firstly, 2 mL of filtrate obtained from RSM were centrifuged at 6000 rpm for 10 min. After centrifugation, pallet was discarded, and supernatant was taken using falcon tubes. About1 mL of this supernatant was taken along with 1 mL of phosphate buffer (pH 7) containing 2 g/L casein. This mixture was placed at 35 °C for 10 mints. The reaction was then stopped by adding 2 mL TCA and then placed at 35 °C for 20 mints [20,21]. Furthermore, this mixture was centrifuged at 8000 rpm for 5 mints, afterward pallet was discarded, and supernatant was taken. About 1 mL of supernatant and 3 mL of Na2CO3 were added in 1 mL Folin's reagent, blue color appeared. Optical density was measured at 660 nm on spectrophotometer (Shimadzu Japan 1900 UV/Vis). To made standard curve of Bovine serum albumin of different concentrations were taken in different test tubes [2,21].

2.6. Assay of Lipase Activity

First prepared fresh phosphate buffer of pH 7. Then, mixed 90ml phosphate buffer with mixture of 2-Propanol, PNPP, Na2CO3 and gum accasia. Took 100µl enzyme and 2ml reaction mixture and incubated at 55 °C for 15 min. Then, added 3ml Na2CO3 and optical density was measured at 410 nm [22]. To make the standard curve of 4-nitrophenol, the stock solution was prepared which contained 1mL of 2-propanol into 10mg of 4-nitrophenol. Then standard dilutions of different concentrations were made by diluting the stock with phosphate buffer [18].

2.7. Assay of Cellulase Activity

Took 0.05g filter paper in a test tube then added 1ml crude enzyme in it. Added a 1ml of sodium citrate buffer pH (4.8-5) and incubated at 50 oC for 30 min. Then, took out 0.01ml solution in a separate test tube and added 1ml DNS and boiled it for 5 min then, added 5ml distilled water after cooling. Took 3ml from test tube inserted into a quartz glass and optical density measured at 540nm [21]. The different concentrations of glucose 0.2mg/mL-1.0mg/mL were used to obtain the glucose standard curve. The 1% stock solution of glucose was prepared. The glucose in the different amounts were taken in separate test tube. Then added 2mL of DNS reagent and boiled it for 10 minutes and then cooled it by adding 10 mL distilled water. The enzyme activity was taken using spectrophotometer (Shimadzu Japan 1900 UV/Vis) at 540nm.

2.8. Optimization of Nonphysical and Physical Parameters

Different temperatures used from 25-45°C for the optimization of fungus while different substrate concentration 3, 7, 12g were also used to optimize enzyme production using R. oryzae [23]. Impacts of different physical and nutritional optimization of lipase, protease and cellulase by RSM After selection of best substrate and fungus strain. Different physical parameters like inoculum sizes (2-6 mL), temperatures (25-45°C), time periods (3-7 days), pH (3-9) and moisture levels (40-100%) optimized through RSM to show maximum enzyme activity.

2.9. Purification of Enzymes

The purification of crude enzymes (lipase, protease and cellulase) were done using following steps [24].

2.10. (NH4)2SO4 Precipitation

Purification of enzymes was done by (NH4)2SO4 precipitation (Salting in and salting out) [25]. 1mL of filtrate from sample of maximum enzyme activity was taken in 5 different test tubes in duplicate while, (NH4)2SO4 was added at 50%-90% in 5 different test tubes, respectively. After placing in freezer, centrifuged at 1500rpm for 15 min, and supernatant was taken and again centrifuged with same time and speed. Activity of enzyme of different samples was measured [26].

2.11. Dialysis

The pallet was obtained then used for purification of enzyme (lipase, protease and cellulase) by dialyzing it overnight against sodium citrate buffer [2].

3. Industrial Applicability

3.1. Washing Test Performance of Lipase

The test fabric was stained with the different stains then air dried. Lipase (0.01mg/mL) used to study of washing test performance against dyes using 1mL, 2mL, 3mL, 4mL, 5mL and 10mL levels. The reaction mixture was taken in 6 beakers.

