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Alkaline Protease Production by Immobilized *Klebsiella aerogenes* Cells from Dairy Effluent Sludge

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Abstract
This study investigated the screening of alkaline protease microorganism from diary effluent sludge and identified by 16S ribosomal RNA nucleotide sequence as *Klebsiella aerogenes* with accession number MF156964.1 and maximum identity 95.45%. The cells were immobilized with coconut pod husks and optimization studies such as the effects of particle sizes, pH, temperature, agitation speed, and incubation time were determined. Out of twenty three microorganisms screened, three were potential protease producers. *K. aerogenes* gave the highest zone of hydrolysis (35 mm) on the skimmed milk agar plate. The particle size (0.075 mm^2) of the immobilization agent gave the highest enzyme activity 176.83 U/mL. The optimum incubation time for the production of protease was 48 h with enzyme activity 143.054 U/mL which further declined. The optimum pH of the protease was pH 9.0 with activity 209.61 U/mL which made it alkaline. The agitation speed 150 rpm resulted in a protease activity 175.83 U/mL and reduced by 56.5% at 250 rpm. The optimal temperature 35 ºC was 183.78 U/mL. This study also confirmed the stability and reusability of the immobilized cells using the coconut pod husks matrix by maintaining from 100% to 76.2% up to six times recycle. Conclusively, the study established the efficiency of low cost, readily available matrix and reusability potentials of coconut pod husks for cells immobilization technology through entrapment at optimal conditions for protease production.
Keywords: *Klebsiella aerogenes*; Alkaline protease; Coconut pod husks; Immobilization; Optimization
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Introduction

Almost 60% of the world enzyme market comprises protease from microbial sources among other important groups of industrial enzymes (Nurullal et al., 2011). The alkaline proteases are of biotechnological significance because of their numerous applications in peptide synthesis for infant formula preparations; meat tenderization process, dehairing and leather processing in textile industry; additives in detergent industry and in pharmaceutical and medical diagnoses (Singh, et al., 2016; Rehman, et al., 2017).

Proteases are enzymes that help in proteolysis (protein catabolism) by peptide bonds hydrolysis. They can be classified according to their pH values as neutral proteases, acid proteases and alkaline proteases (Rupali, 2015). Alkaline protease is a serine endoprotease which has the ability to hydrolyze a wide range of peptide linkage established in plant and animal proteins. Over 350 tonnes of proteases have been synthesized by bacteria and they are attracting more significance above conventional chemicals that cleave peptides because of renewability and low price of cost of production. Microbial proteases can also be produced from fungi and yeast (Potumarthi et al., 2007). There are many alkaline protease producing microorganisms, but those from *Bacillus* strains

possess distinctive ability due to their high activity and stability at high pH, different temperature ranges, ease of purification and wide range substrate specific (Chatterjee, 2015; Asha and Palaniswamy, 2018). Alkaline proteases are mostly used in the detergent industry due to the pH range of detergents (pH 9 – 12) and their ability to degrade protein related strains for example food, blood and grass stains (Asha and Palaniswamy, 2018). As a biocatalyst, immobilized cells show high operation stability, efficiency and are eco-friendly, which makes them profitable for industrial applications (Kumari, *et al,* 2009). However, enzyme immobilization protocol is adopted when the enzyme is either difficult or expensive to extract (Aragao *et al.,* 2014). Whole cell immobilization is used for convenience and on a commercial basis where the need of the product is more demanded (Zaushitsyna *et al.,* 2013).

