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Research Article

CHARACTERIZATION AND OPTIMIZATION OF ALKALINE PROTEASE PRODUCTION FROM *BACILLUS LICHENIFORMIS* HSW-16 ISOLATED FROM SAMBHAR SALT LAKE

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Abstract

Halophilic microorganisms are recognized as potential source of secondary metabolites including enzymes and drugs with wide agricultural and industrial applications. In the present study protease producing halotolerant bacterium *Bacillus licheniformis* HSW-16 was isolated from hypersaline Sambhar lake, Rajasthan India. Protease production was performed by using azocasein as substrate. Confirmation of protease production was also done by amplification of alkaline protease gene and sequencing. The various nutritional factors such as carbon and nitrogen source and other physiological parameters like pH, temperature, incubation time and agitation speed were optimized for optimum protease production. The enzyme was active in pH range 7-10, temperature 25 °C-40 °C and salt concentration of 1.5M. The characteristics demonstrated by this isolate showed that it could be used as a potential source of enzyme.

Keywords: *Bacillus*; enzyme; gene; Protease

Introduction

Proteases belong to the class of hydrolytic enzymes that cleave peptide bonds between amino acid residues, and are the most important commercially used enzymes in the world. They constitute two-thirds of the total enzymes used in various industries and commercially exploited in the detergent, leather, food, pharmaceutical, diagnostics and various other chemical industries (Oberoi *et al.,* 2001). They are also widely employed in agro-processing and food industries for the management of industrial and household waste, preparation of organic fertilizer, meat processing, dairy and poultry production. Proteases can be isolated from all the existing life-forms. It is on the basis of their acid-base nature that they are classified into three groups, that is, acid, base and neutral proteases. Acid proteases belong mostly to fungal origin and performed best at a pH range of 2.0 to 5.0. Proteases having an optimum pH in the range of 7.0 or close are called neutral proteases. This neutral protease belongs mainly to the plant source. Proteases with an optimum activity at a pH range of 8.0 and above are classified as alkaline proteases produced from microorganisms (Alnahdi, 2012). Protease being extensively prevalent in the environment, the most ideal source of obtaining them is from microbes because of their rapid growth, limited space required for their cultivation and ease of genetic manipulation to make new enzymes with desirable properties for various applications (Kocher and Mishra, 2009). The genus "*Bacillus*" is a major and key source of industrial alkaline proteases and is in all probability the only commercialized species for the production of alkaline protease. It was in the 1960′s that the very first detergent containing the first alkaline protease Carlsberg, from *Bacillus licheniformis* was commercialized as a detergent additive (Saeki *et al.,* 2007). Since then, a large number of alkaline proteases derived from different *Bacillus* species have been purified and characterized. These *Bacillus* derived alkaline proteases have been found to be stable at conditions which greatly vary from the physiological conditions. Among them alkaline proteases are of particular interest due to their potent applications in the detergent industry as a cleaning additive in the detergent formulation (Gupta, 2003). Though several microorganisms such as fungi, yeast are known to produce alkaline proteases, microbial proteases especially from *Bacillus sp.* are the most extensively exploited industrial enzymes and play a critical role in various biotechnological applications (Parekh *et al.,* 2000). The protease activity is severely affected by various factors such as pH, temperature, surfactants, bleach systems and mechanical handling which eventually determine its stability. To compensate the industrial demand for an alkaline protease possessing properties to overcome these challenges, there is a constant need to search for new enzyme sources.

Alkaline proteases which are *Bacillus* derived are stable at high pH and temperatures; however a vast majority of

exhibit incompatibility with detergent matrices where applications of such enzymes are most sought after (Gupta *et al.*, 2002). Hence, screening for different protease producing *Bacillus* sp. from widely varying ecological environments can result in isolation of new alkaline proteases with exclusive physiochemical characteristics (Singh *et al.*, 1999). Therefore, we′re still in search of alkaline proteases with enhanced performance for the sole purpose of commercial exploitations keeping its industrial application in mind.