3.2. Dehairing ability of Proteases

Purified protease can be used as hair removal additive. Hair removal efficiency of purified protease was tested on goat skin. The reaction mixture was taken in 6 beakers, first beaker contained the blank (only 50mL buffer and 2 gm goat skin), the other five contained 50mL buffer of pH 5 with 1mL, 2mL, 3mL, 4mL, 5mL and 10mL (0.01mg/mL) purified protease and 2 gm skin was dipped in each reaction mixture, incubated at 35°C for one hour.

3.3. De-sizing of Cotton

This process of de-sizing of cotton performed by enzymatic process at pH 4.8-7 and then the cotton fabrics washed with the help of hot and cold water and then dried in hot air oven desiccated at 80°C and the fabrics was weighted using electronic balance. The washed fabric was de-sized with cellulase (0.01mg/mL) using 1mL, 2mL, 3mL, 4mL, 5mL and 10mL (0.01mg/mL) levels. The reaction mixture was taken in 6 tanks.

4. Results and Discussion

4.1. Strain Identification and Culturing

To produce enzymes (lipase, protease and cellulase), the fungus strain obtained, was one of the novel strains of Rhizopus specie. It was selected by seeing its growth pattern under light microscope. The colonies appeared as black in color. Then, it was maintained on PDA media in Figure 1

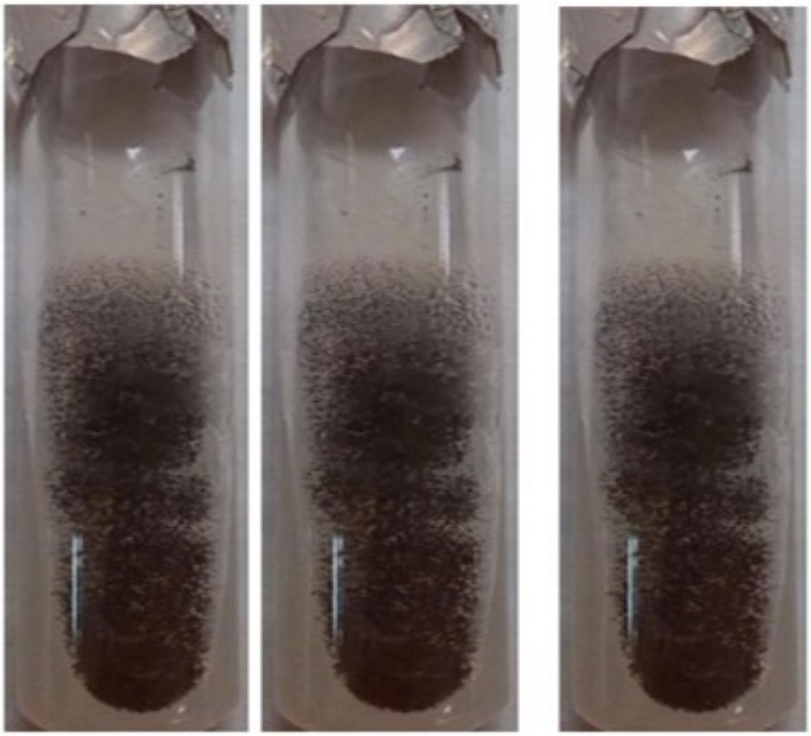

Figure 1: Microscopic Examination of Pure Colonies

4.2. Substrate Screening

Different substrates were used to produce enzymes for example wheat bran and wheat straw. The best substrate was wheat bran for production of enzymes (Table 1). *Rhizopus oryzae* revealed maximum lipase, protease and cellulase activities and selected for further studies.

