Chicken egg shell as a potential substrate for production of alkaline protease by *Bacillus altitudinis* GVC11 and its applications

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Abstract Chicken egg shell and membrane were used as substrate for production of alkaline protease by *Bacillus altitudinis* GVC11. Maltose as additional carbon source enhanced enzyme production up to 13%. Addition of organic nitrogen sources like peptone and yeast extract increased enzyme production by 9% and 5%, respectively and inorganic nitrogen sources did not have any positive effect. The resultant protein hydrolyzate after fermentation was found to have essential amino acids such as leucine, phenyl alanine, isoleucine, lysine, valine, methionine, arginine in considerable quantities and minute concentrations of cysteine. The protein hydrolyzate was also found to have good antioxidant activity.

Keywords Alkaline protease · Egg shell · Collagen · Bioactive compounds · *Bacillus altitudinis* GVC11

Introduction

Bioconversion of waste into usable product is important for economic development and waste management. Efficient utilization of wastes reduces the environmental pollution problems caused by them. Egg shell and membranes are the poultry wastes generated during processing of egg. Hatcheries, fast food centers and domestic sources produce large

quantities of egg shell and membranes. Nearly 250,000 tons of egg shell waste is generated annually during processing (Verma et al. 2012). Outer cover of the egg, the shell, contributes 10–11% of total egg weight. Egg shell is composed of calcium carbonate (96%) and trace elements. Egg shell membrane is composed of collagen type I, V and X, which has diverse applications in cosmetic, food, medical and pharmaceutical domains (Kingori 2011; Wong et al. 1984). Egg shell membrane collagen is an alternative resource to mammalian collagen, can be used for commercial applications in different fields (Zhao and Chi 2009). Egg shell and membranes are non-edible wastes largely disposed but these are reserve of many bioactive compounds which can be extracted by efficient separation (Nakano et al. 2003). Disposal of egg shell and underlying membrane waste contributes to abrasiveness, odor and pollution (Kingori 2011).

Proteases are one of the three largest groups of industrial enzymes which account up to 65% of total world enzyme market share (Ibrahim et al. 2015). Alkaline proteases have various applications in detergent, pharmaceutical, leather, silk, waste management, photographic and dairy industries (Sanathan et al. 2013). Industries producing these enzymes by microbial fermentations are focused on new methods for cheaper production ultimately to decrease the production cost (Mukherjee et al. 2008). Hence, production cost of such enzymes can be reduced by utilizing byproducts or wastes generated during the processing of industrial, agriculture and different aquatic food wastes as substrates. Some of the low-cost byproducts such as wheat bran, rice bran, casein, and soya meal were used as carbon and nitrogen sources which can effectively decrease the cost of protease production. In addition to cost effective production of enzyme, utilization of these largely available wastes as low-cost substrates converts them into value added products (Srividya and Mala 2011).

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Interest on food-derived bioactive peptides, flavonoids, etc., is increasing due to their applications in the field of food and nutritional sciences. These compounds have multiple functional properties such as antioxidant, antihypertensive, antimicrobial, anti-inflammatory and other relevant activities which show positive impact on the human health (Nimalaratne et al. 2015). Bioactive peptides can be produced from precursor proteins by enzymatic hydrolysis and fermentation (Korhonen and Pihlanto 2003). Egg shell membrane derived proteins possess many biochemical and medicinal related properties. Egg shell and membrane hydrolyzate peptides have effective antioxidant activity against oxidative stress (Shi et al. 2014). Present study aimed at utilizing egg shell and membrane (waste) as substrate for the production of alkaline protease by a bacterial organism *Bacillus altitudinis* GVC11 in submerged fermentation.

Materials and methods

Collection, processing of substrates and analysis for nitrogen content

Egg shell and membranes were collected from local hotels (Hyderabad, India), dried under sun light at 35–40 °C, powdered, sieved and used as substrate.

Microorganism and enzyme production

Bacillus altitudinis GVC11, a strain isolated from slaughter house waste dumping soil was used for alkaline protease production (Vijay Kumar et al. 2011a). Inoculum of GVC11 was prepared by growing it in 30 mL nutrient broth in 100 mL flask. The enzyme production was carried out in 250 mL conical flasks containing 100 mL of mineral salts medium (MSM) with egg shell and membrane as substrates at different concentrations. Flasks were inoculated with 2% (v/v) actively growing broth culture of GVC11 (containing 10^8 cells/mL) and incubated at 37 °C, 200 rpm in a rotary shaker. Mineral salts medium composed of (g/L): $(\text{NH}_4)_2\text{SO}_4$ (1), K_2HPO_4 (6), KH_2PO_4 (3), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.01), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.05), $\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$ (0.01), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.0001), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.001), tri-sodium citrate (10), dextrose (1), Na_2CO_3 (10) and pH 10. The fermented broth was used as enzyme source for all studies. All the experiments were carried out in triplicate, twice on different occasions and values presented are mean of the same (Vijay Kumar et al. 2011b).

