

Review Article

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## Protease Production from Biodigested Slurry of Biogas Plants: A Review

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### ABSTRACT

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Protease enzymes are the enzymes that hydrolyse proteins. Each type of protease enzyme breaks specific kind of peptide bonds. These enzymes are of great commercial importance contributing to more than 40% of the world's commercially produced enzymes. Approximately 50% of the enzymes used in industrial processes are proteolytic enzymes. Different substrates used for the protease enzyme production add to the cost and hence makes the process very costly. Different wastes like wheat bran, rice bran, vegetable waste and sweet potato residue are being explored as substrate to be utilized for enzyme production. However, the present review paper mainly focuses on the biodigested slurry either from cattle dung or poultry droppings based gas plant, which has sufficient nutrients and can be used for protease enzyme production. Poultry dropping and cow dung based biodigested slurry has been used first time as a substrate for protease production which may not only pertains to its value addition but will also cures environmental pollution by managing large amount of slurry discharge from biogas plant.

### Introduction

Enzymes are proteins capable of converting a specific compound (the substrate) into another (product) at a high reaction rate. They are also enantio selective catalysts, which enable them to be used either in the separation of enantiomers from a racemic mixture or in the synthesis of chiral compounds. Although the enzymes utilized today are also found in animals (Chymosin, pepsin, trypsin and pancreatin) and plants (ficin, papain and bromelain), still the bulk of them are of microbial origin (pectinases, glucoamylase,  $\alpha$ -amylase, among others). As the

microorganisms present high growing capability, huge amounts of enzymes can be produced in a short period of time as well as strains can be genetically manipulated, in order to improve their enzyme production capability. Moreover, microbial enzymes are well standardized and marketed by several competing companies.

The market for industrial enzymes was valued at about \$1.5 billion in 2015. Blind Carbon Copy (BCC) projects this market to grow at a compound annual growth rate (CAGR) of 6.6% to \$6 billion by 2016 (Anonymous 2016). In the enzyme industry, three types of

enzymes are known: Metabolic enzymes (enzymes which our body produces that work in blood, tissues and organs), digestive enzymes (enzymes that break down food into usable material) and food enzymes (enzymes that are contained in raw food). In addition to these, there are some other industrially important enzymes including cellulolytic, lignolytic and proteolytic etc.

The role of enzymes in many processes has been known for a long time. Their existence was associated with the history of ancient Greece where they were using enzymes from microorganisms in baking, brewing, alcohol production and cheese making etc. Thermostable enzymes, which have been isolated mainly from thermophilic organisms, have found a number of commercial applications because of their overall inherent stability (Demirijan *et al.*, 2001). There are 4 main classes of proteases: serine proteases, cysteine proteases, aspartyl proteases and metalloproteases (Erez *et al.*, 2009). On the basis of pH, proteolytic enzymes are classified as acid, alkaline and neutral enzymes (Sheppard *et al.*, 2007).

### **Production of microbial proteases**

Fermentation is a method of generating enzymes for industrial purposes. It involves the use of microorganisms like bacteria, fungi and yeast to produce the enzymes. Two methods of fermentation have been used to produce enzymes. These are submerged fermentation and solid-state fermentation. Submerged fermentation involves the production of enzymes by microorganisms in a liquid nutrient media. Solid-state fermentation is the cultivation of microorganisms and enzyme production on a solid substrate.

Renge *et al.*, (2012) reported that the SSF uses inexpensive substrates and yields higher

volumetric productivity. The isolated fungal culture *Aspergillus* sps. from soil polluted with abattoir waste secreted extracellular acid proteases in submerged fermentation (Radha *et al.*, 2011). Proteases produced from microorganisms are constitutive or partially inducible in nature. Extracellular protease production in microorganisms is also strongly influenced by media components, e.g. C/N ratio, easily metabolizable sugars, such as glucose (Beg *et al.*, 2002) and metal ions. Protease synthesis is also affected by rapidly metabolizable nitrogen sources, such as amino acids in the medium. Besides these, several other physical factors, such as aeration, inoculum density, pH, incubation and temperature also affect the amount of protease produced (Puri *et al.*, 2002). Chemostat cultures have been successfully used for improving protease production for long-term incubations, using a number of microorganisms. Beg *et al.*, (2002) reported that the overall alkaline protease yield from *Bacillus mojavensis* was improved up to 4-fold under semi-batch and fed-batch operations by separating biomass and protease production phases, using intermittent de-repression and induction during the growth of the organism.