In 2000, the global market for industrial enzymes was 2 billion dollar and continues to increase by 5-10% annually (Nevalainen and Te′o, 2003). Microbial protease accounts for approximately 60% of the total enzyme sale in the world (Banik *et al.*, 2004). Of all proteases, alkaline proteases produced by *Bacillus* species are of significant importance in detergent industry due to their high pH and thermal stability .With this in view, we have isolated a halotolerant bacterium *Bacillus licheniformis* HSW-16 and characterized its production under various physical and chemical conditions. The amplification and sequence analysis of alkaline protease was also performed.

Materials and Methods

Chemicals

Azocasein, sodium hydroxide (NaOH), Tris-HCl, trichloroacetic acid (TCA), phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA) dithiothreitol (DTT) and β-mercaptoethanol was purchased from Sigma Chemical Co. (St. Louis MO, USA). Primers for protease gene amplification were synthesized at Eurofins Genomics Labs Ltd (Eurofin, India). All the culture medium components were purchased from HiMedia laboratories, India and used as per the manufacturer′s instructions.

Sample collection and isolation of bacterial strain

Water samples were collected in a sterile plastic container from the hyper saline Sambhar lake, Rajasthan, India. Collected sample was serially diluted in sterile PBS buffer and the dilutions were plated in LB-agar plates supplemented with 7% sodium chloride and kept for incubation for overnight at 37 °C. Morphologically different colonies were picked based on divergence in morphology, size and colour.

Culture and growth conditions

Bacterial strain was cultivated in medium consists of (g/l): casein (5.0), yeast extract (2.0), KH_2PO_4 (1.0), K_2HPO_4 (1.0), tryptone (3.0), $MgSO_4$ (0.2g). Inoculum cultures were routinely grown in Luria-Bertani broth medium (LB) composed of (g/l): casein peptone 10.0, yeast extract 5.0 and NaCl 5.0, pH 8.0. Media were sterilized by autoclaved at 121 °C for 20 min. Cultivation was performed on a rotary shaker (200rpm) for 24 h at 37 °C in a 250 ml erlenmeyers flask. The culture medium was centrifuged at 12,000g for 15 min at 4 °C and the cell free supernatant was used for protease activity and purification.

Screening assay for protease production

Assay for protease production was screened on Luria-Bertani agar plates supplemented with 7% NaCl and 1% casein. Plates were incubated overnight at 37 °C. The formation of clear zone around the colonies confirms the production of alkaline protease.

Molecular identification and amplification of alkaline protease gene

The protease producing bacterial strain was identified by *16S rRNA* gene sequencing. Genomic DNA of strain was extracted by the Quiagen kit (Quiagen, USA). The *16S rRNA* gene was amplified by using the universal primer 27F1 and 1494 Rc. For the amplification of alkaline protease gene, primers were designed based on the alignments of alkaline protease database available on the NCBI data base. Primers used for the amplification are Forward 5′ (5′-AGGCAGGATCAGGGCTTTTC-3′) and reverse (5′-TTTAGTCTTGCGGCACCGAT-3′). The PCR condition were as follows: initial denaturation for 3 min at 94 °C, 35 cycles each consisting of denaturation for 1 min at 94 °C, primer annealing for 1 min at 54 °C and extension at 72 °C for 5 min and a final elongation of 5 min at 72 °C in a thermal cycler (T100 BioRad, India). Amplicon of size (850bp) was observed on the Gel document system (BioRad, India) and purified with PCR purification kit (Quiagen, USA) and sequenced by Xcelris Genomics Labs Ltd (Xcelris, India).

Protease Assay

Overnight grown culture of strain (1%) inoculums was added in neutrient broth with 7% NaCl and 1% casein and kept for 24 h incubation at 37 °C under shaking condition of 200 rpm. After the incubating time, culture was centrifuged at 12,000 g for 15 min at 4 °C and cell free supernatant was used for protease assay by Anson method (Hagihara, 1958). For the protease assay the range of concentration 50-300 µg of tyrosine was used as standard. The reaction mixture containing 1 ml of enzyme was added to 1 ml of casein (1% w/v in 50 mM potassium phosphate buffer pH 7.5) and mixture was incubated for 10 min at 37 °C. The reaction was terminated by adding 2 ml of 10% trichloroacetic acid (TCA) reagent, kept for incubation at room temperature and then centrifuged for 15 min at 12,000 g. Then 2 ml of filtrate was mixed with 3 ml of 500 mM sodium carbonate solution and absorbance was measured at 280 nm (Jasco, Japan). One unit of enzyme activity is defined as amount of enzyme required to liberate 1 µmol of tyrosine per min under the defined conditions.

