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Extracellular Enzymatic Assays of Lipolytic Bacteria on Lipid-Rich Abattoir Waste water

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Abstract: Wastewater from abattoirs has great impact on the environments, since it is made up of a significant portion of organic contents such as lipids containing high BOD and COD. Amongst other sources of lipid-rich wastewater, in the abattoir wastewater, a significant amount of bacterial capable of utilizing lipids and other organic contents has been found. Exploiting the extracellular hydrolytic capabilities of these microbes is necessary for the overall reduction in the organic contents of the wastewater. Lipolytic bacterial cultures of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *and Bacillus subtilis* were used in the treatment of the lipid-rich wastewater with enrichment nutrient combinations. Thus, the extracellular enzymes; lipase, protease and amylase were assayed at 6hour intervals for a period of 48hours for hydrolytic activity. *Bacillus subtilis* treatment showed the highest amylase and protease activity while *Pseudomonas aeruginosa* showed the highest lipase activity and high amylase and protease activities with *Staphylococcus aureus*, also a good lipase producer. The result indicated extracellular production of the enzymes to cater for the initial breakdown of substrates in the wastewater medium. Owing to the enzymatic activities of lipase, protease and amylase possessed by these lipolytic organisms, they could prove to be beneficial for bringing down the overall organic load of abattoir wastewater.
Keywords: Abattoir, Wastewater, Lipids, Enzymes, Lipolytic bacteria, Degradation, Hydrolytic, Extracellular.

INTRODUCTION

From wastewater sources such as abattoir, a significant portion is made up of organic content such as fats and oils, also known as lipids. The amount of lipid in municipal wastewater is approximately 30-40% of the total chemical oxygen demand [1]. Many oils solidify and form deposits at low temperatures they are still a problem if they remain in a liquid form, often binding to fat and grease deposits and actively increasing the potential for lipids deposit formation [2]. Lipid droplets may also act to concentrate contaminants.

A significant amount of microorganism genera capable of metabolizing lipids in wastewater environments and can degrade wide range of contaminants due to their hydrolytic potentials are presently known and many are yet to be discovered [3-7]. In fact, the lipase-producing microorganisms are found in fat and oil contaminates sources. Thus, they were isolated from bakery, slaughterhouse, kitchen, restaurants and palm oil industry wastewater [8]. Thus, exploiting of the hydrolytic capabilities of microbes, has recently gained attention because of stringent environmental regulations, their metabolically versatility, and a clean and friendly application of enzymes [9-11].

For microbial culture to be effectively applied for biodegradation of lipid-rich effluent, it should be able to produce not only lipase for lipid biodegradation, but also other important hydrolytic enzymes, to bring about an overall reduction in the effluent organic load [12]. Lipids and other macromolecules are too large to be transported directly into microbial cells. Thus, extracellular enzymes are produced by microbes to process and therefore present these substrates in forms that will be nutritionally acceptable to the microbes. Thus, these extracellular enzymes, lipase acts on lipids, protease on proteins and amylase on carbohydrates. The extracellular enzyme production can be demonstrated by presenting the substrate to the test microbial isolate, which helps to evaluate the ability of the test isolate to hydrolyse the test substrate [13]. Hydrolytic enzymes derived from a large variety of microorganisms, degrade a wide range of substrates and macromolecules closely related with the organic pollutants. Hydrolytic activities were responsible for the drastic reduction of

total hydrocarbon from contaminated areas. These hydrolytic enzymes are ubiquitous and catalyze the hydrolysis of their various substrates into the simpler forms [14]. They are particularly important because they specifically hydrolyze pollutants, which are of great interest for different industrial applications, among them the treatment of industrial wastewaters containing high BOD and COD contents, such as dairy wastewaters [15]. The isolation of lipase producing microorganisms capable of degrading fat and oil in wastewater are interesting in that they utilize fat and oil and eliminate the pre-treatment processes.

However, the natural microbial degradation of lipid-rich wastewater is slow due to their low hydrolytic activity. Hence, choice of microbial cultures with high hydrolytic activity for lipid-rich abattoir wastewater and their application for the removal of pollutants may be one of the ways to enhance biodegradation.

METHODOLOGY

Collection of wastewater sample and physicochemical analyses of the sample

Abattoir wastewater sample was collected aseptically from major point of discharge of the abattoir located along the Aba River, Abia State, Nigeria. The physicochemical qualities of the abattoir wastewater samples were examined.

