Screening & Optimization Study on Fungal Proteases and their Applications

Riya M. Bhanushali¹, Hetal K. Panchal²

¹,² Dolat-Usha Institute of Applied Sciences and Dhiru-Sarla Institute of Management & Commerce, Valsad, 396001, India

ABSTRACT

The present study focuses on production of extracellular proteases by a local fungal isolate through solid state fermentation. All the fungal isolates were screened for their protease producing ability & two isolates showing highest protease production were selected for further studies. Optimization of different fermentative variables like carbon and nitrogen sources, pH, temperature and various solid substrates was done to enhance enzyme production. OVAT method was used to study optimization. Protein concentration and enzyme activity was measured. The maximum protease production was found when the medium was supplemented with corn seed meal as a solid substrate, glucose as a carbon source, peptone as a nitrogen source. The optimum pH & temperature was 8 and 37°C respectively. The enzyme was further purified by ammonium sulphate fractionation and dialysis at 60%, 70% & 100% saturation. Where, 100% saturation showed best results. Then proteases was resolved by SDS-PAGE analysis. Various applications like, Antioxidant activity, Milk Clotting Activity (MCA), Efficiency to remove blood stains & feather degradation activity was successfully performed using fungal protease. Proteins from germinating seeds of chick pea, mung bean, soybean and cowpea were hydrolysed for the production of amino acids. Amino acids were recovered, estimated & utilized for chelation of metalnutrients viz., Cu & Mn. The resultant chelates were employed to detect with Fourier Transform Infra-Red Spectrophotometer (FTIR) analysis. The resultant amino acids-metal nutrient chelates can be utilized as organic fertilizer.

Keywords: Fungal protease, Optimization, Antioxidant activity, Milk clotting activity, seed proteins- amino acids- chelates, FTIR

1. INTRODUCTION

Proteases are among the oldest and most diverse families of enzymes known and are involved in every aspects of organism’s function. Proteases are complex group of hydrolytic enzymes which are responsible for the hydrolysis of protein molecules into small peptides & amino acids. Because of their broad substrate specificity, proteases have a wide range of applications such as in leather processing, detergent formulations, baking, brewing, meat tenderization, peptide synthesis, cheese manufacturing, soya sauce production, protein hydrolysate, pharmaceutical industry, waste treatment, silk industry, organic synthesis, recovery of silver from waste photographic film, as well as analytical tools in basic research and have high commercial value [1, 2, 3, 4]. They can be synthesized by plants, animals, and microorganisms constituting around 60% of the worldwide enzyme market [5]. Microorganisms are the most common sources of commercial enzymes due to their physiological and biochemical properties, facile culture conditions and ease of cell manipulation [6]. Microbial proteases are preferred to animal and plant sources because of various advantages. A variety of microorganisms such as bacteria, fungi, yeast and actinomycetes are known to produce these enzymes [7]. Among them fungi exhibit a wider variety of proteases than bacteria. Protease
produced by SSF have greater economic feasibility. SSF is more advantageous as compared to SmF because of low fermentation technology, low cost, higher yields-concentration of enzymes and reduced waste output. In the present investigation, protease producing fungi were isolated & screened. Various optimization parameters were studied to maximise the protease production. And the ability of the selected isolate was tested by performing different activities.

2. MATERIALS AND METHODS

2.1 Collection & identification of Fungi

About Twenty-five different fungi were directly collected from the Microbiology Laboratory of Dolat-Usha Institute of Applied Science and Dhiru-Sarla Institute of Management & Commerce, Tithal Road, Valsad. The samples were inoculated onto sterile Sabouraud’s agar plates (Peptone – 10gm, Glucose – 40gm, NaCl – 5gm, Agar – 24gm, D/W – 1000ml, pH – 5.2). The plates were incubated at room temperature for 6-7 days. After incubation, fungal morphology were studied macroscopically by observing colony features (Colour and Texture) and microscopically by staining with lacto phenol cotton blue and observe under compound microscope for the conidia and arrangement of spores.