The use of a natural suitable matrix (coconut pod husk) will help to improve the time of action of the microorganism, stability, reusability and the overall performance of the enzyme. Also, it will help in the industries as a more and cheaply available matrix replaces the expensive and less available hereby helping companies save cost while improving profit (Chatterjee, 2015; Duman and Tekin, 2020). The aim of this present work was to produce alkaline protease by immobilized cells of *Klebisiella aerogenes* using a suitable matrix (Coconut pod husk) and characterizations of various condition parameters. To determine the stability and reusability efficiency of the coconut pod husks as a suitable substrate for cell immobilization of *K. aerogenes.*

Materials and Methods

Microorganisms

Potential protease degrading microorganisms were isolated from the effluent sludge of a soy milk company Lagelu Industrial Estate, Lagos-Ibadan Expressway, Lagelu Local Government Area, Ibadan, Oyo State and they were tested for their ability to produce protease and ferment lactose.

Media/Substrates/Reagents

Nutrient agar (NA) (HiMedia Laboratories Pvt, India); Nutrient Broth (NB) (Biolab Budapest, Hungary). Potato Dextrose agar (PDA) (Microexpress India). Coconut pod husk (Ikotun market, Alimosho Local Government Area, Lagos).

Methods

Sample collection

Effluent sludge was collected from a dairy company at Ibadan, Oyo State. Bacterial and fungal isolations were carried out using serial dilution and spread plate procedures. Pure cultures were maintained in Nutrient agar and Tryptone soy agar at 27 °C (for fungi) and 37 °C (bacteria) as well as sub-cultured from time to time to maintain its viability in the laboratory.

Screening for Protease Production

Protease production by the bacterial strain was screened on agar plates supplemented with skimmed milk agar (SMA) which contains 1% skimmed milk, 1% peptone, 0.5% sodium chloride and 2 % agar powder. Screening media were adjusted to pH 7.2, inoculated with *Klebsiella aerogenes* and incubated at 37 °C for 24-48 h. Protease production was confirmed based on the zone of clearance (hydrolysis) formed.

Inocula Preparation

The Inocula were prepared by adding a loop full of pure culture to sterilized nutrient broth under aseptic conditions.

Preparation of Tyrosine Standard Solution

Tyrosine standard solutions were prepared with ten different concentrations of tyrosine in 100 mL of water. Tyrosine solutions of 0 mg to 100 mg were added in 100 mL of water and then checked for their optical density

Molecular Identification (16S ribosomal RNA nucleotide sequence)

Total genomic DNA extraction, Polymerase Chain Reaction (PCR) and DNA sequencing using standard protocols were investigated at the Bioscience Laboratory, International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. The bacterium isolate was subjected to the extraction of total genomic DNA according to the protocol of Zymo Research Bacterial/Fungi DNA MiniPrepTM Instruction Manual and kit. DNA amplification was carried to evaluate phylogenetic grouping of the genomic DNA. The PCR cocktail mix consist of 2.5ul of 10x PCR buffer,1ul of 25mM MgCl2, 1ul each of forward primer (16SF: GTGCCAGCAGCCGCGCTAA) and reverse primer (6SR: AGACCCGGGAACGTATTCAC).

Analysis of fluorescent labeled 16s RNA products generated by PCR cocktail mix on an AB 373a Stretch (short gun) DNA sequencer was used to determine the nucleotide sequences. Primers 16SF: GTGCCAGCAGCCGCGCTAA and 16SR: AGACCCGGGAACGTATTCAC were used for sequencing processes. The 16sRNA sequences were submitted to the non-reductant nucleotide database at Genbank using the BLAST program to determine the identity of the microorganisms (https://www.ncbi.nlm.nch.gov)

Immobilization Techniques

Small pieces of coconut pod husks were caught into different sizes (Plate 1) soaked and washed severally in distilled water and then autoclaved in 100 mL flasks. Then 6 mL nutrient broth, containing 5.3 x 10^4 cells/ml of microbial suspension added were incubated at 37 ºC, 150 rpm for 24 h.