Enzyme extraction and assay

The fermented broth was centrifuged at 15,000 rpm for 15 min at 4 °C. Supernatant obtained was used as enzyme

source. Alkaline protease activity was determined with alkali soluble casein as substrate according to the method described previously (Vijay Kumar et al. 2011a, b). One unit (U) of alkaline protease activity is defined as the amount of enzyme which releases 1 μg tyrosine per minute under standard assay conditions.

Effect of carbon and nitrogen sources on alkaline protease production

Effect of different carbon (glucose, lactose, maltose and xylose) and nitrogen (ammonium sulphate, ammonium nitrate, yeast extract and peptone) sources on alkaline protease production was studied by adding these to mineral salts medium at 1% (w/v) by uni-dimensional method.

Analysis of free amino acids by HPLC

Free amino acids released on degradation of egg shell and membrane by proteolytic activity of *Bacillus altitudinis* GVC11 were derivatized using phenyl-isothiocyanate (PITC) and quantified by HPLC (Shi et al. 2013).

Fig. 1 Alkaline protease production by *Bacillus altitudinis* GVC11 using egg shell and membrane as substrate in mineral salts medium (MSM)

Fig. 2 Effect of additional carbon sources on alkaline protease production by *Bacillus altitudinis* GVC11 using egg shell and membrane as substrate

Fig. 3 Effect of additional nitrogen sources on alkaline protease production by *Bacillus altitudinis* GVC11 using egg shell and membrane as substrate

Analysis for antioxidant activity of protein hydrolyzate (fermented broth)

Fermented broth (protein hydrolyzate) was centrifuged at 15,000 rpm for 15 min and supernatant collected was tested for antioxidant activity. Protein concentration of supernatant was determined by Lowry's method. DPPH radical scavenging activity (RSA) of protein hydrolyzate was determined by the method of Memarpoor-Yazdi et al. (2012) with minor modifications. Sample of 200 μ L mixed with 600 μ L of methanol and 200 μ L of DPPH (0.15 mM in methanol). The mixture was shaken vigorously for 2 min and kept for 60 min in the dark at room temperature. The absorbance of the mixture was measured at 517 nm using a UV-Vis spectrophotometer. The control contained 800 μ L

of methanol and 200 μ L of DPPH. Ascorbic acid was used as standard reference antioxidant.

DPPH radical scavenging ability was calculated using the following equation

$$\text{DPPH radical scavenging activity (\%)} = \frac{[\text{blank absorbance} - \text{sample absorbance} / \text{blank absorbance}] \times 100.}$$

Results and discussion

Egg shell and membrane as substrate for alkaline protease production

Disposal of egg shell and underlying membrane contributes to environmental pollution (Phil and Zhihong 2009). Utilization of CaCO_3 present in the egg shells as neutralizer in farm fields and as a source of calcium, can reduce the pollution problem to certain extent. However, most of the egg shell membrane (ESM) is discarded because of its insolubility in water and organic solvents which makes its applications restricted. The effective solubilization of egg shell membrane proteins will provide further exploration of its possible uses. Earlier studies reported that CaCO_3 induces the enzyme production significantly (Nilegaonkar et al. 2007). In the present study, egg shell and membrane was used as substrate for production of alkaline protease by *Bacillus altitudinis* GVC11. Maximum alkaline protease production of 13,226 U/mL was observed with 20% substrate at 48 h (Fig. 1). Enzyme production increased as the

Table 1 Free amino acids present in egg shell and membrane control (unfermented) and fermented broth for alkaline protease by *Bacillus altitudinis* GVC11 after enzyme extraction (protein hydrolyzate)

Type of amino acid	Concentration of free amino acids in unfermented (control) broth ($\mu\text{g/mL}$)	Concentration of free amino acids in fermented broth ($\mu\text{g/mL}$)
Phosphoserine	0.972	2.836
Glutamic acid	0.265	0.902
Amino adipic acid	1.137	1.642
Asparagine	0.084	–
Histidine	11.52	1.577
Carnosine	0.260	0.207
3-Methyl histidine	0.418	0.267
Valine	0.139	0.210
Leucine	1.018	–
Aspartic acid	–	0.253
Arginine	–	2.755
Hydroxy proline	–	0.118
Phosphoenolamine	–	0.434
Serine	–	0.187
Glycine	–	0.103
Cystathione	–	0.880

– indicates absence in the broth

Fig. 4 **a** Free amino acids present in egg shell and membrane unfermented (control) broth as analyzed by HPLC (also includes non standard amino acids). **b** Free amino acids present in egg shell and

membrane fermented (by *Bacillus altitudinis* GVC11) broth as analyzed by HPLC (also includes non standard amino acids)

substrate concentration increase from 4% to 20% and later decreased. With all substrate concentrations used in the study (4, 8, 12, 16, 20 and 24%), enzyme production was found to be increased from 24 to 48 h and subsequently decreased on further incubation. Enzyme production was also studied only in minimal medium, where it was found to be constitutive (Fig. 1). Cheng et al. (2009) reported the egg shell membrane decomposition by a protease producing *Pseudomonas* sp. High enzyme production was obtained in our study than the above report.