Optimization of pH, temperature, and incubation period for protease production

The optimum pH on protease production was determined by growing the bacterial culture in the nutrient broth by

varying the pH from 5.0 to 12.0 with 1% (w/v) casein. The effect of temperature on protease activity was studied from 25 °C-40 °C using casein as a substrate. Loss of enzyme stability at temperatures above 50 °C suggests conformational instability at high temperatures which is said to be dependent on composition and structure of amino acids (Xu *et al.* 2013). Protease activity was examined by incubating the purified enzyme for 120 min at different temperature. The non-heated enzyme was considered as control.

Effect of Salt (NaCl), carbon and nitrogen sources on protease production

The effect of different salt concentration on the enzyme activity was evaluated by varying salt concentration from 0-3M with 0.5 M variation in the nutrient broth. After overnight growth of the culture the cell free supernatant was evaluated for protease activity. For the effect of carbon sources on protease activity, nutrient broth media was supplemented with different carbon sources such as sucrose, dextrose, fructose, xylose, galactose, lactose, maltose (1% w/v). The nitrogen sources used were urea, potassium nitrate, sodium nitrate, ammonium chloride, ammonium nitrate (1% w/v). After 24 h incubation, the cell free supernatant was quantified for protease assay.

Effect of different metal ions on protease activity

The effect of different metal ions on the enzyme activities was evaluated by incubating the reaction mixtures with $MgCl_2$, $FeSO_4$, $FeCl_3$, $CuSO_4$ and $MnCl_2$. Protease activity was determined by Anson method.

Results and Discussion

Isolation and identification of strain

A halophilic bacteria *Bacillus licheniformis* was isolated from Sambhar salt lake and screened for protease production on casein agar plate for protease production. Presence of zone of hydrolysis around the streaked colony was considered as positive for protease production. The obtained PCR product of amplified alkaline protease gene (Fig. 1) showed 100% identity with alkaline serine protease AprX of *Bacillus licheniformis* 9945A and 99% identity with alkaline serine protease of *Bacillus licheniformis* strain BL-09.

Effects of pH, temp and incubation period on enzyme activity

The protease activity using casein as a substrate, was examined at various pH values between 5 and 12 with a maximum protease production observed at pH 9 (145.55U/mg) followed by a pH of 8 (135.28 U/mg). Increase in pH from 5 to 9 causes the increase in protease activity upto 75% (Fig. 2a). A decreasing trend of protease production is seen on either side of the graph. Reduction in enzyme activity of gram positive halophilic bacteria at higher pH was also observed by (Oren *et al.*, 1999). Most of the *Bacillus sp.* has optimum pH from 7.0 to 11.0 for maximum protease production (Jayaraman and Shivanand, 2009).

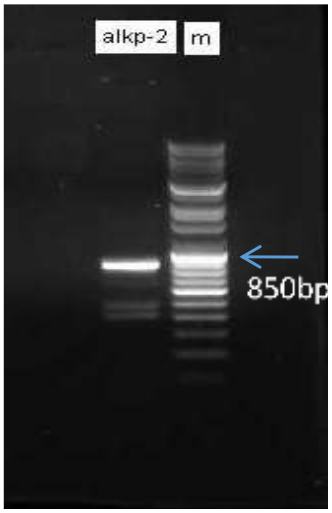
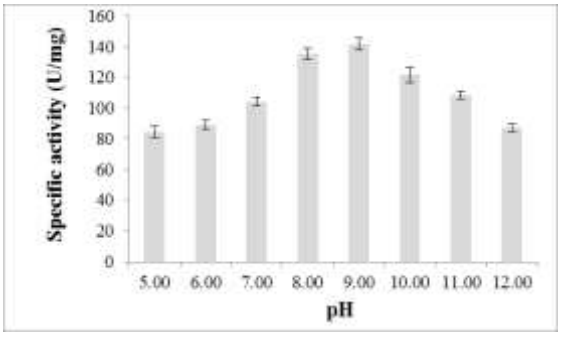
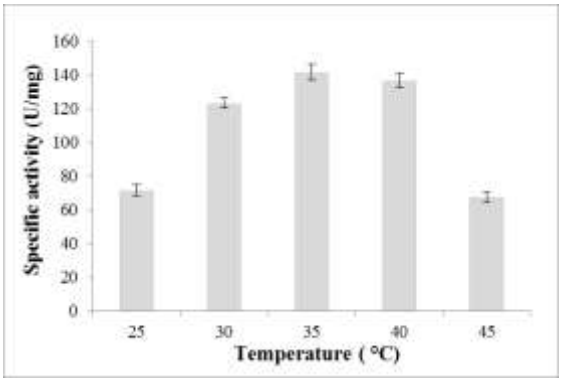