Protease Enzyme Production

Protease production medium (g/100 mL) containing glucose-0.1, peptone-1, yeast extract-0.02, K_2HPO_4-0.05, $CaCl_2$-0.01, $MgSO_4$-0.01 (pH 7.0). Sterile medium (100 mL) was measured into 250 mL shake flask and inoculated with 5 mL of overnight *K. aerogenes* strain culture, incubated at 37 °C, 150 rpm for 24 h in a shaking incubator (UI-Qadar *et al.,* 2009).

Proteolytic Activity Assay

Fermentation culture was centrifuged at 10,000 rpm, 4 °C for 15 min. Free cell supernatant was used for protease assay (Sevinc *et al.,* 2011). Enzyme (1 mL) as reaction mixture was added to 1 mL casein (1% w/v in 50 mM potassium phosphate buffer, pH 7.5) and incubated at 37 °C for 10 min. The reaction was terminated by adding 2 mL of 10% trichloroacetic (TCA) acid reagent kept for 30 min incubation at 4 C and then centrifuged for 15 min at 10,000 rpm. Trichloroacetic (TCA) (2 mL of 10%) acid reagent kept for 30 min incubation, centrifuged at 4 °C at 10,000 rpm for 15 min. Then filtrate (2 mL) was mixed with sodium carbonate solution (500 nM) and optical density was taken at 660 nm. One unit of enzyme activity is defined as the amount of protease required to release 1 µmol of tyrosine per minute under the defined assay conditions. The prospective protease producer was used for further optimization parameters to improve the enzyme production.

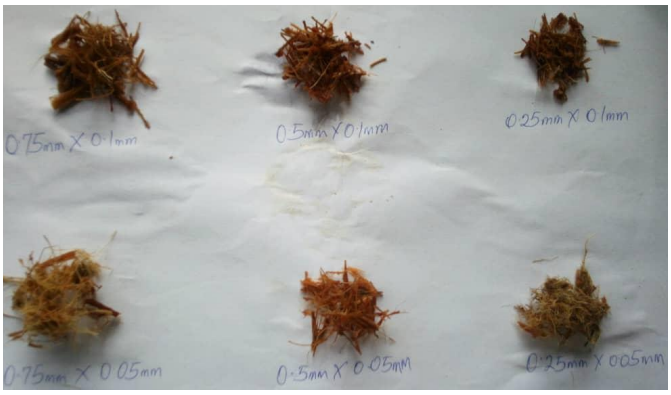

Plate 1: Coconut pod husks matrices used for the study

Optimization of Production Conditions for Protease Production using Immobilized cells of K. aerogenes

The effect of particle sizes of coconut pod husk (0.075 mm^2, 0.05 mm^2, 0.025 mm^2, 0.0375 mm^2, 0.0125 mm^2) on production of protease was investigated by culturing *K. aerogenes* in fermentation media at 37 ºC, pH 7.6 and agitation 150 rpm for 24 h while the pH effect was conducted at different pH value of 4.5, 7.6, and 9.0 using appropriate buffers at 150 rpm, 37 ºC and particle size of 0.075 mm^2 at 24 h. The effect of temperature on protease production was obtained by culturing the bacteria in the production medium at temperature ranges 25–55 °C (10 °C interval) at 150 rpm, pH 7.6 and particle size of 0.075 mm^2 at 24 h. The effect of agitation on protease production was carried out by incubating bacteria in fermentation media at 37 °C, pH 7.6 under shaking conditions; 50, 100, 150, 200, 250 rpm at 24 h.

Repeated use of Coconut pod husks for Protease Production

The potential ability of repeated use of immobilized cells of *K. aerogenes* using the coconut pod husks was carried out at optimized conditions (temperature of 37 °C; 150 rpm, pH 7.6 and particle size of 0.075 mm^2 at 24h) in the protease production medium and was assayed to determine the activity. It was subsequently changed in batches at every 48 h within twenty (20) days.

