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Research Article

NOVEL KERATINOLYTIC ACTIVITY OF CYBERLINDNERA FABIANII NRC3 AZA AS A PLANT GROWTH PROMOTING AGENT (PGPA)

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Abstract

Two field experiments had been conducted in Nubaria sandy soil, Behaira Governate, Egypt to show the effect of keratinase enzyme produced by the novel microbial isolate (*Cyberlindnera fabianii* NRC3 Aza) on plants.The trials had been conducted in the two successive summer seasons (2011/2012 and 2012/2013) to show the effect of keratinase enzyme from degraded feather–waste on the morphology and chemical composition of peas pods (*Pisumsativum* L.)–family *Fabaceae* (*Leguminasae*). In 2011/2012 season, only the chemical analysis of the dried powdered beads was studied. In 2012/2013 season, the morphological studies of the yield were considered beside the chemical ones. The results depicted significant effects of the sprayed enzyme (keratinase) on peas as plant growth promoting agent (PGPA), compared with the blank (sprayed with water). Electrophoreses and amino acid analysis were carried out for the characterization of the partial pure keratinase enzyme.

Keywords: Keratinase enzyme; Chicken feather; Plant growth promoting; Electrophoresis; Peas pods' Amino acid analysis.

Introduction

Keratin-containing materials (feather, hair, wool, bristles, horns, hoofs, beaks, claws, etc.) as by-products of a number of industrial processes are abundant in nature. They have limited applications by the common proteolytic enzymes. The high mechanical stability of keratins depends on the presence of large quantities of salts, disulfide and hydrogen bonds linkages and other cross-linking (Vignardet *et al.*, 2001). Previously, keratinous materials together with other animal wastes were baked, milled and subsequently used as feed additives for domestic animals (Shih *et al.*, 1993). After the danger of bovine spongiform encephalopathy (BSE) has been recognized, the recycling of poultry and animal waste products for animal feed is no longer advantageous (Tsiroulnikov *et al.*, 2004). Since the incineration of animal wastes entails large expenses, these wastes are now predominantly disposed of in landfill sites. Storage and disposal of these unrecyclable wastes create important ecological and sanitary problems. Thus, environmental friendly, economically sound, and safe methods of decontamination and disposal are badly needed. For this purpose, destruction of the rigid keratin structure is necessary. Degradation of keratin waste (feather) is usually achieved by specific enzymes (keratinases) (Tsiroulnikov *et al.*, 2004; Jou *et al.*, 1999). During the last few decades, research has been conducted to improve the agronomic utilization of organic wastes, and keratin wastes in particular. At this stage the simplest and most appropriate uses are as bio-fertilizers and plant growth promoters (PGP) (Cabeza *et al.*, 1998; Ros *et al.*, 2003; Fuchs *et al.*, 2004; Freitas *et al.*, 2007). During the past couple of decades, the use of plant growth promoting rhizobacteria (PGPR) for sustainable agriculture has increased tremendously in various parts of the world. Significant increases in growth and yield of agronomically important crops in response to inoculation with PGPR have been repeatedly reported (Das *et al.*, 2013). In the last few decades , a large array of bacteria including species of *Pseudomonas*, *Azospirillium*, *Azotobacter*,*Klebsiella*, *Enterobacter*, *Alcalisens*, *Arthobacter*,*Burkholderia*,*Bacillus* and *Serratia* have reported to enhance plant growth (Sakthivel and Karthikeyan 2012). These mechanisms can be activated simultaneously or independently at different stages of plant growth. Among these, phosphate solubilization, biological nitrogen fixation, improvement of other plant nutrients uptake and phytohormoneproduction (like indole -3- acetic acid) are some of the regulators that profoundly influence plant growth (Zaidi *et al.*, 2009).

Degradation of feather and keratin by indigenous microbial culture are very important practice and necessary for

environment. Feathers are produced in large amounts as a by-product at poultry processing plants, reaching millions of tons annually.

These feathers produce a big disposal problem in environment. Feathers are almost composed of over 90% protein, having keratinas the main component (Cabeza *et al.*, 1998). Keratins, a major class of animal proteins, which are constituents of vertebrate skin, nail, hair, feather, wool, etc.., are abundant in nature and hard to degrade but have limited uses in practice.Since they are insoluble and resistant to degradation, feathers create a problem of solid waste management (Ros *et al.*, 2003) and also largely responsible for their high degree of recalcitrance. Little attention has been given to the utilization or recycling of these wastes.