### **Fungal proteases**

Fungal proteases magnetized the interest of researches due to high diversity, broad substrate specificity and stability under extreme conditions; it offers an advantage of separation of mycelium by simple filtration. Fungal proteases can conveniently be produced in solid-state fermentation process. Fungal proteases are also used in for modifying food proteins. The different alkaline protease producing fungal species are *Aspergillus candidus*, *Aspergillus flavus*, *Aspergillus fumigates*, *Aspergillus melleus*, *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus sojae*, *Aspergillus sydowi*,

*Cephalosporium sp. KSM 388, Paecilomyces lilacinus, Scedosporium apiospermum Rhizopus oligosporus.* (Velooralappil *et al.*, 2013)

The enzymes of fungi are largely acid proteases however many fungi have also been reported to produce neutral and alkaline proteases (Tremacoldi *et al.*, 2004). Acid proteases are the most important group of proteases with respect to use in food industry. The acid proteases are low in basic amino acid content and hence have low isoelectric points. However, some strains of *Aspergillus niger* (Heneri *et al.*, 1988) and *Aspergillus flavus* also produce alkaline protease (Malathi and Chakraborty, 1991).

### **Bacterial proteases**

Bacterial alkaline proteases have more commercial importance in laundry, food, leather and silk industries due to their high production capacity and catalytic activity. Bacterial alkaline proteases are characterized by their high activity at alkaline pH (8 - 12), with optimal temperature between 50°C and 70°C.

These properties of bacterial alkaline proteases make them suitable for use in the detergent industry. Prominent bacteria producing proteases are *Alteromonas sp.*, *Arthrobacter protophormiae*, *Brevibacterium linens*, *Hyphomonas jannaschiana VP 3*, *Lactobacillus helveticus*, *Microbacterium sp.*, *Nocardiopsis dassonvillei*, *Oerskovia xanthineolytica TK-1*, *Pimelobacter sp. 2483*, *Pseudomonas aeruginosa*, *Pseudomonas maltophilia*, *Pseudomonas sp. SJ320*, *Salinivibrio sp. Strain AF-2004*, *Staphylothermus marinus*, *Streptomyces isolate EGS-5*, *Streptomyces microflavus*, *Streptomyces moderatus*, *Streptomyces rectus* and *Streptomyces rectus var. proteolyticus* (Velooralappil *et al.*, 2013).

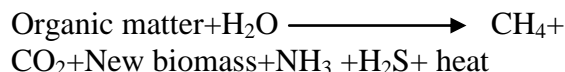
### **Substrates for proteases production**

Substrates play an important role in production of enzymes using microbial source. A novelty in fermentation medium design is necessary to increase protease enzyme production with low cost solid substrates. Magdi *et al.*, (2009) reported that *Bacillus subtilis* KO strain was isolated from molasses that was obtained from the industrial products of sugar factory. Different experiments were performed to optimize the cultivation conditions of *Bacillus subtilis* KO strain and the medium contents to reduce the cost of protease enzyme production. Optimum protease enzyme production of 30-32 IU/ml was reported after 48 hr incubation period at 40-45°C, pH from 7-7.5, 0.1% (w/v) gelatin concentration in the presence of ammonium phosphate as inorganic source. Rauf *et al.*, (2010) reported optimal conditions for protease enzyme production using sunflower meal as a substrate with inoculum size of (1%), at pH 3.0 and incubation temperature of 35°C after cultivation period of 72 hr. The following table 1 depicts various substrates used for protease production by various researchers.