Results

Isolation and identification of microorganisms isolates

Potential microorganisms were identified from the dairy effluent sludge. These organisms were found to be able to degrade lactose and produce protease as observed from the zone of clearance on skimmed milk agar (Not shown). *Klebsiella aerogenes* with accession number MF156964.1 revealed maximum identity (Fig. 1) when similar sequences were downloaded at NCBI using the BLASTn tool.

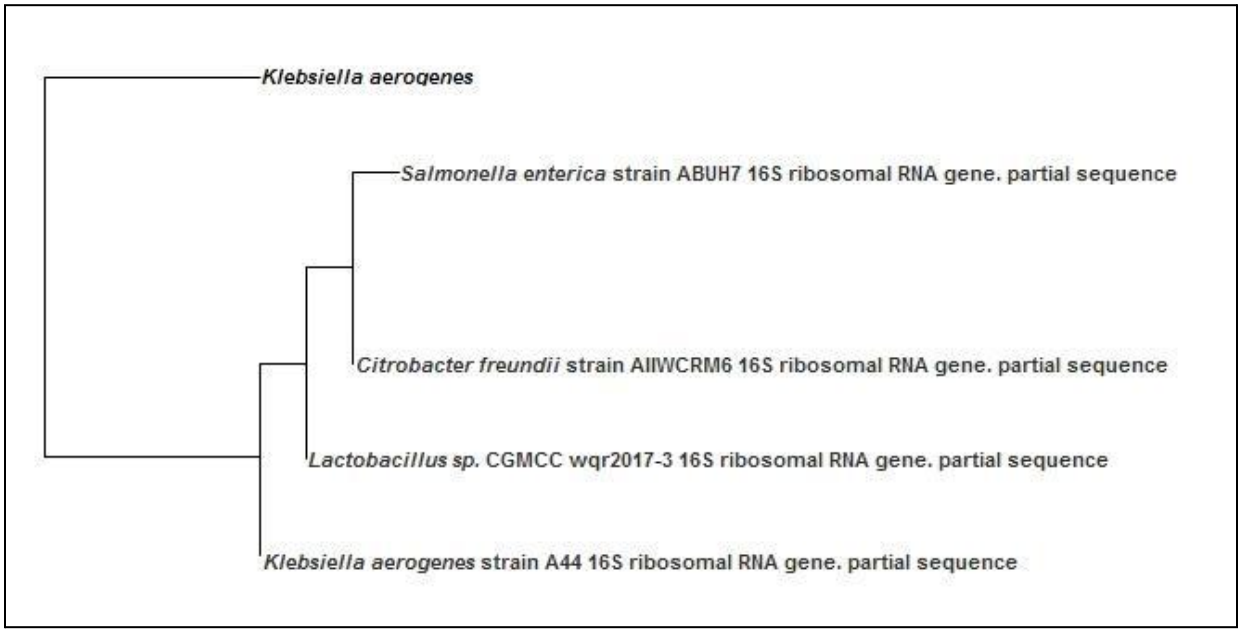

Fig. 1: The Phylogenetic Tree as Identified by FINCH TV

Optimization Conditions of Protease Production using immobilized cells of K. aerogenes

It was discovered that the best particle size to be used for the experiment is 0.075mm^2 with protease activity of 176.83 U/mL (Fig. 2). This particle size was used throughout the experiment.

Over the period of 5 days, assays of the proteolytic activity were carried out to find the best incubation time for the production of protease using the 0.075 mm^2 particle sizes with the parameters were 24 and 120 h. The best time for protease production was 48 h with protease activity of 143.054 U/mL (Fig. 3).

The pH of the protease medium was altered to determine the optimum pH for the production of protease. 0.75mm x 0.1mm coconut pod husks, with 6mL of inoculum were incubated for 48 h using pH of 4.5, 7.6 and 9.0 regulated with appropriate medium. The study revealed the optimum pH for the production of protease is 9.0 making it an alkaline protease. At pH 9.0 the protease activity was 209.61 U/mL (Fig. 4).