2.2 Screening by Plate Assay Method

Production of proteolytic enzymes by fungal isolates was detected by using the Plate Assay Method [8], in which gelatin is act as the protein source of that medium. The fungal isolates were spot inoculated on the medium supplemented with 1% gelatin (Peptone – 5gm, Beef extract – 3gm, NaCl – 5gm, Agar – 24gm, D/W – 1000ml, pH – 6). After inoculation, plates were incubated at Room temperature for 3-4 days. Following incubation, gelatin degradation was observed as a clearing zone around the fungal colonies. The zone of gelatinolysis was clearly seen when plates were flooded with aqueous solution of Frazier’s reagent (15gm HgCl\textsubscript{2} mixed in 20ml 7M conc. HCl and 80ml D/W). Mercuric chloride solution react with gelatin to produce a white precipitate which made the clearing zone visible. The zone indicates the extracellular protease activity of the fungal strain. Enzyme activity was measured by the following formula: \( \text{EA} = D - d \). Where, \( D \) = diameter of colony plus clearing zone, \( d \) = diameter of colony. Two most potent isolates were chosen for further production, optimization & application studies.

2.3 Protease Production

The selected two fungal isolates were inoculated in sterile protease specific broth (Yeast extract – 1gm, MgSO\textsubscript{4} – 0.02gm, glucose – 2gm, K\textsubscript{2}HPO\textsubscript{4} – 0.1gm, D/W – 100ml, pH – 5.0). Flasks were inoculated & incubated at room temperature for 5-6 days on rotary shaker. At the end of incubation, the contents of flasks were filtered through Whatmann filter paper No. 1 and then filtrates were centrifuged at 8,000 rpm for 10 minutes.

Pellet were discarded after centrifugation and clear supernatant was used as a source of protease. The supernatant was further used for subsequent studies [9].

2.4 Measurement of Protein and Enzyme activity

2.4.1 Protein estimation:

Protein estimation of crude enzyme extract was determined according to the Folin-Lowry’s method.

2.4.2 Enzyme activity:

Protease activity in the crude enzyme extract was determined according to the method of Carrie Cupp-Enyard by using casein as substrate. Two test tubes were taken and labelled as
test (T) and blank (B). 5ml of 0.65% casein solution was added in test and blank, were placed at 37°C for 30 minutes to occur enzymatic reaction. The reaction was terminated by addition of 5% Trichloro acetic acid (TCA) solution in both the tubes. 1ml of enzyme solution was added in test (T) tube only and it was allowed to stand for 15 minutes at room temperature. Then solution from both the tubes were filtered by using Whatmann no. 1 filter paper.

2ml of filtrate from both the tubes were taken into two new tubes. 5 ml sodium carbonate was added in both test tubes followed by addition of 1 ml of 2 fold diluted Folin Ciocalteu phenol reagent. Then resulting solution were incubated in dark for 30 minutes at room temperature for the development of blue colour. The absorbance of the blue colour compound was measured at 660nm against a reagent using tyrosine standard [10].

2.5 Optimization of process parameters by One Variable At a Time (OVAT) method

2.5.1 Effect of various types of Carbon source: Various carbon sources were investigated for their effect on enzyme production. The carbon sources tested were Glucose, Maltose, Fructose & Sucrose [11].

2.5.2 Effect of various types of Nitrogen source: Protease production was investigated using different nitrogen sources like Peptone, Yeast extract and Beef extract [12].

2.5.3 Effect of various pH: The effect of pH was studied at pH 5, 6, 7, & 8 [13].

2.5.4 Effect of various Temperature: The effect of different temperatures like- -4°C, Room temperature and 37°C were studied [14].

2.6 Effect of various solid substrates (SSF): Solid substrates like Cotton seed meal, Peanut seed meal, Corn seed meal and Soybean husk were checked for the maximum production of protease as they are easily available at low cost [15].

2.7 Extraction and Purification

The crude protease extract obtained by culturing fungal isolate in optimized protease production medium was subjected to Ammonium sulphate fractionation (60%, 70%, & 100%). The precipitates so obtained by centrifugation were dissolved 0.1M phosphate buffer (pH 6.7) and dialyzed against the same buffer for overnight at room temperature. The samples were then tested for protease activity & protein estimation. Then specific activity, fold purification and % yield were calculated.