### **Biodigested Slurry**

Biogas represents a mixture of different gases produced as a result of the action of different anaerobic microorganism on domestic and agricultural waste (Ezeonu *et al.*, 2005). It usually contains more than 50% methane and other gases namely carbon dioxide, hydrogen, nitrogen and oxygen in relatively low proportions. The mixture of the gases is combustible if the methane content is more than 50% (Agunwamba, 2001). Biogas has proven to be a modern energy source that has improved the life of people (Sasse, 1988). In India initially, cowdung or gohar was used as main fermentative material. Thus, these are mostly popular by the name of gohar gas

plant. Biogas plant consist of mainly two parts: A digester or fermentative tank with an inlet into which the fermentative mixture (cowdung and water in the ratio of 1:1) is introduced in the form of slurry and a gas holder to collect biogas and helps to cut off air to the digester with an overflow pipe or outlet chamber to lead out the spent slurry or digested slurry.



The organic components of biomass can be classified into broad biological groups: proteins, carbohydrates and lipids or fats. Carbohydrates make up the bulk of the biodegradable material and include cellulose, starch and sugars. Proteins are large complex organic materials composed of hundreds of thousands of amino acid groups. Lipids or fats are materials containing fatty acids. The anaerobic treatment of biomass involves two distinct stages (William, 1999). In the first stage, complex components, including fats, proteins and polysaccharides are hydrolysed and broken down to their component subunits. This is facilitated by facultative and anaerobic bacteria, which then subject the products of hydrolyses to fermentation and other metabolic processes leading to the production of simple organic compounds. This first stage is commonly referred to as acid fermentation. In this stage, organic material is simply converted to organic acids, alcohols and new bacterial cells. The second stage involves the conversion of the hydrolysis products to gases (mainly methane and CO<sub>2</sub>) by several different species of strictly anaerobic bacteria and is referred to as methane fermentation. Anaerobic digestion is a relatively efficient conversion process for poultry dropping producing a collectable biogas mixture with an average methane content of 60%. Systems are usually site specific but must have a certain minimum amount of poultry dropping to supply a given system. The methane produced

by this process can be used as a fuel for boilers, as a replacement for natural gas or fuel oil and can also be fired in engine-generators to produce electricity for on-farm use or sale to electricity companies. Biodigested slurry produced in the plants have various applications particularly for increasing soil fertile.

### **Poultry droppings**

Kelleher *et al.*, (2002) reported that waste from the poultry industry includes a mixture of excreta (manure), bedding material or dropping (e.g. wood shavings or straw), waste feed, dead birds, broken eggs and feathers removed from poultry houses. Other wastes include those from cage, conveyer belt and water flushing systems. The dropping and manure component of this waste has a high nutritional value and is used as an organic fertiliser, thus recycling nutrients such as nitrogen, phosphorous and potassium. These components (poultry litter) have traditionally been land spread on soil as an amendment. However, over-application of this material can lead to an enriching of water nutrients resulting in eutrophication of water bodies, the spread of pathogens, the production of phytotoxic substances, air pollution and emission of greenhouse gases. Bitzer and Sims, (1988) reported that excessive application of poultry dropping in cropping systems can result in nitrate (NO<sub>3</sub>) contamination of ground water. High levels of NO<sub>3</sub> in drinking water can cause methaemoglobinaemia (blue baby syndrome), cancer, and respiratory illness in humans and fetal abortions in livestock. Alternative, environmentally acceptable, disposal routes, with potential financial benefits, may lie in large-scale biomass to energy schemes that can also provide an easier to handle fertiliser as a by-product. Three options have been considered and in some cases implemented: centralised anaerobic digestion, composting and direct combustion with combined heat and

power. The cost of transporting feedstock has, in all cases, been the limiting factor.

### **Cow dung**

Bains *et al.*, (1973) investigated principles of treatment of animal slurries. Biological systems are considered more suitable for recycling purposes, minimising the possibilities of added toxic chemical residues entailed in chemical treatment.