Fig 1: Amplified gel image of alkaline protease gene of *Bacillus licheniformis*. Lane M denotes the Marker, Lane alkp: amplified alkaline protease gene of isolate HSW-16





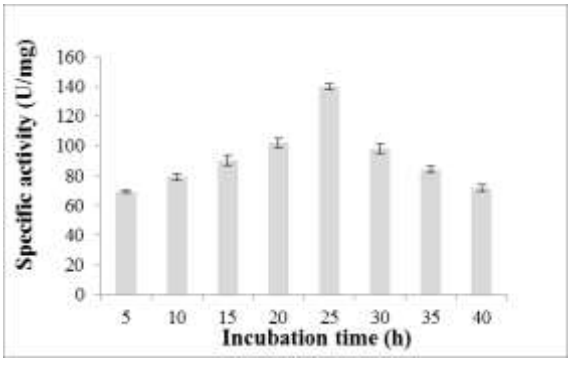


Fig 2(a, b, c): Effect of varying pH, temperature and incubation time on activity of alkaline protease of Bacillus licheniformis HSW-16.

The influence of temperature on protease activity was investigated by varying temperature within the range of 25 °C-45 °C. It is evident from Fig. (2b) that maximum protease production (141.47 U/mg) was observed at 35 °C followed by 40 °C (136.59 U/mg). Decresae in temperature from 35 °C to 30 °C reduced the protease production of 15%. However increase in temperature from 25 °C to 35 °C increased the enzyme production upto 98%.

Considering the incubation time as a factor for protease production, maximum protease production (139.80 U/mg) was obtained after 25 h of incubation (Fig. 2c). However it is difficult to correlate between time of cellular growth and extracellular enzyme production as it varies with the test organism and conditions of growth.

Effect of salt (NaCl), carbon and nitrogen sources on protease production

Salt at a concentration of 1.5mM supplemented in the media yielded highest amount of protease production (136.92U/mg) after 24 h of incubation. Further increase in salt concentration caused reduction in protease production. Increase in salt concentration from 0M to 1.5M caused the increase in protease production upto 120.63%, whereas reduction in protease production of 86.60% was observed further increase in salt concentration from 1.5M to 3.0M (Fig. 3a). Previous report of (Jayaraman and Shivanand, 2009) also observed similar NaCl concentration for maximum protease production.

Among the various carbon sources tested, highest protease yield (138.49 U/mg) was observed in media containing fructose, closely followed by lactose (120.25 U/mg) and maltose (117.10 U/mg). Less production of enzyme was observed in the medium containing dextrose (91.40 U/mg), galactose (93.75 U/mg) and xylose (88.01 U/mg) (Fig. 3b). Previous report of Chakraborty and Malathi, (1991) and Srinivasan *et al.* (1993) have observed maximum protease production while using lactose and maltose as carbon sources.

Effect of various nitrogen sources on enzyme production by *Bacillus licheniformis* was studied (Fig. 3c). Maximum protease production was observed with ammonium chloride (141.50 U/mg) followed by ammonium nitrate (118.30 U/mg) and urea (108.64 U/mg) used as nitrogen source. *Bacillus licheniformis* produced markedly less protease production in the presence of potassium nitrate (100.28 U/mg) and sodium nitrate (89.05 U/mg) (Fig. 3c).

Effect of different metal ions on protease activity

In the present study, maximum protease production (140.11 U/mg) was observed in media supplemented with $ZnSO_4$ followed by $MgSO_4$ (132.28 U/mg) and $FeSO_4$ (121.70 U/mg) (Fig. 4). Addition of other metal like $CaCl_2$, $CaCO_3$ and $FeCl_2$ had no enhanced effect on protease production.