The accumulation of some of these wastes in nature is considered to be a serious source of pollution and health hazards. A group of proteolytic enzyme which is able to hydrolyze insoluble keratins more efficiently than other proteases and produced by some microorganisms, is called keratinases. Keratinolytic enzymes may have potential roles in biotechnological processes that involve keratin containing wastes from the poultry and leather industries.

Keratinase is produced by some microorganisms like bacteria such as *Bacillus sp*. (Manczinger *et al.*, 2003), *Thermoanaerobacter* (Riessen and Antranikian2001), *Chryseobacterium* (Riffle and Brandelli, 2002), *Flavobacterium* (Riffle *et al.*, 2003), *Vibrio* (Sangali and Brandelli, 2000); fungi such as *Aspergillus*, *Absidia* and *Rhizomucor*(Friedrich *et al.*, 1999); some species of dermatophytes, including *Trichophyton mentagrophytes*, *T. rubrum*, *T. gallinae*, *Microsporium canis* and *M. gypseum* (Bockle *et al.*, 1995); a few actinomyccetes such as *Streptomyces pactum*, *S. albus* (Bockle *et al.*, 1995). Keratinase has several applications in feed, fertilizers, detergents, leather and pharmaceutical industries (Brandelli *et al.*, 2010).The isolation of some microorganisms can be used to get rid of keratinous wastes and increasing crop productivity (Anwar *et al.*, 2014).

This work was devoted to isolate a microorganism with versatility and potential uses in the environment. It will be used as a Plant Growth Promoting Agent (PGPA) with the benefit of increasing crop productivity as well as the cleaning of the environment (feather wastes hydrolysis).

Materials and Methods

Isolation and Screening of a Splendid Microbe for Keratinase Production

Microbial samples were isolated from feather and leather wastes obtained from different local dumping sites in Egypt as well as a marine isolate from sea water (Mediterranean).

Medium for Isolation (g/l)

NaCl, 0.5; KH_2PO_4, 0.7; K_2HPO_4, 1.4; $MgSO_4$, 0.1; prepared milled white chicken feather, 10; and agar, 20 at pH 8 (Letourneau *et al.*, 1998).Using serial dilutions technique, fourteen isolates were selected from the plates containing the above medium. Microbiological survey for keratinase enzyme had been carried out for the most appropriate fourteen isolates. They were eleven bacterial isolates, one fungus and two actinomycetes species. For the eleven bacterial isolates, the screening had been performed on shaking liquid cultures. The remaining fungal and actinomycetes isolates were subjected to both static and shaking liquid cultures. The most potent feather degrading isolate was selected and designed as NRC3 aza.

Strain Identification

Taxonomic studies and 16S rDNA sequencing

The isolatewas identified according to Bergey's Manual of Systematic Bacteriology (Williams *et al.*, 1989).DNA extraction was done using protocol of Gene JET^{TM} genomic DNA Purification Kit (Thermo) # K0 721. Then PCR was made using Maxima Hot Start PCR Master Mix (Thermo) # K1061. PCR conditions were: 35 cycles–denaturation at 94^{o}C for 1min., annealing at 50^{o}C for 2min., and extension at 74^{o}C for 1.5min. An initial 3min. denaturation at 94^{o}C and a final 5 min. extension at 74^{o}C were used. Primer Design set was

NL1 :(5'-GCATATCAATAAGCGGAGGAAAAG-3')

NL4 :(5'–GGTCCGTGTTTCAAGACGG-3').

Then PCR clean up for the PCR product was made using Gene JET^{TM} PCR Purification Kit (Thermo) # K0701. A phylogenetic tree was constructed employing Biology Work Bench program.

Feather processing

Chicken feathers were collected and soaked for 12hr in a washing liquid containing 1% detergent for degreasing, then washed thoroughly with distilled water. The feathers were then dried completely at 60^{o}C, milled and sieved by 1mm pore sieve.