Effect of additional carbon and nitrogen source on alkaline protease production

Synthesis of proteolytic enzymes is controlled by availability of carbon and nitrogen sources in the fermentation medium. Effect of different additional carbon sources such as glucose, lactose, maltose and xylose and nitrogen sources such as ammonium nitrate, ammonium sulphate, soya peptone and urea on the enzyme production was studied by supplementing the medium at 1% (w/v) concentration of each. Among

the different carbon sources, maltose was found to be enhancing the enzyme production significantly than others (Fig. 2). Increase in enzyme production on supplementation with maltose was 13% giving to a maximum of 15,062 U/mL whereas a decrease in enzyme production up to 54% was observed with the addition of xylose, followed by lactose and glucose by 13% and 7% respectively (Fig. 2). Enhanced production of enzyme with maltose could be due to its preferred utilization to other carbon sources. Among the nitrogen sources, organic nitrogen peptone and yeast extract increased enzyme production by 9% and 5% respectively, whereas inorganic nitrogen sources, ammonium sulphate and ammonium nitrate decreased the enzyme production by 26% and 25% respectively (Fig. 3). Similar observation was found with maltose, yeast extract and peptone in the case of protease production by *Bacillus cereus* strain AT (Vijayaraghavan et al. 2014). Addition of galactose and peptone resulted in increased protease production by *Bacillus subtilis* as reported by Pant et al. (2015).

Free amino acids in egg shell and membrane protein hydrolyzate (fermented broth)

Growth of *Bacillus altitudinis* GVC11 in fermentation medium with egg shell and membrane as substrate resulted in degradation/hydrolysis of membrane proteins and fermented broth was analyzed for its free amino acids, in addition to alkaline protease. It was observed that a total of 14 amino acids were found to be present in the fermented broth (Table 1). Egg shell and membrane protein hydrolyzate were rich in some essential amino acids such as histidine, arginine and valine. In addition, non-essential amino acids such as aspartic acid, glutamic acid, serine and glycine were also found to be present. The protein hydrolyzate also found to have good quantity of non standard amino acids, phosphoserine, phosphoenolamine and low levels of amino adipic acid, hydroxyl proline, carnosine, 3-met-histidine, cystathione (Table 1; Fig. 4a, b). It is important that, the egg shell and membrane protein hydrolyzate can be used as animal feed supplement and/or organic fertilizer due to the presence of such amino acids. Using egg shell and membrane as substrate resulted in low-cost production of alkaline protease and also the formation of essential amino acids rich protein hydrolyzate which ultimately reduces environmental pollution caused by this poultry waste and also gives value addition to the fermented broth.

Fermented broth for antioxidant activity

Antioxidant activity of peptides depends on the amino acid sequence and enzyme used for the digestion (Shahidi and Zhong 2008). Fermented broth was tested for antioxidant activity by DPPH free radical scavenging property at

Fig. 5 Antioxidant activity of egg shell and membrane fermented broth (protein hydrolyzate) of *Bacillus altitudinis* GVC11

various concentrations of protein ranging from 300 to 1200 µg/mL. As the protein concentration of hydrolyzate increased from 300 to 900 µg/mL, DPPH free radical scavenging activity increased. Further increase in the protein concentration resulted in stabilized antioxidant activity. The protein concentration required for 50% of DPPH free radical scavenging activity (A_{50}) was found to be 900 µg/mL (Fig. 5), which is a significant activity than the previous reports available (Shi et al. 2014), but less than the ascorbic acid as standard. Further studies are required for purification and characterization of the antioxidant peptides present in the protein hydrolyzate.

Conclusions

Microbiological conversion of waste into valuable products enhances not only the sustainable economic development but also helps in management of waste. The poultry egg processing waste, egg shell and membrane, was effectively used as substrate for the production of alkaline protease in bacterial fermentation which resulted in significant enzyme production. Supplementation with maltose, yeast extract and/or peptone as carbon and nitrogen sources enhanced the enzyme production. The fermented broth containing protein hydrolyzate was rich in essential amino acids which can serve as value added byproduct to be used as animal feed supplement and/or fertilizer. Significant antioxidant activity of fermented broth is an additional value to it.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest for this research work.

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