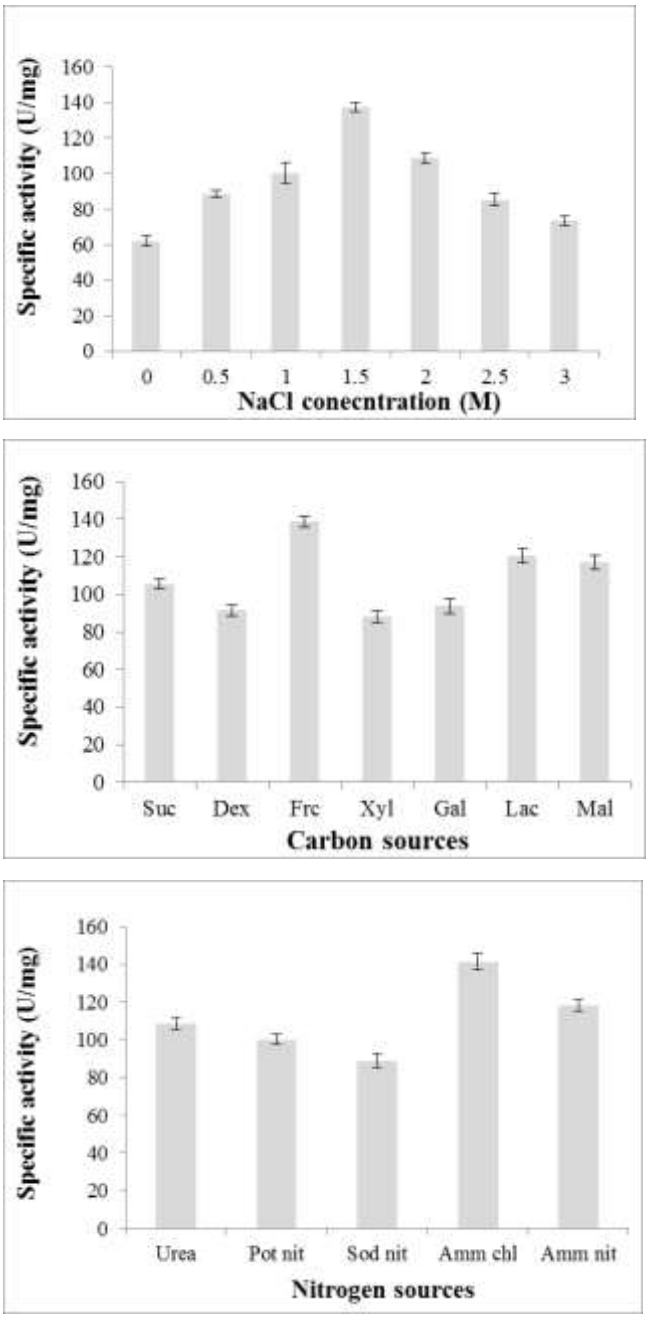


Fig. 3(a, b, c): Alkaline protease activity of strain Bacillus licheniformis HSW-16 under different salt concentrations, carbon and nitrogen sources.

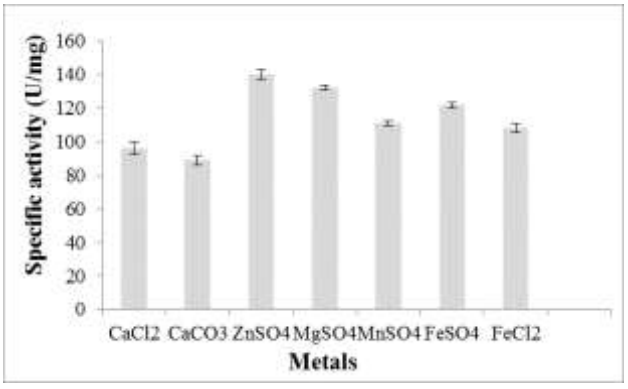


Fig. 4: Effect of various metal ions on protease activity of isolate Bacillus licheniformis HSW-16.

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Conclusion

The present study demonstrated that *Bacillus licheniformis* HSW-16 isolated from Sambhar lake have good source of enzyme production with various industrial applications. The isolated microorganism was investigated with various media components for higher protease production. The produced enzyme has properties such as optimal salt concentration of 1.5M, pH at 9.0 and stability toward various metals makes enzyme potentiality for various applications. Further studies are required to investigate the application of produced enzyme for industrial applications.