Fermentation of the Selected Strain for Keratinase Production

Inoculum Preparation

Optimization of keratinase production by the selected potent isolate *Cyberlindnera fabianii* NRC3 aza was studies using chicken feather as sole substrate. The organism was maintained on tryptic soy agar (TSA) slants and incubated at 37^{o}C for 5 days. The spores were scratched from the 5-days- old slants with sterile distilled water containing 0.1% tween 80 (spore suspension containing 105 spores ml^{-1}).Then transferred into the inoculum medium which was composed of (g/L): glucose, 10; peptone, 10; yeast extract, 3; Ca Cl_2. $2H_2O$, 2 (modified from Chitte*etal*.) prepared in

250ml Erelenmeyer flasks and incubated on a shaking incubator at 37ºC ,180rpm for 2 days (Chitte *et al.*, 1999). 2% of the 2 days-old inoculum was used for the inoculation of each submerged fermentation (SmF) flask and incubated for 3days in a shaker incubator at 180 rpm.

Culture Media

Basic liquid medium used for the enzyme production composed of (g/l): chicken feather, 20; NaCl, 0.5; KH_2PO_4, 0.7; K_2HPO_4, 1.4; MgSO4, 0.1; pH 7.2 (Cai C. et al 2008). Fermentation was carried out by seeding 3- days inoculum of (NRC3 aza) (6% v/v) in a 250ml Erlenmeyer flasks with 50ml of the basic liquid medium containing chicken feather as the sole N and C source at 37ºc and incubated in a shaker incubator at 180 rpm. Prior to assay, the fermentation broth was centrifuged to separate the filtrate.

Preparation of Keratin Solution

Keratinolytic activity was measured with keratin (0.5 % w/v) as substrate. Soluble keratin was prepared from white chicken feathers by the method of Wawrzkiewicz *et al.*, (Wawrzkiewicz *et at*., 1987).

Native chicken feathers (10gm) in 500ml of dimethyl sulfoxide were heated by a reflex condenser at 100ºc for 2hr. Soluble keratin was then precipitated by addition of cold acetone (1L) at -70ºC for 2hr. followed by centrifugation at 1000 x g for 10min.The resulting precipitate was washed twice with distilled water and dried at -40ºc in a vacuum dryer. One gram of quantified precipitate was dissolved in 20ml of 0.05 mol/L NaOH. The pH was adjusted to 8.0 with 0.1 mol/ L Tris and 0.1 mol/L HCl and the solution was diluted to 200ml with 0.05 mol/L Tris–HCl buffer (pH 8.0).

Enzyme Assay

Keratinolytic activity was assayed as follows: 1.0 ml of crude enzyme properly diluted in Tris- HCl buffer (0.05 mol/L, pH 8.0) was incubated with 1ml keratin solution at 50ºc in a shaker water bath for 10min., and the reaction was stopped by adding 2.0ml 0.4mol/L trichloroacetic acid (TCA). After centrifugation at 1450 x g for 30min, the absorbance of the supernatant was determined at 280nm (UV-2102, UNICO Shanghai Corp, China) against a control. The control was prepared by incubating the enzyme solution with 2.0ml TCA without addition of keratin solution (Gradisar *et al.*, 2005).One unit (U/ml) of keratinolytic activity was defined as an increase of corrected absorbance of 280 nm (A_{280}) with the control for 0.01 per minute under the conditions described above and calculated by the following equation:

U= 4 x n x (A_{280} /0.01 x 10), where n is the dilution rate, 4 is the final reaction volume (ml); 10 is the incubation time (min).

Determination of Protein Concentration

The soluble protein concentration was determined by the Lowry method using bovine serum albumin as a standard (Lowry *et al.*, 1951).

Production of Keratinase by Solid State Fermentation (SSF)

This was achieved by the fermentation of (NRC3aza) on solid waste media composed of 10gm feather , 10 gram wheat bran, 10gm feather + 0.05gm wheat bran and 10gm wheat bran +0.05gm feather, each was prepared in 250ml Erlenmeyer flasks and moistened with 10ml of the basal medium at pH 7.2 (100% moisture content). The flasks were incubated with freshly prepared (30%) inoculum and incubated for 72hr. at 37ºC (optimum time for keratinase activity) (EL- Gendy 2010).

