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Research article

Keratinase production and biodegradation of polluted secondary chicken feather wastes by a newly isolated multi heavy metal tolerant bacterium-*Alcaligenes* sp. AQ05-001

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ABSTRACT

Biodegradation of agricultural wastes, generated annually from poultry farms and slaughterhouses, can solve the pollution problem and at the same time yield valuable degradation products. But these wastes also constitute environmental nuisance, especially in Malaysia where their illegal disposal on heavy metal contaminated soils poses a serious biodegradation issue as feather tends to accumulate heavy metals from the surrounding environment. Further, continuous use of feather wastes as cheap biosorbent material for the removal of heavy metals from effluents has contributed to the rising amount of polluted feathers, which has necessitated the search for heavy metal-tolerant feather degrading strains. Isolation, characterization and application of a novel heavy metal-tolerant feather-degrading bacterium, identified by 16S RNA sequencing as *Alcaligenes* sp. AQ05-001 in degradation of heavy metal polluted recalcitrant agricultural wastes, have been reported. Physico-cultural conditions influencing its activities were studied using one-factor-at-a-time and a statistical optimisation approach. Complete degradation of 5 g/L feather was achieved with pH 8, 2% inoculum at 27 °C and incubation period of 36 h. The medium optimisation after the response surface methodology (RSM) resulted in a 10-fold increase in keratinase production (88.4 U/mL) over the initial 8.85 U/mL when supplemented with 0.5% (w/v) sucrose, 0.15% (w/v) ammonium bicarbonate, 0.3% (w/v) skim milk, and 0.01% (w/v) urea. Under optimum conditions, the bacterium was able to degrade heavy metal polluted feathers completely and produced valuable keratinase and protein-rich hydrolysates. About 83% of the feathers polluted with a mixture of highly toxic metals were degraded with high keratinase activities. The heavy metal tolerance ability of this bacterium can be harnessed not only in keratinase production but also in the bioremediation of heavy metal-polluted feather wastes.

1. Introduction

As a delicacy, the daily consumption of chicken increases annually as one of the cheapest and healthiest sources of protein. In the rearing and processing of these chickens, millions of tonnes of feathers are produced. These feather-wastes are made up of fibrous and structural keratin polymers that are highly cross-linked by cysteine disulphide bonds, hydrogen bonds, and hydrophobic interactions (Korniłowicz-Kowalska and Bohacz, 2011; Eslahi et al., 2014). This property of feathers and other keratins renders them

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relatively recalcitrant to degradation by soil microbes and proteolytic enzymes such as trypsin, pepsin, and papain (Bach et al., 2012). However, the high protein content in feathers can be used as an alternative source of protein and essential amino acids in animal feed formulation, fertilisers and in other industrial applications (Fakhfakh et al., 2011). In addition, as cheap agricultural wastes, feathers possess high biosorbent property which makes them useful in the removal of heavy metals from surface water (Al-Asheh et al., 2003). However, their repeated use in bioremediation of heavy metals from industrial effluents and other chemically polluted wastes has led to the accumulation of heavy metal polluted feathers – a secondary pollutant that is highly toxic to many feather-degrading microbes.

The conventional methods used for disposing or converting them to protein hydrolysates such as landfilling, incineration, burning or the use of chemicals are costly, not environmentally friendly, and pose a danger to living organisms (Sharaf and Alharbi, 2013; Sharma and Sharma, 2013) in addition to the loss of wanted essential amino acids such as methionine, lysine, and cysteine (Farag and Hassan, 2004; Rajput and Gupta, 2013). As an alternative to conventional methods, microbial degradation of unpolluted feathers (primary feather wastes) has been introduced and is attracting attention as it is turning feather-waste to wealth (Sivakumar and Shankar, 2011; Sivakumar and Raveendran, 2015; Yusuf et al., 2015). But for secondary heavy metal polluted feathers, no report on their biodegradation is available to date in the literature, therefore there is strong need for isolation of heavy metal tolerant microorganisms that can utilise the polluted wastes as substrate to produce high amounts of valuable by-products such as keratinase in addition to clearing the environment of toxic wastes.

A number of feather-degrading microbes (FDMs) such as bacteria, *Streptomyces* and fungi (Demir et al., 2015; Ghasemi et al., 2012; Sivakumar and Raveendran, 2015) have been isolated from various environmental sources and are available in the literature. FDMs produced extracellular keratinase in the presence of keratin substrate to support them in the bioconversion of feathers to protein mass. This enzyme has many applications in leather, cosmetics and other industries (Agasthya et al., 2013; Fakhfakh et al., 2011). The amount of keratinase produced by various FDMs varies from one species, keratin substrate and culture condition to another. However, the growth of a particular FDM in a keratin-containing media and subsequent keratinase production are grossly influenced by various physical and medium factors. These factors can be optimised to achieve faster bacterial growth, feather degradation, and maximum keratinase production.