2.8 Electrophoresis SDS-PAGE analysis

The molecular weight of fungal proteases are generally in the range of 20 and 50 kDa. In order to evaluate the proteolytic enzyme profile, crude enzymatic extracts corresponding to fungal strains were analysed by polyacrylamide gel electrophoresis. Hence, SDS- PAGE analysis allowed to distinguish a clear difference between protein patterns of the examined fungal strains [16].

2.9 Applications of fungal protease

2.9.1 Antioxidant activity:

The reducing power of the fungal extract was determined by the method described by Oyaizu (1986) with modifications [17]. Various concentration of fungal extract that is protease, were mixed with 1 ml of 0.2M phosphate buffer (pH 6.6) and 1 ml of 1% potassium ferricyanide.
The reaction mixture was incubated at 50°C for 20 minutes followed by addition of 1 ml of 10% trichloro acetic acid (TCA) and centrifuged at 2000 rpm for 10 minutes. An aliquot of supernatant (1 ml) was mixed with 1 ml of deionized water and 250µl of 0.1% (v/v) ferric chloride.

Absorbance was measured at wavelength 700nm against the diluent as blank. Higher absorbance indicates greater reducing power. Butylated Hydroxy Toluene (BHT) was used as the standard antioxidant.

2.9.2 Milk Clotting activity:

Milk clotting activity was measured using a modified method of Sousa and Malcata. The milk substrate was prepared by dissolving 12gm of skimmed milk powder in 100cm³ CaCl₂ solutions (0.01 mol L⁻¹). The pH value of the milk was adjusted to 6.0 with 1M HCl before use. The milk substrate (2 cm³) was heated at 60°C, and thoroughly mixed with 2 cm³ of the enzyme solution. The time for formation of fragments was measured with stopwatch. One unit of milk clotting activity (MCA) is equal to the amount (mg) of enzymes required to coagulate 1cm³ of reconstituted skimmed milk in 1 min at 60°C and pH 6.5. The MCA was calculated by using Equation: MCA = 2400 / (r × F). Where, r is the time for the formation of fragments (s), F is the dilution coefficient [18].

2.9.3 Feather degradation activity:

Feathers were microscopically observed before the treatment. And then feather was subjected to the crude enzyme extract. Which is incubated at 37°C, for 14 days. After incubation the feather was microscopically examined for the feather degradation [19]. Distilled water blank containing feather tube was used as control.

2.9.4 Silk degumming activity of the protease:

Degumming of silk was done by using commercial process [20]. In which enzyme bath was prepared by adding protease enzyme 50 ml, 0.5 g sodium bicarbonate, 1 g non-ionic detergent (Tween 80) with M: L ratio 1:20 & phosphate buffer (pH 7). 35.15 g of silk yarn washed with soft water & then immersed into the prepared enzyme bath.

The temperature was maintained at 90°C for 70 minutes. Then the silk yarn was removed, washed & dried. After drying, weight loss was compared to calculate the percentage of silk degumming. And increased protein concentration in the solution of enzyme bath was indicated through performing protein estimation by Folin Lowry’s method.

2.9.5 Removal of blood stain from the cotton cloth:

Fibrinolytic potential of the purified enzyme was determined by incubating the pieces of cotton fabric impregnated with blood [21]. The dried blood stains were fixed with 2% (v/v) formaldehyde for 30 min and the excess formaldehyde is removed by rinsing with water. The cotton fabrics were then allowed to dry.

After drying, the cotton fabrics were incubated separately with 2ml of partially purified protease along with detergent, 2ml of D/W with detergent and 2ml of distilled water at 37°C for 1 hour. The cotton fabrics were then rinsed with water and checked for the removal of blood stains after drying.

2.9.6 Production of amino acids-metal nutrient chelates:

2.9.6.1 Production of free amino acids- Fungal spore suspension was inoculated in modified production media suggested by [22] (malt extract 1gL⁻¹, glucose 6gL⁻¹, yeast extract 1gL⁻¹,
peptone 2 gL⁻¹, K₂HPO₄ 0.5 gL⁻¹, MgSO₄·7H₂O 0.5 gL⁻¹, FeSO₄·7H₂O 0.01 gL⁻¹, pH-7.5) and incubated on rotary shaker at 37°C for 9 days. After incubation the filtered broth was subjected to 100% ammonium sulphate precipitation & dialysis. Then this dialyzed sample were used for protease activity & also utilized for the digestion of seed proteins.