References

Alnahdi HS (2012) Isolation and screening of extracellular proteases produced by new Isolated Bacillus sp. *Journal of Applied Pharmaceutical Science* **2**(9): 071-074.

Banik RM and Prakash M (2004) Laundery detergent compatibility of the alkaline protease from Bacillus cereus. *Microbiol. Res.* **159**(2): 135-140.

Bhaskar N Sudeepa ES, Rashmi HN and Selvi AT (2007) Pratial purification and characterization of protease of Bacillus proteolyticus-CFR3001 isolated from fish processing waste and its antibacterial activities. *Bioresour Technol.* **98**: 2758-2764.

Chakraborty R and Malathi S (1991) Production of Alkaline Protease by a new *Aspergillus flavus* isolate under Solid Substrate fermentation condition for use as depletion agent. *Applied Environmental Microbiology* **57**: 712- 716.

Gupta R (2003) Purification and characterization of an oxidation stable thiol-dependent serine alkaline protease from *Bacillus mojavenesis*. Enzyme. *Microbial. Technol.* **32**: 294-304.

Gupta R, Beg QK and Lorenz P (2002) Bacterial alkaline proteases: molecular approaches and industrial applications. *Appl. Microbiol. Biotechnol*. **59**: 15–32.

Hagihara B (1958) The Enzymes, vol. 4, Academic Press Inc. Newyork.

Jellouli K, Bougatef A, Manni L, Agrebi R, Siala R, Younes l and Nasri M (2009) Molecular and biochemical characterization of an extracellular serine-protease from vibrio metschnikovii J1. *J. Ind. Microbio. Biotechnol.* **36**: 939-948.

Kocher GS and Mishra S (2009) Immobilization of Bacillus circulans MTCC 7906 for enhanced production of alkaline protease under batch and packed bed fermentation conditions. *Internet. J. Microbiol*. **7**: 2.

Laemmli UK (1970) Cleavage of structural proteins during assembly of head of bacteriophage T4. *Nature* 227: 680-685.

Nevalainen KMH and Te′o VSJ (2003) Enzyme production in industrial fungi-molecular genetics strategies for integrated strain improvement. *Appl. Mycol. Biotechnol.* **3**: 241–259.

Oberoi R, Beg QK, Puri S, Saxena RK and Gupta R (2001) Characterization and wash performance analysis of an SDS-resistant alkaline protease from a Bacillus sp. *World J. Microbiol. Biotechnol.* **17**: 493-497.

Oren A, Ventosa A, Gutiérrez MC and Kamekura M (1999) Haloarcula quadrata sp. nov., a square, motile archaeon isolated from a brine pool in Sinai (Egypt). *Int. J. Syst. Bacteriol.* **49**: 1149–1155.

Parekh S, Vinci, VA and Strobel, RJ (2000) Improvement of microbial strains and fermentation processes. *Applied Microbiology and Biotechnology* **54**(3): 287-301.

Saeki K, Ozaki K, Kobayashi T and Ito S (2007) Detergent alkaline proteases: enzymatic properties, genes, and crystal structures. *J. Biosci. Bioeng.* **103**: 501–508.

Sareen R and Mishra P (2008) Purification and characterization of organic solvent stable protease from Bacillus licheniformis RSP-09-37. *Appl. Microbiol. Biotechnol.* **79**: 399-405.

Shivanand P and Jayaraman G (2009) Production of extracellular protease from halotolerant bacterium, *Bacillus aquimaris* strain VITP4 isolated from Kumta coast. *Process Biochemistry* **44**: 1088–1094.

Singh J, Vohra R and Sahoo D (1999) Alkaline protease from a new obligate alkalophilic isolate of *Bacillus sphaericus*. *Biotech. Lett.* **21**: 921-924.

Xu D, Li C, Wang Y, Sun L, Zhao H and Zhao M (2013) Characterisation of acid proteases from a fusant F76 and its progenitors *Aspergillus oryzae* HN3042 and Aspergillus niger CICC2377. *Int. J. Food Sci. Technol.* **48**(4): 678-684.