Enzyme Extraction

The content of each flask was mixed with 100ml of 0.1% tween 80 distilled H_2O and shaken for 1hr. at 180 rpm and at room temperature. The solid mat was separated using a cloth mesh; then, the cell extracts were centrifuged in a cooling centrifuge at 10000 rpm for 20 min (Abdel-Fattah 2013). The supernatant was then used for the enzyme and protein assay as well as the horticultural application as plant growth promoting enzyme (Paul et al.2013).Culture filtrate from solid state fermentation was symbol as (S), the one from liquid or submerged state fermentation as (L) and the water blank as (B).

Electrophoretic Studies

Polyacrylamide gel elecrophoresis of the Keratinase was carried out to determine the protein profile of the enzyme (Jayalakshmi *et al*., 2011).

Description

The Page Ruler™ plus Prestained Protein Ladder is a mixture of 9 recombinant, highly purified colored proteins with apparent molecular weights of 10 to 250 kDa. It contains two orange proteins as reference bands. Other proteins are coupled with a blue chromophore. The Page Ruler™ plus Prestained Protein Ladder is ready- to – use; supplied in a loading buffer for a direct loading on SDS-plyacrylamide gels.

Composition

0.1– 0.2 mg/ml of each protein in 62.5 mM Tris- H_2PO_4 (pH 7.5at 25ºC), 1mM EDTA, 2% SDS, 10 mM DTT, 1 mM NaN_3 and 33 % glycerol.

Land Preparation and Systems Used

On 20.11.2012 peas were seeded in 5 rows of 12 meters long. First spray was on 26.12.2012 (36 days of the plant age).Concentration of culture filtrate was 5ml/1000ml water to be sprayed with. The first 4 meters of the row were sprayed with water only (Blank), the second with the submerged or liquid state fermentation culture filtrate (L)

and the last with the solid state fermentation culture filtrate(S).

The second spray was 73 days after seeding. The crop was harvested 160 days after seeding.The plant was irrigated by drip irrigation system. Peas varieties were 5, each in a raw.

Table 1: Distribution system of peas cultivation

Peas variety No.	1st. 4 meters of the row	Middle 4 meters of the row	Last 4 meters of the row
1	(B)blank (water)	(S) solid state c.f.	(L) SmF c.f.
2	(B)	(S)	(L)
3	(B)	(S)	(L)
4	(B)	(S)	(L)
5	(B)	(S)	(L)

S: solid, L: liquid

Results and Discussion

Microbial keratinolytic enzymes have been described for various biotechnological applications in food, detergents, textiles, and leather industries, and yet the growing demand for these enzymes necessitates the screening for novel keratinolytic microorganisms with potential applications (Gupta *et al.*, 2002). A total of 14 pure cultures were isolated and purified which obtained from different samples collected from Governorates of Egypt. All isolates were screened for their keratinolytic activities. In this way we can probe the process of feather degradation under variable biological and enzymatic conditions (Jayalakshmi *et al.*, 2011).All isolates were grown on the basal medium and have the ability to degrade feather. Preliminary screening showed that one isolate exhibited the highest keratinolytic activity (1706.67 U/ml) in its culture supernatant. The promising keratin-degrading strain was isolated from leather. Moreover, it showed pronounced growth and complete hydrolysis of native chicken feathers. This isolate was identified as *Cyberlindnera fabianii* NRC3 aza.

Identification and Characterization of the Selected Strain

Phylogenetic Analysis of 16S rDNA

Phylogenetic analysis based of the 16S rDNA sequence of strain NRC3 aza exhibited a high level of homology (96%) with the sequence of *Pichia fabianii* 26S ribosomal RNA gene, (Fig.1). The sequence (1,496 bp) was submitted to Gen- Bank under the accession number EF 550321.1.The isolate was suggested to be a novel species of candida and was designated as *Cyberlindnera fabianii* NRC3 aza.