Various agitation speed was tried on the protease production medium to determine the optimum speed for protease production. Agitation employed ranged from 50 to 250 rpm at 50 rpm interval. Each was inoculated at a pH of 9.0 for 48 hours. It was discovered that the agitation speed of 150 rpm was the best for production of protease with a protease activity of 175.84 U/mL (Fig. 5).

To determine the optimum temperature for the production of protease selected temperatures between 25-65 ºC were used. Each of these was inoculated at a pH of 9.0, agitation speed of 150 rpm and incubated for 48 h. It revealed the optimum temperature for protease production to be 35 ºC with activity of 183.78 U/mL (Fig. 6).

Repeated use of coconut pod husks immobilized protease of Klebsiella aerogenes

The coconut pod husks immobilized cells of *K. aerogenes* at optimum conditions (Temperature 35 ºC, Agitation 150 rpm, Incubation Time 48 h, Particle size 0.075 mm_2 and pH 9.0) was indicated in Fig.7. The immobilized cells exhibited more than 75% of their initial relative activities after it was used for six (6) times. The protease activity gradually dropped till 19.9% at the 10th trial.

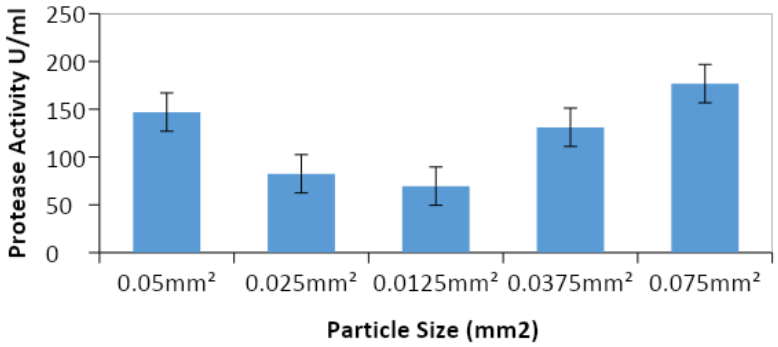

Fig. 2: Effect of Particle Sizes of Coconut pod husk on Protease Production. The error bar represent the standard deviation of three samples).

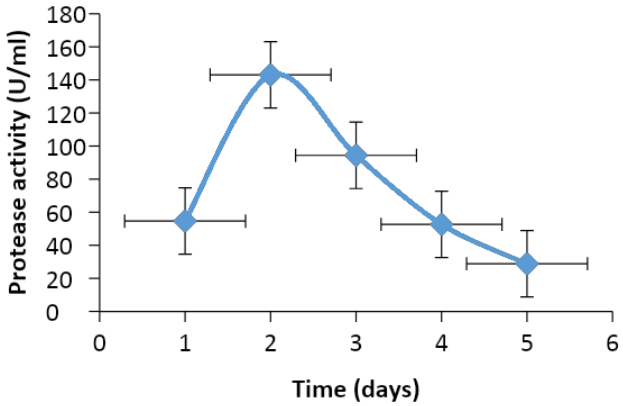

Fig 3: Effect of Incubation Period on Production of Protease on *K. aerogenes* immobilized cells. The error bars represent the standard deviation of three samples)

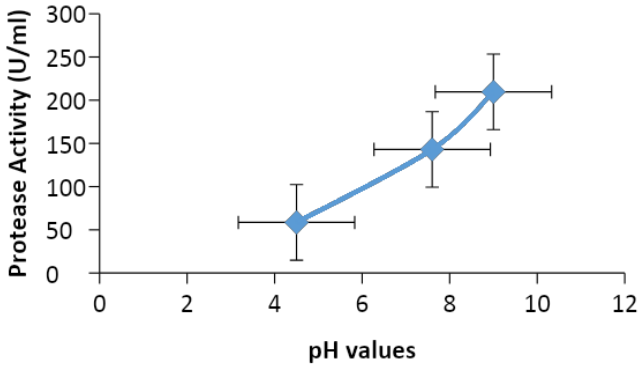

Fig. 4: Effect of pH on Protease Production by immobilized cells of *K. aerogenes* The errors bar represent the standard deviation of three samples).