Physical treatment cannot meet *al.*, the objectives and is primarily concerned with disposal such as incineration and dumping. The solid removal in pig and cattle dung slurries was 25-30% at low loading rates and a greater reduction of the order of 40-50% was obtained in the treatment of poultry slurry. COD reduction of about 60-90% in the treatment of pig slurry, 75-90% with cattle slurry & 50-67% in poultry slurry was obtained.

The equipment and methods for the solid liquid separation of slurries are now widely used. Rotary screen with press rollers, combined brushed screen, vibrating screen, & roller press, vacuum filtration and pressure filtration are some of the methods for the treatment of animal slurries. Application of sedimentation principles for the treatment of animal waste slurry is effective. Deans and Evans (1987) examined gamma irradiation as a possible method for treating slurry. The chemical treatment with metal salts and organic polymers for solid liquid separation of animal manure has also been done.

Table 2 summarizes various substrates used for biogas production, thus producing large amount of slurry. This biodigested slurry is a big menace and needs proper management and value addition otherwise causes environmental pollution.

The proximate and chemical composition of Cow dung, poultry dropping and biodigested slurry determined as per the table 3 which shows that biodigested slurry nitrogen content decreased from cow dung and poultry droppings. So, these all analysis show that biodigested slurry it a great substrate for protease production.

### **Optimization of cultural parameters**

Incubation period, pH, incubation temperature and size of inoculum are important parameters for maximum yield of enzyme varies among various species or even in the same species isolated from various sources. Many workers have reported a broad incubation period ranging from 36 to 96 h for the maximum yield of protease enzyme by *Bacillus* strains (Gupta *et al.*, 2002; Olajuyigble and Ajele, 2008).

Initial pH of the medium required for obtaining maximum production depends not only upon the bacterium but also upon the ingredients of the medium. Maximum alkaline protease production has been reported from different pH ranges (7-11) by different *Bacillus* species (Khosravi *et al.*, 2008). Temperature is one of the most critical parameters that have to be controlled in any bioprocess (Chi and Zhao, 2003). Optimum production temperature for different *Bacillus* species have been reported as, 25° C for *Bacillus circulans* (Jaswal and Kocher, 2007).

### **Characterization of proteases enzyme**

Usharani and Muthuraj (2009) reported that optimum pH for enzyme stability found to be 7.0. At pH 6.0 only 63% of the maximum enzyme activity was obtained, increasing to 87% at pH 7.0 and the enzyme activity was declined gradually after pH 7.0.100% of enzyme activity was still detectable at pH 7.0 after 4 h at 37°C.

**Table.1** Different substrates used for protease production

| Substrate                  | Micro-organisms                                | References                          |
|----------------------------|--|-------------------------------------|
| Wheat bran                 | <i>Aspergillus flavus</i>                      | Muthulakshmi <i>et al.</i> , (2011) |
| Rice bran                  | <i>Rhizopus</i> strains                        | Sumantha <i>et al.</i> , (2006)     |
| Waste hair                 | <i>Paecilomyces lilacinus</i>                  | Cavello <i>et al.</i> , (2012)      |
| Pigeon Pea                 | <i>Bacillus sp.</i> JB 99                      | Johnvesly <i>et al.</i> , (2002)    |
| Agro waste storage compost | <i>Bacillus laterosporus</i> -AK1              | Arulmani <i>et al.</i> , (2007)     |
| Herbivorous dung           | <i>Mucor sp.</i>                               | Alves <i>et al.</i> , (2005)        |
| Rabbit dung                | <i>Beauveria sp.</i> MTCC 5184                 | Shankar <i>et al.</i> , (2011)      |
| Waste water                | <i>Aspergillus clavatus</i> ES1                | Hajji <i>et al.</i> , (2007)        |
| Vegetable waste            | <i>Bacillus circulans</i>                      | Jaswal <i>et al.</i> , (2008)       |
| Sewage sludge              | <i>Geobacillus caldoproteolyticus, sp. nov</i> | Chen <i>et al.</i> , (2004)         |