S. No	Substrate	Lipase activity (IU/mL)	Protease activity (IU/mL)	Cellulase activity (IU/mL)
1	Wheat bran	4.6±0.05	1.1±0.02	21.4±0.05
2	Wheat straw	2.3±0.03	0.91±0.01	12.7±0.01

Table 1: Substrate Screening

4.3. Optimization of Temperature and Substrate

Different temperatures used from 25-45°C as trails. The optimum temperature was achieved at 25°C for the maximum production of lipase, protease and cellulase (Table 2). Highest enzyme production was observed when 12g of substrate was used from R. oryzae. When the large amount of substrate was used then the surface area for fungus spores to penetrate was increased. The maximum enzyme activity occurred in the range of 3-12g of substrate by fungus strain. It had been studied that fungus showed maximum enzyme production in the range of 25-37°C [23]. When the temperature decreased or increased from this range then a metabolic change occurred in the microbes [18,23]. To produce enzyme, the experiment was done by utilizing sugarcane bagasse and oyster mushroom at temperature 20°C. This temperature was difficult to maintain at industrial level [2]. In present study, the enzymes revealed maximum activities at temperature 25°C which was more than 20°C [23].

S.NO	Temperature °C	Lipase activity (IU/mL)	Protease activity (IU/mL)	Cellulase activity (IU/mL)
1	25	4.7±0.01	1.94±0.02	21.4±0.01
2	35	2.4±0.03	0.94±0.04	18.3±0.04
3	40	2.2±0.04	0.89±0.05	13.6±0.01
4	45	1.1±0.01	0.22±0.03	2.7±0.05
	Substrate levels (g)	**Lipase activity (IU/mL)**	**Protease activity (IU/mL)**	**Cellulase activity (IU/mL)**
1	3	4.7±0.01	0.91±0.02	21.4±0.01
2	7	5.4±0.03	0.45±0.04	18.3±0.04
3	12	5.9±0.04	1.98±0.05	23.6±0.01

Table 2: Optimization of Temperature and Substrate

4.4. Impacts of Different Physical and Nutritional Parameters

Different parameters like inoculum size, temperature, period, pH, substrate concentration and moisture contents were optimized for maximum enzyme production through Response Surface Methodology (RSM) [27].

4.5. Optimization of pH

To produce enzyme from fungus strains, pH was a very crucial parameter which revealed a steep graph. Different pH ranging from (3, 5, 9) were used as trails using RSM. The optimum pH required by the fungus for the maximum production of lipase, protease and cellulase was pH 5. It had been already studied that fungus showed maximum enzyme production in the range of 5-9. When the pH decreased or increased from this range then denaturation of enzyme would occur [28]. The experiment was performed to produce enzymes using co-culture of *Aspergillus* and Fusarium while optimum pH (pH 7) was achieved using forest wastes [29]. This pH was more than pH 5 which was optimum in recent study (Table 3).

Time Period (Days)	Temperature °C	pH	Moisture Content %	Inoculum size (10^7 spores/mL)	Lipase Activity (IU/mL)	Protease Activity (IU/mL)	Cellulase Activity (IU/mL)
3	35	5	40	2	2.4±0.01	0.64±0.03	18.3±0.02
3	35	7	40	2	2.7±0.03	0.34±0.02	16.7±0.02
3	35	9	40	2	3.0±0.03	0.14±0.04	15.0±0.05
3	40	5	70	4	2.0±0.04	0.43±0.05	12.9±0.03
3	40	7	70	4	1.5±0.01	0.42±0.04	13.2±0.05
3	25	9	70	4	3.8±0.02	1.1±0.02	12.8±0.04
3	**25**	**5**	**100**	**6**	**6.6±0.02**	**2.9±0.01**	**27.4±0.04**
3	45	7	100	6	1.5±0.05	0.93±0.02	12.7±0.02
3	45	9	100	6	0.9±0.01	0.55±0.01	9.8±0.03