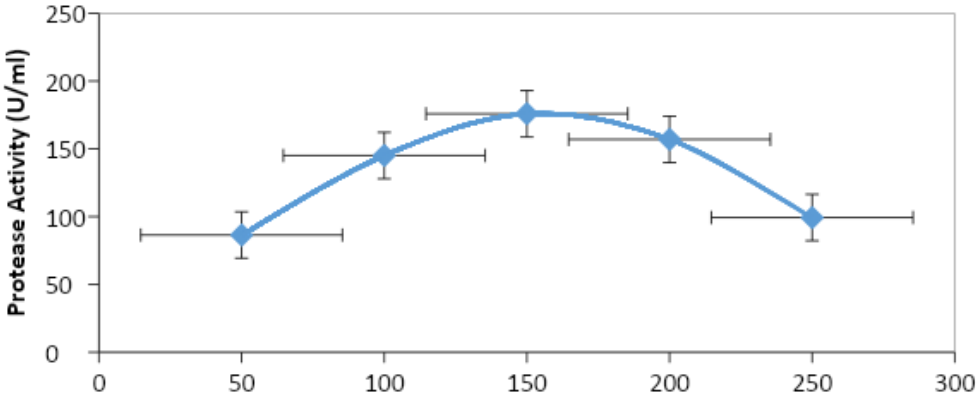

Fig. 5: Effect of Agitation on Protease Production by immobilized cells of *K. aerogenes.* The error bars represent the standard deviation of three samples)

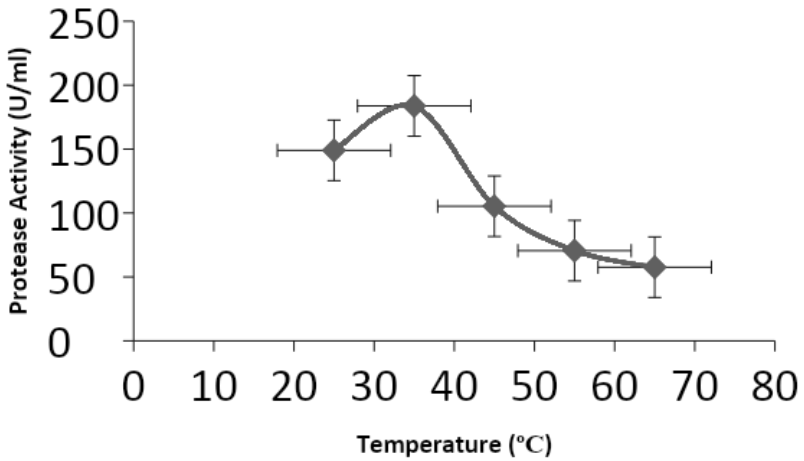

Fig. 6: Effect of Temperature on Protease Production by immobilized cells of *K. aerogenes.* (The error bars represent the standard deviation of three samples).

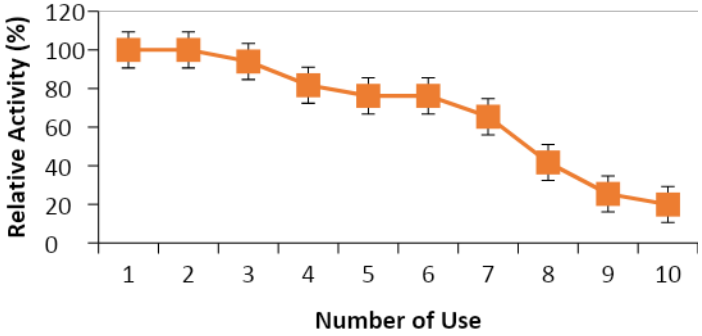

Fig. 7: Repeated Use of Coconut pod husk immobilized protease of *K. aerogenes*. Reaction Conditions – Temperature 35 ºC, Agitation 150 rpm, Incubation Time 48 h, Particle size 0.075 mm^2 and pH 9.0. The error bars represent the standard deviation of three samples)