**Table.2** Potential Biogas production from various substrates

| Substrate                 | *HRT (days) | Solid concentration (%) | Temperature (°C) | Biogas yield added (m <sup>3</sup> / Kg VS*) | Methane (%) |
|---------------------------|-------------|-------------------------|------------------|--|-------------|
| Sewage sludge             | 25          | 6.0                     | 35               | 0.52   | 68          |
| Domestic garbage          | 30          | 5.0                     | 35               | 0.47   | -           |
| Piggery waste             | 20          | 6.5                     | 35               | 0.43   | 69          |
| Poultry waste             | 15          | 6.0                     | 35               | 0.50   | 69          |
| Cattle waste              | 30          | 10.0                    | 35               | 0.30   | 58          |
| Canteen waste             | 20          | 10.0                    | 35               | 0.60   | 50          |
| Food market waste         | 20          | 4.0                     | 35               | 0.75   | 62          |
| Mango processing waste    | 20          | 10.0                    | 35               | 0.45   | 52          |
| Tomato processing waste   | 24          | 4.5                     | 35               | 0.63   | 65          |
| Lemon waste               | 30          | 4.0                     | 37               | 0.72   | 53          |
| Citrus waste              | 32          | 4.0                     | 37               | 0.63   | 62          |
| Banana peel               | 25          | 10.0                    | 37               | 0.20   | 55          |
| Pineapple waste           | 30          | 4.0                     | 37               | 0.37   | 60          |
| Mixed feed of fruit waste | 20          | 4.0                     | 37               | 0.62   | 50          |

\*HRT: Hydraulic retention time; VS: Volatile solids (Somayaji, 2005)

**Table.3** Proximate and chemical analysis of dung and biodigested slurry

| S. No | Parameters                                       | Substrate        |   |            |                                   |
|-------|--|------------------|---|------------|-----------------------------------|
|       |  | Poultry dropping | Poultry dropping based Biodigested slurry | Cow Dung   | Cow Dung based Biodigested slurry |
| 1.    | pH   | 6.2±0.7          | 8.1±0.05                                  | 6.97±0.03  | 7.12±0.05                         |
| 2.    | Total Solids (TS% dry weight basis)              | 38.23±0.40       | 7.46±0.70                                 | 18.32±0.22 | 8.41±0.23                         |
| 3.    | Volatile Solids (VS% dry weight basis)           | 63.70±0.76       | 54.35±1.32                                | 84.22±0.20 | 66.34±0.25                        |
| 4.    | Ash (% dry weight basis)                         | 37.82±1.08       | 46.41±1.87                                | 15.31±0.30 | 33.03±0.16                        |
| 5.    | Acid Detergent Fibre (ADF % dry weight basis)    | 16.23±1.17       | 41.51 ±0.94                               | 46.96±0.12 | 55.23±0.28                        |
| 6.    | Neutral Detergent Fibre (NDF % dry weight basis) | 33.75±0.80       | 37.76±0.30                                | 64.65±0.35 | 56.99±0.12                        |
| 7.    | Cellulose (%)                                    | 9.33±0.41        | 6.09±0.90                                 | 21.16±0.30 | 10.26±0.28                        |
| 8.    | Hemicellulose (%)                                | 18.48±0.80       | 10.07±0.10                                | 18.05±0.14 | 0.79±0.03                         |
| 9.    | Lignin (%)                                       | 27.49±0.46       | 22.31±1.11                                | 16.43±0.25 | 38.01±0.34                        |
| 10.   | Silica (%)                                       | 1.77±0.17        | 2.75±0.39                                 | 5.18±0.12  | 16.10±0.08                        |
| 11.   | Nitrogen (%)                                     | 7.56±0.85        | 6.28±1.05                                 | 4.51±0.03  | 3.37±0.04                         |
| 12.   | Protein (mg/ml)                                  | 0.61±0.85        | 0.48±0.09                                 | 1.09±0.03  | 0.41±0.02                         |

(Goyal *et al.*, 2016) (Kaur and Phutela, 2017)

This might be the neutral protease. The optimum temperature of enzyme was 35°C. Enzyme activity was stable with temperature within the range of 30°C to 60°C.