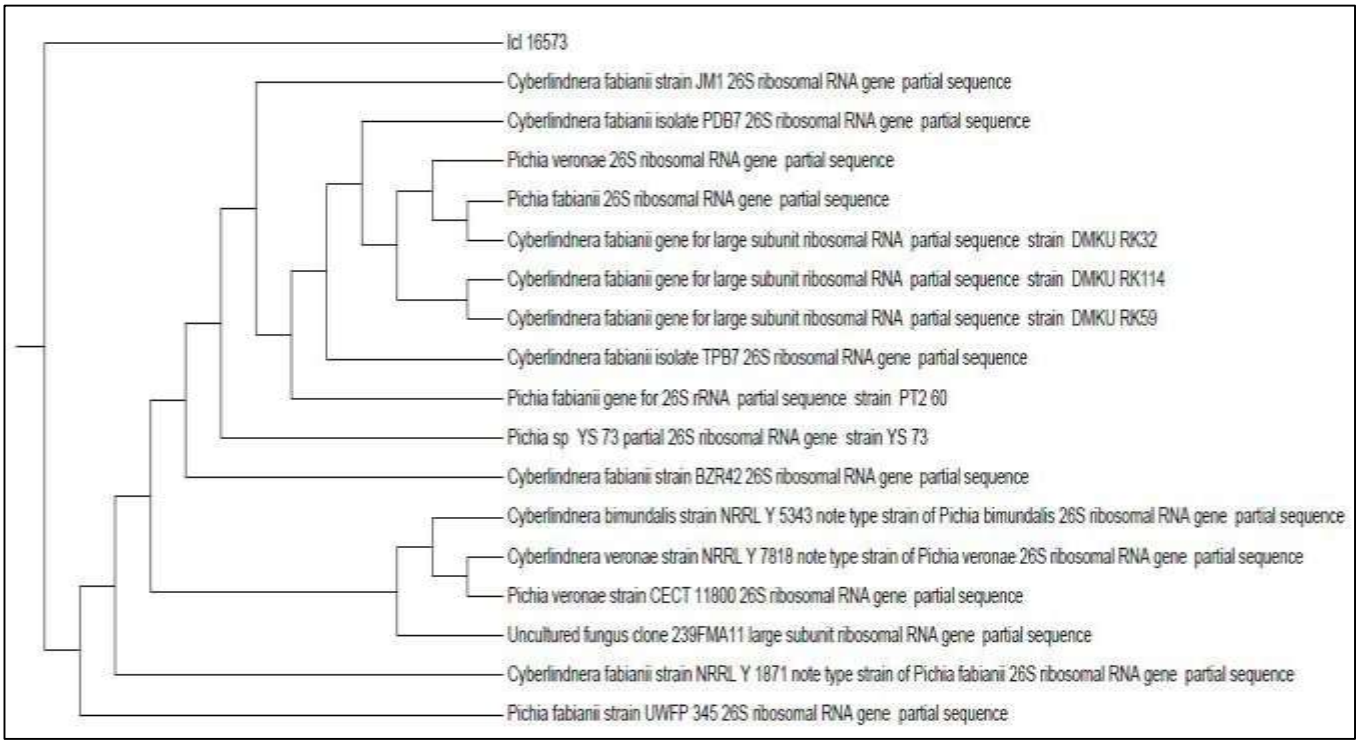

Fig. 1: Phylogenetic tree based on the 16S rDNA sequence of strain *Cyberlindnera fabianii* NRC3 aza within the genus Candida. The tree was constructed by analyzing approximately 1496 bp of the sequence with CLUSTRAL W 16S rDNA sequence alignments using the program Biology Work Bench

The time course of keratinase production by *Cyberlindnera fabianii* NRC3 aza culture grown in minimal liquid medium with 1% (w/v) whole feather as its primary source of carbon, nitrogen and energy is presented in Fig. 2.

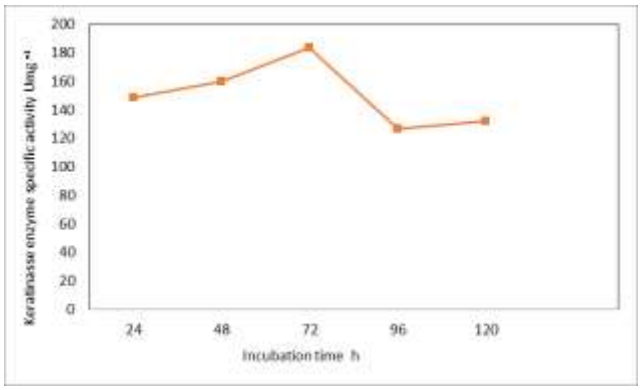

Fig. 2: The average of keratinolytic specific activity during growth of*Cyberlindnera fabianii* NRC3 aza in whole feather medium at 37 °C and initial pH 7.5