Immobilized and Leakage Cells of K. aerogenes

The percentage of immobilized cells of *K. aerogenes* and leaked cells from the medium at the optimum conditions were determined using the total plate count method. It was discovered that 74.6% of cell was immobilized by the coconut pod husks and

82% of free cells were leaked into the protease production medium at the end of the fermentation period.

Discussion

Klebsiella aerogenes rod shaped bacterium is a gram negative, catalase and indole positive, but oxidase negative with peritrichous flagella for motility (Sanders and Sanders 1997). Protease production from *K. aerogenes* was influenced by factors like pH, temperature and incubation periods. They are good producers of hydrogen and consume different sugar unlike strict aerobes (Asadi and Zilouei 2017).

Although there has been a lot of work done on utilizing organic matrices in immobilization procedures of enzymes and cells, to the best of our knowledge no research study has been found using coconut pod husks. A related work report that was found involved the use of chitosan, corn cob and corn tassel in alkaline protease production by immobilized cells using *Bacillus licheniformis* (Vida *et al.,* 2013). Coconut pod husks are inexpensive, eco-friendly and contain about 26.72% and 17.73% cellulose and hemicellulose respectively (Muharja *et al.,* 2020), alongside other potentially active functional groups and superficial charges on their surface. Particle size, structure and porosity of the diffusing molecule is also an important factor determining the degree of immobilization of microorganisms (Osho *et al.,* 2014; Zur *et al.,* 2016). This study revealed that the larger the surface area of the immobilization agent the more the microorganism in the medium and hence the higher the protease activity. This yield could also be a result of the solubility and particle size of the immobilization agent, which supported the availability of sufficient oxygen for microorganism growth in the pore spaces (Gorecka and Jastrzabska, 2011).

In the optimization studies carried out in this study, the maximum protease activity obtained was 209.61 U/mL at pH 9 which was in agreement with the work of Vida *et al, (*2013) having the highest activity of 176.7 U/mL at the same pH. Vida *et al.,* (2013) and Vishakha *et al., (*2013) also noted that protease activity increases steadily until it gets to the maximum protease activity then it begins to drop gradually. The pH indicated that *Klebsiella aerogenes* was an alkaline protease producing organism like *Bacillus licheniformis* as the protease activity of the microorganisms increased with an increased pH. Temperature is an important factor for enzyme synthesis. For the effect of temperature, the temperature was in the mesophilic range mostly between 30 – 40 ºC (Vida *et al.,* 2013). However, studies utilized high temperatures to increase the rate of biotransformation and the solubility of water immiscible substrates (Kongpol *et al.,* 2009). For the effect of incubation time, the optimum incubation time was 48 h, after this time the protease production reduces, this is due to the depletion of nutrients in the protease production medium. In other studies bacteria producing high level of protease have an incubation period of 48-72 h (Tang *et al.,* 2008, Badoei-Dalfard and Karami, 2013, Anbu, 2016). Coconut pod husks have a high percentage of cell immobilization with 74.6% being immobilized into the coconut pod husks. In comparison to chitosan corn tassel and corn hub (Vida *et al,* 2013) which all had lower percentages of immobilized cells.

Cell immobilization helps to restrict microorganisms and this helps microbiologists and biotechnologists in many aspects, one of them is in continuous fermentation. This research gives an insight on how protease is produced by *K. aerogenes* which can find various applications in industries due to its stability in pH (as detergents are usually made with high pH) and other favorable parameters. It also shows that coconut husks are a very good immobilization agent having 74.6% of cells being immobilized.

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