Each seed samples chick pea, mung bean, soybean and cowpea (10gm each) were germinated with sterile D/W for 48 hours in incubator at constant temperature (37°C) and humidity (80%) and extracted in distilled water.

The seeds were crushed together using blender and crushed material was adjusted to pH- 7.5 with 0.1N NaOH and subjected to fungal protease for the enzymatic digestion at 37°C for 4-5 days to recover maximum amount of amino acids. Then the digested material was treated with 95% ethanol for 30min and centrifuged at 10,000xg for 10 min and extracted with D/W [23].

Seed crush without digestion were used as control. Total amino acids were estimated from the filtrate by Ninhydrin method and utilized for synthesis of metal-nutrient chelate.

2.9.6.2 Production & analysis of amino acids-metal nutrient chelate

The amino acids (Chelating solution) recovered from enzymatic digestion were utilized for production of chelates [24]. One gram each of metal nutrients like, copper sulphate and manganese sulphate was added separately to 100ml of chelating solution and incubated for 2 hour on rotary shaker at 37°C. After 2 hours of incubation it was employed to detect chelate using FTIR spectrophotometer.

3. RESULTS AND DISCUSSION

3.1 Identification of fungi

Among most isolates the genera *Aspergillus* were dominant. The colony pigmentation was white, yellow-brown to black shades of green, black grown evenly with dense & erect conidiophores. Morphology of conidia was observed by Lacto-phenol cotton blue (LPCB) mounting.

3.2 Screening by plate assay method

Screening of fungi for their protease activity was carried out by the hydrolysis of substrate incorporating in the medium by plate assay method. After incubation period, enzyme activity were detected by appearance of zones around the fungal colonies. Two fungal sp. (F 5 & F 15) showed highest zones around the colony, were used for further study. Among fungal species of certain genera, *Aspergillus, Penicillium, Paecilomyces, Rhizopus* and *Rhizomucor* are well known producers of proteases [25, 26].

3.3 Measurement of Protein and Enzyme activity

By performing protein estimation and enzyme activity. Specific activity was calculated by using formula: Specific activity = Enzyme activity/Protein concentration

From the above results it can be said that F 15 has high protease activity than F 5, but in case of Specific activity F 5 has good results (Table 1).

Table 1. Protease activity of selected fungal isolates

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>Enzyme activity</th>
<th>Protein conc.</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>F 5</td>
<td>6.5 µg/min/ml</td>
<td>1.8 mg/ml</td>
<td>3.61 Unit/ml</td>
</tr>
<tr>
<td>F 15</td>
<td>6.7 µg/min/ml</td>
<td>1.9 mg/ml</td>
<td>3.52 Unit/ml</td>
</tr>
</tbody>
</table>
3.4 Optimization by OVAT method

3.4.1 Effect of Carbon sources: Different carbon sources were investigated to get maximum enzyme production & activity. And promising results were obtained when medium was supplemented with Fructose and Glucose as a carbon source for F 5 & F15, respectively. (Fig.1) Increased yield of alkaline protease production were reported by several other researchers [21] using different sugars such as Dextrose, Sucrose, Lactose, Maltose.

3.4.2 Effect of Nitrogen sources: The growth of the microorganisms and enzyme production requires nitrogen source. Maximum protease activity found when medium was supplemented with peptone as Nitrogen source. (Fig. 2) Researchers [12] observed that yeast extract showed promising results.

3.4.3 Effect of pH: pH of the fermentation medium is one of the most important factor affecting enzyme production. In the present study a pH of 8.0 was found to be optimum for protease production. (Fig. 3) An optimum pH of 8.0 and 7.0 was reported for protease produced by Aspergillus flavus and Aspergillus niger respectively [13].