Microbial analyses

Lipolytic bacterial species from the lipid-rich abattoir wastewater sample were properly identified and screened for their lipolytic activity according to Ononiwu and Ekwenye [7]. The lipolytic bacterial species isolated were *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Bacillus subtilis.*

Wastewater treatment

Wastewater treatment was done according to Mongkolthanaruk and Dharmsthiti [16] with modifications. Wastewater sample was distributed into five portions of 2 litres each contained in 2 litres flasks. Enriching the oil waste supplemented minimal medium with other carbon and nitrogen sources may be helpful to spur microbial growth and activity. Hence, the sampled wastewater was enriched with the following nutrients combinations: yeast extract (1.5g/l), peptone (1.0g/l) and glucose (2.0g/l). In four containers, 20ml was inoculated of each bacterial culture (OD ~600nm of 2Mcfarland standard equivalent approximately cell density 6×10^{8}cfu/ml) to make 1% (v/v) while one container was left uninoculated, thus control and incubated in shaking incubator at 35˚C with 200 rpm. Some samples were drawn from each of the flasks at intervals of 6hours for a period of 48hours and later centrifuged 5000 revolutions per minute (rpm) for 30 minutes at 4˚C. Cell free supernatant corresponding to growth phase was used as the crude enzyme to assay the extracellular enzymatic activities of lipase, protease and amylase. The storage period was 4 days.

Microbiological extracellular enzymatic assay

Lipase assay

Lipolytic activity was determined by colorimetric method based on the activity in cleavage of p-nitrophenyl palmitate (p-NPP) at pH 8.0 [17]. The reaction mixture contained 180µl of solution A (0.062g of p-NPP in 10ml of 2-propanol, sonicated for 2min before use), 1620µl of solution B (0.4% triton X-100 and 0.1% gum arabic in 50mM Tris-HCl, pH 8.0) and 200µl of properly diluted enzyme sample. The product was detected at 410 nm wavelength after incubation for 15 min at 37◦C. Under this condition, the molar extinction coefficient (Ɛ410) of p-nitrophenol (p-NP) released from p-NPP was 15,000 M^{-1}. One unit of lipase activity will be defined as 1µmol of p-nitrophenol (p-NP) released per minute by 1ml of enzyme.

Protease assay

Protease activity was determined by the method of Anson [18]. Briefly, 1 ml of 1.5% casein solution, pH 7.0 was placed at 37˚C and, then, 1ml of properly diluted enzyme sample was added. The reaction was incubated for 10 min prior to the addition of 2ml of 0.4M trichloroacetic acid. The solution with precipitates was filtered and to 0.5 ml of the clear filtrate, 2.5ml of 0.4M Na_2CO_3 and 0.5ml of Folin reagent were added. After further 10min of incubation, the colour density developed was determined at 660nm. One unit will be defined as 1µmol of tyrosine released per minute by 1 ml of enzyme.

Amylase assay

Amylase activity was assayed according to Berfeld [19]. Briefly, 0.5ml of properly diluted enzyme was added into a tube containing 1.5ml of 2% (w/v) of potato starch solution and 1ml of 0.05M acetate buffer, pH 5.0. The reaction mixture was incubated at 40˚C for 15min. Then, 1 ml of the mixture was transferred to a new tube containing 1ml of 3, 5-dinitrosalicylic acid and boiled for 10min. The colour density was determined spectrophotometrically at 520nm. One unit was defined as 1µmol of glucose released per minute by 1ml of enzyme.

RESULTS

Table-1: Physicochemical qualities of the abattoir wastewater sample

Parameters	**Mean value** ± ***standard deviation***
Colour	Pale red
Appearance	Clouded
pH	5.92 *±0.14*
Temperature (^{o}C)	29.02 *±0.04*
Turbidity (NTU)	418.55 *±1.18*
Conductivity (µs/cm)	394 *±6.24*
Total Soluble Solute (mg/l)	1560.8 *±0.67*
Total Dissolved Solute (mg/l)	981.1 *±0.26*
Biological Oxygen Demand (mg/l)	1075.4 *±1.86*
Chemical Oxygen Demand (mg/l)	1904.3 *±0.61*
Lipid Contents (mg/l)	17006 *±2.08*
Protein Determination (mg/l)	744.37 *±1.48*
Reducing Sugar (mg/l)	461.89 *±0.03*

Enzyme assays

Illustrations of the enzyme activities of the lipolytic bacteria at different incubation periods of 6 hours intervals for 48 hours are shown below. The species of *Staphylococcus aureus* is a slow carbohydrate fermenter producing the first trace of amylase at 6hours. However, amylase production got to its peak at 24hrs (11.40mM/min/ml). *Pseudomonas aeruginosa* produced its peak at 12hrs (10.24mM/min/ml), *Klebsiella pneumoniae* showed an early activity and its peak of amylase production at 6hrs (7.21mM/min/ml), as *Bacillus subtilis* produced the showed amylase activity at 12hrs (13.8mM/min/ml) and some appreciable activity after 18, 24, 30 hours (12.22, 11.31 and 9.1 mM/min/ml) respectively.