5	35	5	100	2	2.2±0.03	0.35±0.03	8.7±0.05
5	35	7	100	2	2.4±0.01	0.72±0.05	8.8±0.05
5	35	9	100	2	2.1±0.02	0.66±0.02	7.0±0.03
5	40	5	40	4	1.2±0.04	0.49±0.01	3.9±0.01
5	40	7	40	4	0.5±0.05	0.90±0.05	1.5±0.02
5	25	9	40	4	1.0±0.02	0.22±0.03	2.9±0.01
5	25	5	70	6	4.4±0.01	0.77±0.01	11.8±0.03
5	45	7	70	6	1.2±0.02	0.27±0.04	10.7±0.02
5	45	9	70	6	1.1±0.04	0.85±0.05	2.7±0.03
7	35	5	70	2	1.9±0.05	0.32±0.01	6.2±0.03
7	35	7	70	2	0.3±0.02	0.43±0.03	1.1±0.05
7	35	9	70	2	0.7±0.02	0.54±0.02	2.1±0.05
7	40	5	100	4	2.2±0.01	0.23±0.05	13.6±0.02
7	25	9	100	4	2.7±0.03	0.31±0.01	12.1±0.02
7	25	5	40	6	3.6±0.04	0.64±0.04	0.7±0.04
7	45	7	40	6	0.2±0.04	0.52±0.03	9.1±0.05
7	45	9	40	6	0.7±0.02	0.23±0.02	7.1±0.03

Table 3: RSM Trails and Results

4.6. Optimization of Time Period

To produce enzyme from fungus strains period was another crucial factor. Different period ranging from (3, 5, 7 days) were used as trails using RSM (Table 3). The period of 3 days required by the fungus for interaction with substrate and for the maximum production of lipase, protease and cellulase. It had been already studied that fungus showed maximum enzyme production when it was given the 3 days for proper interaction with substrate. In previous study, the incubation time of 12 days revealed maximum enzyme activity using fungus [30].

4.7. Optimization of Inoculum Size

Another crucial factor that effects the production of enzyme from fungus strains time was inoculum size. Different inoculum sizes (2, 4, 6 mL) were used as trails using RSM (Table 3). When the fungal spores were properly spread on the surface of substrate then the proper interaction occurred which helped in the maximum production of enzyme. The 6mL inoculum size was enough to high concentration of enzyme. The lower amount of inoculum size produced less amount of enzyme [31,32].

4.8. Optimization of Substrate Moisture

The most important parameter is moisture level under RSM trials. Different substrate moisture concentrations 40, 70, 100% were used for enzyme production from Rhizopus oryzae. Maximum enzymes activities were achieved at 100% moisture level which was due to hit and trial method of RSM (Table 3).

4.9. Statistical Graphs (Contour and Surface Plot)

Design Expert software was used to design statistical graphs. The graphs were of two types: the one was surface contour and the other was surface plot. These graphs showing relationships among various variables such as temperature, time, pH, inoculum, and moisture contents with respect to protease, lipase and cellulase activity were described. Graphs showed that by increasing one variable also increased enzyme activity up to optimum level but after this optimum level, it started to decline. The sample showed maximum protein precipitation for 90 % $(NH_4)_2SO_4$ concentration [26].

5. Industrial Applicability

5.1. Washing Test performance of Lipase

The maximum washing efficiency was obtained when 10 mL of enzyme culture was used. These results were quite effective in research especially use of lipase in washing powders (Figure 2).