3.4.4 Effect of Temperature: Incubation temperature also plays an important role in enzyme production and growth of microorganisms, which can be activated at one temperature and inhibited at another. The optimum temperature for protease production was 37°C for both the fungi. (Fig. 4) Similarly, [27] reported the optimum temperature for protease production by the mesophilic fungi Synergistes species was at 35°C.
3.5 Enzyme production using Solid state fermentation: The selection of solid substrates for enzyme production in SSF process depends upon several factors, mainly related to cost and availability of the substrate. Out of four solid substrates corn seed meal gave good results as compared to others. (Fig. 5) [28] got good results using wheat bran as the solid substrate.

![Figure. 5 Enzyme production using various solid substrates](image)

3.6 Extraction and Purification: For both the fungi maximum activity was found in 100% saturation in Ammonium sulphate as well as in Dialysis (Table 2, 3). According to [29], the highest protease activity was found with 60 to 80% ammonium sulphate saturation.

Table 2: Results of enzyme activity after Ammonium sulphate precipitation

<table>
<thead>
<tr>
<th>Parameters (After Ammonium sulphate precipitation)</th>
<th>F 5</th>
<th>F 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Saturation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 %</td>
<td>2.87</td>
<td>2.76</td>
</tr>
<tr>
<td>70 %</td>
<td>4.67</td>
<td>3.02</td>
</tr>
<tr>
<td>100 %</td>
<td>5.47</td>
<td>4.77</td>
</tr>
<tr>
<td>Specific activity (Unit/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 %</td>
<td>0.795</td>
<td>0.784</td>
</tr>
<tr>
<td>70 %</td>
<td>1.293</td>
<td>0.857</td>
</tr>
<tr>
<td>100 %</td>
<td>1.515</td>
<td>1.355</td>
</tr>
<tr>
<td>Fold purification</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Yield</td>
<td>32.9%</td>
<td>31.2%</td>
</tr>
<tr>
<td>% Saturation</td>
<td>53.3%</td>
<td>40.8%</td>
</tr>
<tr>
<td>60 %</td>
<td>68.6%</td>
<td>72.6%</td>
</tr>
</tbody>
</table>

Table 3: Results of enzyme activity after Dialysis

<table>
<thead>
<tr>
<th>Parameters (After Dialysis)</th>
<th>F 5</th>
<th>F 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Saturation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 %</td>
<td>1.98</td>
<td>1.88</td>
</tr>
<tr>
<td>70 %</td>
<td>3.44</td>
<td>2.02</td>
</tr>
<tr>
<td>100 %</td>
<td>3.48</td>
<td>2.93</td>
</tr>
<tr>
<td>Specific activity (Unit/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 %</td>
<td>0.548</td>
<td>0.534</td>
</tr>
<tr>
<td>70 %</td>
<td>0.952</td>
<td>0.573</td>
</tr>
<tr>
<td>100 %</td>
<td>0.963</td>
<td>0.832</td>
</tr>
<tr>
<td>Fold purification</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Yield</td>
<td>19.8%</td>
<td>28.6%</td>
</tr>
<tr>
<td>% Saturation</td>
<td>41.5%</td>
<td>31.3%</td>
</tr>
<tr>
<td>60 %</td>
<td>42.3%</td>
<td>50.7%</td>
</tr>
</tbody>
</table>
3.7 Electrophoresis SDS-PAGE analysis:

The molecular weight of protease was determined by comparing it with the standard protein marker (6.5 kDa – 200 kDa). The molecular weight of fungal proteases are generally in the range of 20 and 50 kDa [30].

The development of clear bands on the blue background of the gel indicated the presence of protease activity. SDS-PAGE was done from the supernatant extracted from both the isolates F 5 & F 15. Thicker bands in gel means there is more of that particular size molecule in the sample.

From the Fig. 6 for F 5 isolate band was observed near 40 kDa. Moreover F 15 also gave good proteolytic activity as it shows bands near 44.3 kDa marker.

3.8 Applications of fungal protease:

3.8.1 Antioxidant activity - From the Fig. 7, protease of F 15 has more antioxidant activity than the protease of F 5. Darker the colour, higher is the reducing potential.