The keratinolytic activity of the culture filtrate appeared on the first day and achieved its maximum level (1706.67 U/ml 183.51U/mg proteins) on the third day of semi liquid fermentation. Complete solubilization of the solid feather occurred by day 4 of cultivation as the culture solution appeared as a suspension Fig. 3. However, the 4 days required for chicken feather degradation by NRC3 aza should facilitate its industrial use and offer tremendous potential for development of biotechnological methods for the hydrolysis of feather and its utilization as a feedstuff. It is evident that *Cyberlindnera fabianii* NRC3 aza very efficiently degraded chicken feather with maximum enzyme activity after 72 h incubation time. This is in agreement with Jeong*et al.*(Jeong *et al.*, 2010). Moreover, it is shorter than that of *Streptomyces lavendulae* which required 7 days for maximum keratinase production (Demina and Lysenko 1995) and *Bacillus licheniformis* PWD-1 which degraded intact feather completely at 50°Cin 10 days (Cheng *et al.*, 1995).

Table 2: Production of keratinase enzyme bythe local isolate *Cyberlindnera fabianii* NRC3 aza based on solid-state fermentation using feather and wheat bran wastes.

Waste g	Protein content Mg ml^{-1}	Keratinase activity U ml^{-1}	Specific activity U mg^{-1}
F	2.16	15333.3	709.88
F+W	3.15	15853.4	503.28
W	4.81	15453.3	321.27
W+F	4.25	1638607	385.57

F = 10 g feather, F+W = 10 g feather + 0.05 g wheat bran, W= 10g wheat bran, W+F = 10g wheat bran + 0.05 g feather

Solid State Fermentation (SSF)

The results in Table 2, illustrated the production of keratinase enzymes using the solid poultry feather waste and the agroindustrial waste (wheat bran) as the sole C and N source. Complete feather degradation was achieved when *Cyberlindnera fabianii* NRC3aza was grown on different combinations of feather and wheat bran as the substrate, producing maximum keratinase activity on the whole-feather substrate (15333.3 Ug^{-1}, 709.88 Umg^{-1}).

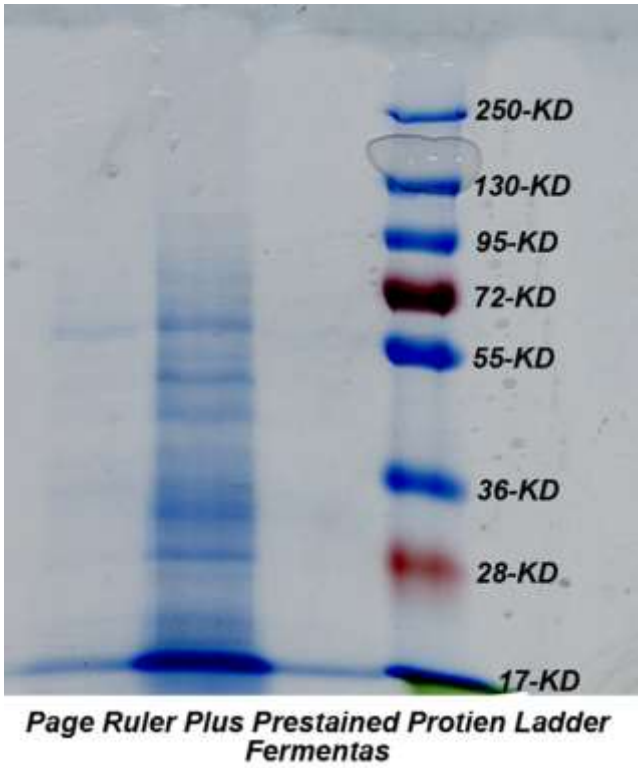

Fig. 3: SDS–polyacrylamide gel electrophoresis of the precipitated keratinase enzyme (partial pure fraction). Lane 1: The marker proteins. Lane 2: partial pure keratinase. Lane 3: Crude keratinase extract (before ammonium sulphate fractionation) with 100% Ammonium sulphate.