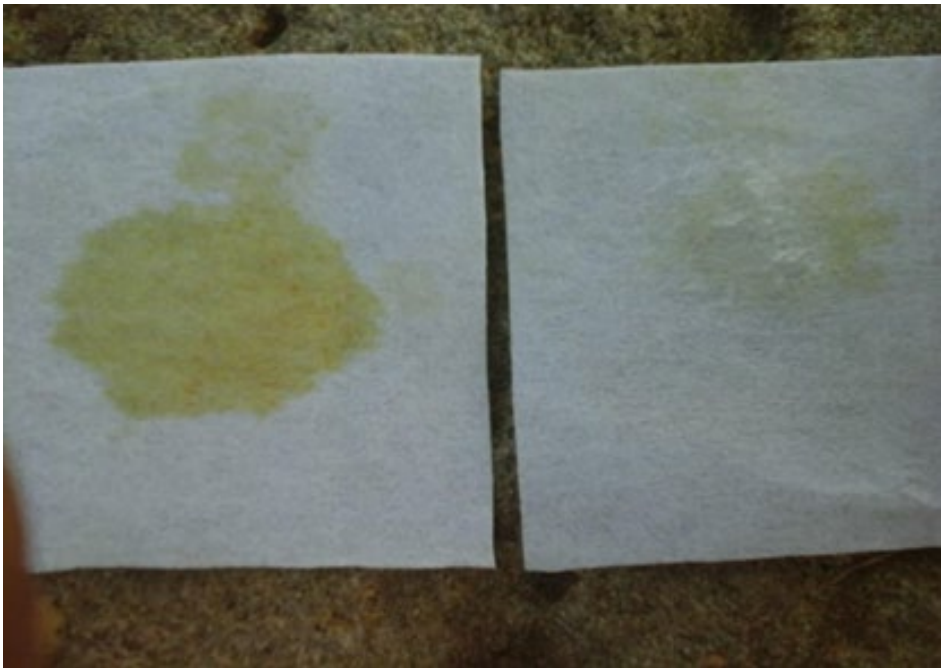

Figure 2: Lipase Washing Performance

5.2. Dehairing Ability of Proteases

The best hair removing was achieved when 5ml enzyme solution used over paper (Figure 3). These results were comparable to previous studies but in different way [4].

Figure 3: Dehairing of Skin

5.3. DE Sizing of Cotton

The maximum de-sizing efficiency was obtained at 60°C and at 4mL enzyme concentration and these results were good from previous studies [33]. The de-sizing parameter is particularly important in pulp and paper industry (Figure 4).

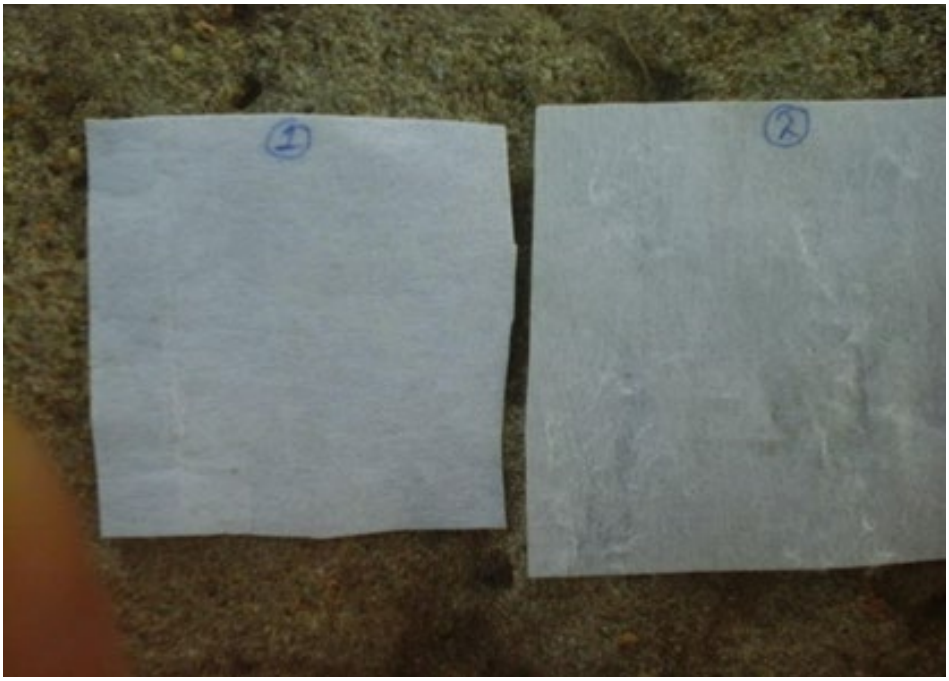

Figure 4: De-Sizing of Cotton

6. Conclusion

The major purpose of this research was to produce enzymes (lipase, protease and cellulase) by utilizing a cheap agricultural waste like wheat bran and wheat straw through solid-state fermentation using newly isolated Rhizopus oryzae. Microbial enzymes obtained from agro-industrial wastes are of great importance. This work will also be beneficial for many industrial applications such as in detergent industry as detergent additive, in leather industries in hair removing creams and de-sizing in paper industry.

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