<table>
<thead>
<tr>
<th>Fungal sample</th>
<th>Time (in sec)</th>
<th>MCA (Units/cm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F 5</td>
<td>360 sec</td>
<td>6.66 Unit/cm³</td>
</tr>
<tr>
<td>F 15</td>
<td>387 sec</td>
<td>6.20 Unit/cm³</td>
</tr>
</tbody>
</table>
3.8.3 *Feather degradation activity*- Degradation rate was examined during 7 days with day to day observation. From the Fig. 8 it has been said that, there was no degradation in control tube.

![Figure 8 Microscopic examination of feather degradation](image)

3.8.4 *Silk degumming activity*- Degumming of silk by using fungal protease shows difference in colour as well as weight loss. Weight loss was calculated in form of percentage. And protein concentration was increased in enzyme bath. Which was detected by Folin Lowry’s method. From the above results it can be said that protease of F 5 has more efficiency to remove the protein (sericin) layer from the silk fibre.

<table>
<thead>
<tr>
<th>Fungal isolate</th>
<th>Protein concentration</th>
<th>Weight loss (in %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F 5</td>
<td>2.49 mg/ml</td>
<td>5.14 %</td>
</tr>
<tr>
<td>F 15</td>
<td>2.45 mg/ml</td>
<td>2.13 %</td>
</tr>
</tbody>
</table>

3.8.5 *Removal of blood stain from cotton cloth*- Microbial proteases are widely used in detergent industry. Proteases from both the fungal isolate F 5 and F 15 gave better results of washing with/without detergent (Fig. 9).

![Figure 9](image)
3.8.6 Production of amino acids- metal nutrient chelates

- Production of free amino acids

Results show that the amount of amino acids released from undigested seed crush and seed crush digested with protease of F 5 and F 15 noted gradual increase in amino acid with increase in incubation period. Amino acid estimation was performed by Ninhydrin method. L-lysine was used as standard amino acid. Amino acids released from treated & untreated seed crush were recorded at regular interval. The maximum release of amino acids 2.49 mg/ml was recorded at 96 h of incubation in F 5 and 1.79 mg/ml at 96 h of F 15.

- Production and analysis of amino acid- metal nutrient chelate

The amino acids (chelating solution) recovered from enzymatic (F 5 & F 15) digestion were utilized for the production of chelates. The metal nutrients like Cu$^{2+}$ and Mn$^{2+}$ chelated with organic chelating agents were vary in colours. In present investigation, the detection of metal nutrient chelate has been confined by FTIR spectrophotometer.

Figure. 9

A. Distilled water + Stained cloth(Control)
B. Distilled water + Stained cloth + Enzyme of F 5
C. Distilled water + Stained cloth + Enzyme of F 5 + Detergent
D. Distilled water + Stained cloth + Enzyme of F 15
E. Distilled water + Stained cloth + Detergent
F. Distilled water + Stained cloth + Enzyme of F 5 and F 15 + Detergent

Figure. 10 (a)
FTIR can be used to monitor the modification in the vibrational absorption bands of a ligand due to metal-ligand complex formation. The simple stretching vibrations in the 1600 cm\(^{-1}\) to 3500 cm\(^{-1}\) region are the most predictable, and absorptions in this region are used to identify
functional groups in molecules. During chelation, binding of the carboxylic groups and primary amine groups of amino acids to the metal nutrients is indicated by the broadening of peak and changes of the peak position of hydroxyl groups.

In present investigation, detection of metal nutrient chelate has been confined to the higher frequency region in which internal vibrations of the chelating solutions were observed.

4. CONCLUSION

Based on the results obtained in this study, various optimization conditions like carbon-nitrogen source, pH, temperature and solid substrate for SSF were identified to get maximum protease production. Enzyme purification and characterization had been performed. Various applications like, Antioxidant activity, Milk Clotting Activity (MCA), feather degradation activity, Efficiency to remove blood stains & Silk degumming activity was successfully performed using fungal protease.

The amino acids produced from leguminous seeds can chelate metal nutrients. This amino acid-metalnutrient chelates can be utilized as liquid organic fertilizer which can be useful to overcome the metalnutrient deficiency in short term vegetable crops. Hence, these enzyme have number of commercial applications at various places like: Dairy industry, Detergent industry, Laundry, Poultry farm, Slaughter house, Textile industry, fertilizer manufacturing companies, etc.

5. REFERENCES