The conventional method for achieving this is the one-factor-at-a-time (OFAT) technique. This technique is, however, tedious, laborious, time-consuming and often ignores the interactive effect between different factors (Okoroma et al., 2012). The use of statistical approach of optimisation such as Plackett–Burman factorial designs (PBFD) and response surface methodology (RSM) can correct the disadvantages of OFAT (Kumar et al., 2014). PBFD are important in reducing the number of experimental runs by screening out the most significant factors from a large number of factors screened using OFAT. RSM is used for studying the effect of several factors that influence various responses. Its advantage over OFAT is that it allows for simultaneous variation of factors, and limits the number of experimental runs (Fakhfakh-zouari et al., 2010).

In this study, isolation and selection of a novel Gram-negative bacterium, *Alcaligenes* sp. AQ05-001, which shows high potential of feather-degrading ability and heavy metal tolerance were reported. However, information on its bioconversion potentials as well as factors that control its ability to synthesise and release extracellular keratinase remains inadequate. The objective of this study is therefore to evaluate the potentials of whole living cells of *Alcaligenes* sp. AQ05-001 in bioconversion of highly toxic and recalcitrant chicken feather wastes to safer and valuable products. This is in addition to evaluating their ability to optimise the physical and medium conditions influencing both keratinase production and degradation of both heavy metal polluted and unpolluted feather wastes using one-factor-at-a-time and the response surface methodology approach.

2. Materials and methods

2.1. Isolation of heavy metal tolerant feather-degrading and keratinase-producing bacterium

The bacterium was isolated from one of the soil samples obtained from feather dumping sites in Johor, Malaysia. One gram of the soil sample was dissolved in sterilized distilled water and diluted five folds with sterilized distilled water. The medium used for the growth of this bacterium was adopted from Joshi et al. (2007) with slight modifications. About 50 μl aliquot from 10^{-3}, 10^{-4}, and 10^{-5} dilutions of the soil samples were spread on the freshly prepared skim milk agar (SMA) plates pH 7.5 containing (g/L): 0.5 peptone, 0.3 yeast extract, 0.1 dextrose, 1.0 skim milk powder, and 1.5 agar. Colonies of bacteria that showed high hydrolysis zone on SMA were inoculated in feather meal agar (FMA) pH 7.5 which contained (g/L): 1.0 feather, 0.5 NaCl, 0.7 K_2HPO_4, 1.4 KH_2PO_4, 0.001 $MgSO_4$. $6H_2O$ and 1.5 agar. One percent (v/v) suspension of pure colonies of bacteria from FMA was inoculated into feather meal broth (FMB) which contains (g/L): 1.0 feather, 0.5 NaCl, 0.7 K_2HPO_4, 1.4 KH_2PO_4 and 0.001 $MgSO_4$. $6H_2O$. Other sets of FMB containing 1 part per million (ppm) of nine different heavy metals were also prepared. Stock solutions (1000 ppm) of the following heavy metals – silver (Ag), arsenic (As), cadmium (Cd), cobalt (Co), copper (Cu), mercury (Hg), nickel (Ni), lead (Pb), and zinc (Zn) – were used to prepare the 1 ppm solution in the FMB. Bacterial growth in the two sets of FMB was compared. The bacterium that grew, degraded feather faster, and showed higher keratinolytic activity in the presence of two and above heavy metals at 1 ppm was selected and inoculated onto fresh FMA.

2.2. Morphological, biochemical and molecular identification of the strain

The identity of the selected bacterium was detected by studying its morphological, biochemical and molecular characteristics. The gram stain technique was used to reveal its morphology (Cappuccino and Sherman, 1996) while Bergey's manual of systematic bacteriology method was used for its biochemical characteristics (Brenner et al., 2005). Molecular confirmation of its identity was performed by 16S rDNA sequence analysis which involves: genomic DNA extraction, amplification of the genomic DNA using polymerase chain reaction (PCR), and sequencing of the 16S genes. Genomic DNA extraction was carried out from 18 h old culture of the bacterium using GeneJET Genomic DNA purification kit (Thermo Fisher Scientific, USA) according to the manufacturer's instruction. Amplification of the 16S rDNA was carried out using a Biometra Thermal Cycler (Labrepco, Germany) using two universal 16S rDNA primers with the following sequences: 1492R (5′-tacggttacgttacgactt-3′) and 27F (5′-agagtttgatcctggctcag-3′). PCR reaction mixture was carried out using Ready Mix polymerase master mix (Sigma-Aldrich) and was run for 35 cycles. The PCR machine was set at the following conditions: initial denaturation 98 °C for 5 min, denaturation 95 °C for 30 s, annealing 49.8 °C for 30 s, extension 72 °C for 90 s, and final extension 72 °C for 8 min.