Bacillus subtilis had the highest protease activity (102.69mM/min/ml) after 12hrs, while *Staphylococcus aureus* exhibited 98.41mM/min/ml at the same period. Both *Bacillus subtilis* and *Staphylococcus aureus* also show some considerable activities at 18hrs. *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* exhibited protease activities of 70.50 and 60.31mM/min/ml respectively at 6hrs.

Pseudomonas aeruginosa exhibited a prominent lipase activity over other isolates (0.074mM/min/ml) at 18hrs and some appreciable activities at 24 and 30hrs (0.066 and 0.050mM/min/ml respectively). *Staphylococcus aureus* got its peak at 12hrs (0.068mM/min/ml) and appreciable activity at 18 and 24hrs (0.059 and 0.051 respectively). *Bacillus subtilis* showed high lipase activity at 18 and 24 hrs (0.057 and 0.053 mM/min/ml respectively), while *Klebsiella pneumoniae* at 12hrs exhibited its peak (0.054mM/min/ml).

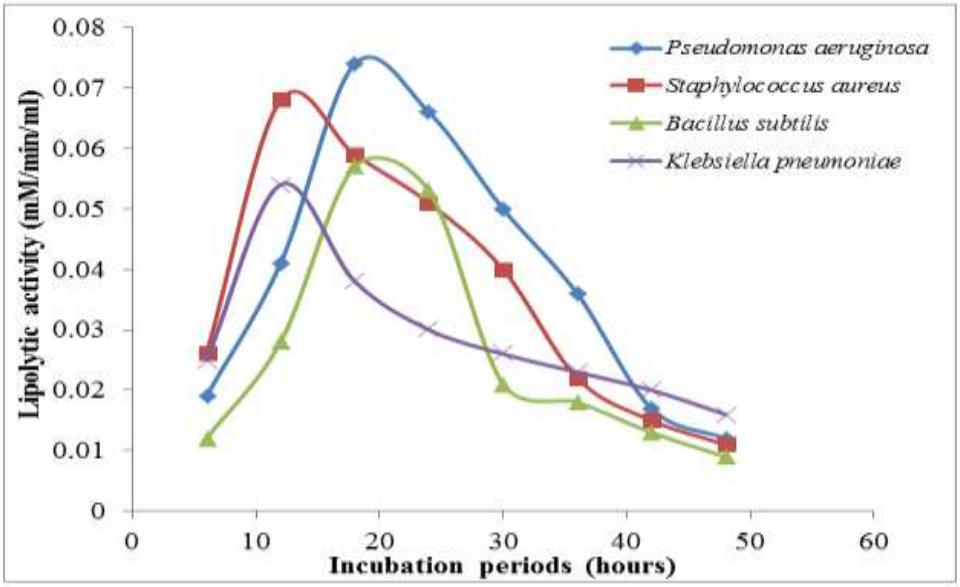

Fig-1: Lipolytic assay for different isolates at different incubation periods

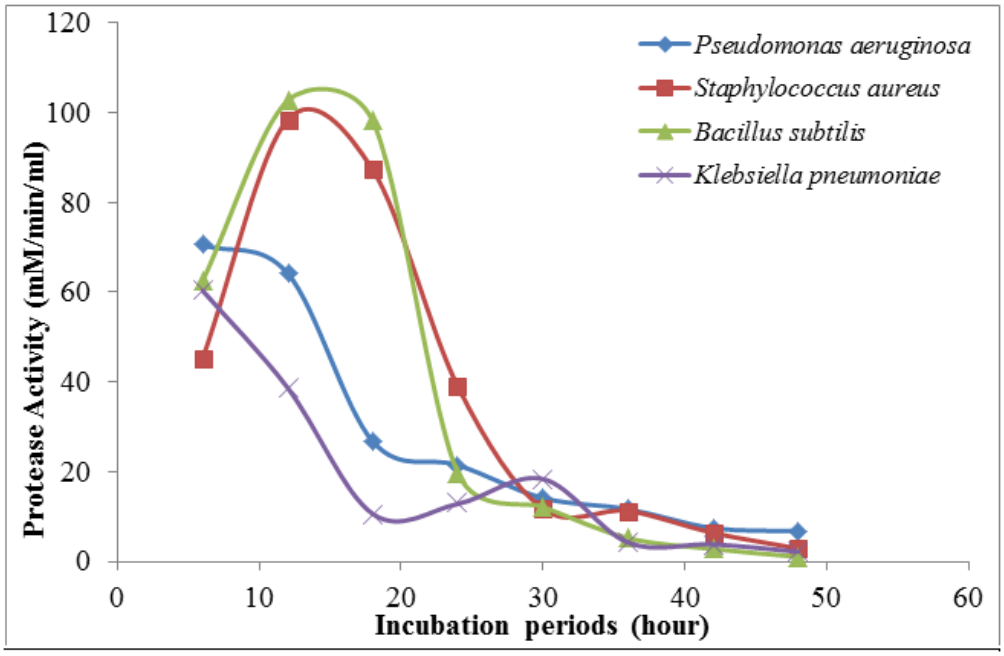

Fig-2: Protease assay for different isolates at different incubation periods

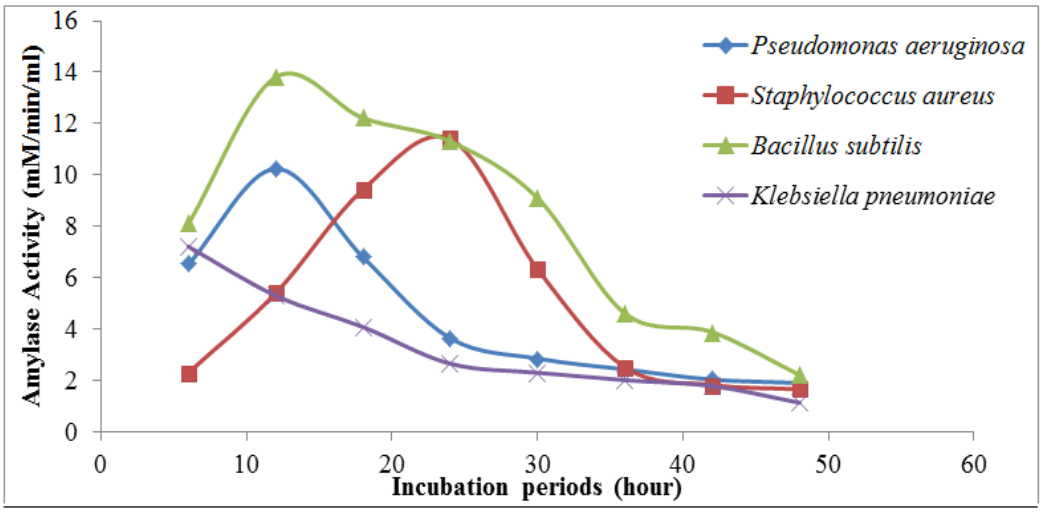

Fig-3: Amylase assay for different isolates at different incubation periods

DISCUSSION

Enzyme activity

As observed each organism produced specific and different amount of hydrolytic enzymes (amylase, protease, lipase), which reflects on how the bacterial isolates degraded/ utilized the various substrates in the wastewater samples. The report of Dunhaupt *et al.*, [20] supports this finding, which stated that, experiments which indicate the very different degradation efficiency might be due to the different reaction system of enzymes from each culture. Also the variation in the enzyme activities of the organisms might be due to the bacterial cell contacts with the substrate and could be explained further by the enzyme regulation mechanism [8]. The use of shaking incubator was able to achieve this contact between cells and substrate molecules.