Kamoun *et al.*, (2008) reported that enzyme was active at temperature from 50 to 75°C and had an optimum at 65-70°C, while activity decreased rapidly above 70°C. Addition of 2mM CaCl<sub>2</sub> resulted in a shift in the apparent optimal temperature for activity from 65-70°C to 70°C.

Oseni (2011) reported that protease from *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus fumigatus* showed the highest activity at day 4 ( $7.5 \times 10^{-4}$  U/ml/min), ( $1.97 \times 10^{-3}$  U/ml/min), ( $4.30 \times 10^{-3}$  U/ml/min) respectively. *Penicillium italicum* showed its highest activity at day 3 ( $1.87 \times 10^{-3}$

U/ml/min) and lowest activity at day 5 ( $8.3 \times 10^{-4}$  U/ml/min).

#### **Protease enzyme production from biodigested slurry**

Protease enzyme profile of biodigested slurry of poultry dropping based biogas plant was analysed which showed that biodigested slurry has more enzyme activity of 157 U/g and 163 U/g by *Humicola fuscoatra* MTCC 1409 and *Aspergillus* (T-14) respectively (Goyal *et al.*, 2016).

Protease activity of biodigested slurry of cow dung based biogas plant showed the activity of 64.39 U/g and 54.37 U/g in biodigested slurry from *Trichoderma harzianum* MTCC 792 and *Aspergillus* (BL4) (Kaur and Phutela, 2017)

## Industrial applications of proteases

### Detergent additives

Proteases are one of the standard ingredients of all kind of detergents ranging from those used for household laundering to the reagents used for cleaning contact lenses or dentures. The enzymes must be stable at high temperature and pH and have compatibility with bleaches, surfactants, chelating and oxidizing agents present in the detergents (Oberoi *et al.*, 2001). Alkaline proteases from *Bacillus mojavensis* (Beg and Gupta, 2003), *Bacillus sp.* L21 (Tari *et al.*, 2006) and *Bacillus licheniformis* RP1 (Sellami-Kamoun *et al.*, 2008) have also been reported useful for detergent industry.

### Leather tanning

Proteases are used in the soaking, dehairing and bating stages of preparing skins and hides. The enzymatic treatment destroys undesirable pigments, increases the skin area and thereby clean hide is produced. Bating is traditionally an enzymatic process involving pancreatic proteases. Varela *et al.*, (1997) reported the use of *Bacillus subtilis* IIQDB32 alkaline protease for unhairing sheep skin.

### Management of industrial and household waste

Proteases solubilize proteinaceous waste and thus help lower the biological oxygen demand of aquatic systems. The use of keratinolytic protease for food and feed industry waste, for degrading waste keratinous material from poultry refuse (Ichida *et al.*, 2001) and as depilatory agent to remove hair from the drains (Takami *et al.*, 1992) has been reported. A formulation containing proteases from *Bacillus subtilis*, *Bacillus amyloliquefaciens* and *Streptomyces sp.* and a disulfide reducing agent (thioglycolate), that

enhances hair degradation and helps in clearing pipes clogged with hair-containing deposits, is currently available in market.

### Photographic industry

Alkaline proteases play a crucial role in the bioprocessing of used X-ray or photographic films for silver recovery. These waste films contain 1.5–2.0% silver by weight in their gelatin layer, which can be used as a good source of silver for a variety of purposes. Ishikawa *et al.*, (1993) have reported the use of alkaline protease of *Bacillus sp.* B21-2 for the enzymatic hydrolysis of gelatin layers of X-ray films to release silver particles.

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