To access the various roles of each bacterial cell used for the consortium in the wastewater treatment, the activity of the three major enzymes that were considered critical for the wastewater treatment was evaluated [21]. *Bacillus subtilis* has the highest amylase and protease activity while *Pseudomonas aeruginosa* produced the highest lipase activity and appreciable high amount of amylase and protease activities with *Staphylococcus aureus*, also a good lipase producer. All the isolated lipase producers produced better enzyme activity than *Klebsiella pneumoniae*, thus were considered best for consortium [7]. This might be due to the strains isolated. Although, some strains of *Klebsiella*, *Pseudomonas* and *Staphylococcus* have been reported [8] to show prominent enzyme activities, while some strains do not. Also the extracellular enzyme production was during

the growth phases of the test organisms which experienced a decline in enzyme production and activity, an indication of substrates utilization or degradation by the bacterial cells. In other words, the result probably indicated extracellular production of the enzymes (which are proteins) to carter for the initial breakdown of substrates in the wastewater medium. This result corresponds with the report of Odeyemi *et al.,* [8] that decline in the amount of protein was probably due to the loss of enzyme activity when all the available substrates were used up by the growing cells.

CONCLUSION

Owing to the combined hydrolytic activities of lipase, protease and amylase possessed by these lipolytic organisms, they could prove to be beneficial for bringing down the overall organic load of abattoir wastewater. Moreover, pre-treatment of high lipid content wastewater using hydrolytic enzymes is a proven pre-treatment strategy that makes them more amendable to anaerobic treatment. Also, the enzymatic assays carried out could serve as valuable tools in augmenting the isolates performance when applied in bioremediation scenarios. Hence, it appears that bacteria associated with abattoir lipid-rich wastewaters are the novel source of environmental enzymes for possible commercial applications and may play an important role in enzyme-catalyzed organic matter cycling in domestic and industrial environments.

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