However, the Candida isolate,produced active keratinase on the whole wheat bran substrate (15453.3 Ug^{-1}, 321.27 Ug^{-1}). Wheat bran as one of the important agro-industrial wastes, was used for the production of microbial enzymes on SSF (George *et al.*, 1995 and Purushotham *et al.*, 1996). The *Cyberlindnera fabianii* NRC3 aza produced keratinase enzyme both inducibly and constitutively; which was similar to the result obtained by El-Gendy (2010) who produced keratinase enzyme constitutively on solid rice straw. In consideration of economics and technology, it was shown that SSF has several advantages over the SmF in producing products for the food, feed, pharmaceutical, and agricultural industries, especially for yeast cultures. In addition, it is a simple, cost-effective, and environmental friendly process for the solid waste management of feather and other keratinous wastes; it is also a potential method to improve the nutritional value of keratinous wastes as feed supplements. However, SSF include high volumetric productivity, relatively high concentration of product, generation of less effluent, simple fermentation equipment, and purification procedures, which are less costly.

Additionally, the advantages of using low-cost natural material in SSF processes, which greatly reduces pollution problems, are of special economic interest for the countries with abundance of biomass, agro industrial, and poultry residues.

keratinase Enzyme Electrophoresis

Electrophoresis was carried out for the batch precipitated keratinase enzyme (partial pure fraction) with 100% Ammonium sulphate (Eman and Neveen, 2011). Electrophoresis was illustrated in Fig. 3.

SDS‑PAGE is an excellent method for rapidly assessing the purity and molecular weight of proteins (Roe, S., 2001).The Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of the partial pure alkaline keratinase (lane 2) of *Cyberlindnera fabianii* NRC3 aza revealed four sharp bands with different molecular weights of extracellular keratinases compared with the marker proteins (Lane 1). The apparent molecular weights of the partial pure enzyme were 28 kDa for KI and 65 kDa for KII this was in agreement with the molecular weight of the enzymes from *Trichophyton mentagrophytes* – 28–65 kDa (Siesenop and Bohm, 1995). On the other hand, the partial pure enzyme showing a single Protein band in SDS–PAGE with a molecular weight of 45–50 kDa for KIII which is similar to the molecular weight of the enzymes from *Trichophyton mentagrophytes* (Malviya *et al*., 1992).

Amino Acid Profile of Cyberlindnera Fabianii NRC3 Aza Keratinase

Amino acid analysis of the present partial pure keratinase from *Cyberlindnera fabianii* NRC3 aza showed that it is composed of 14 different amino acids, Table 3.

Table 3: Amino Acid Analysis of the keratinase Enzyme

Pk no.	Name of amino acid	Conc. µg/ ml	Relative concentration, %
4	Aspartic	179.04	3.938
5	Threonine	54.8	1.205
6	Serine	118.16	0.026
7	Glutamic acid	1904.32	41.891
8	Glycine	107.76	2.370
9	Alanine	192.88	4.243
10	Valine	131.36	2.89
11	Isoleucine	59.28	1.304
12	Leucine	209.84	4.616
13	Tyrosine	173.44	3.815
14	Phenylalanine	282.72	6.219
15	Histidine	221.28	4.868
17	Lysine	113.2	2.490
18	NH_4^+	473.76	10.422
19	Arginine	324.08	7.129

Pk. No. ≡ Peak No.

The enzyme contained high proportions of glutamic acid (41.891%), arginine (7.129%) and phenylalanine (6.219%). Histidine (4.868%), leucine (4.616%) and alanine (4.243%), aspartic (3.938%), tyrosine (3.815%) was of moderate amounts. Valine, lysine, glycine and threonine constituted less than 3%, each. isoleucine constituted (1.304%) whereas Serine was 0.026%.

In this relation, Farag and Hassan (2004) demonstrated that the purified keratinase enzyme of *A. oryzae* is composed of 17 different amino acids with high quantities of glycine, glutamic acid and serine as compared with moderate amounts of aspartic acid, histidine, arginine and lysine. On the other hand, keratinase of M. canis was recorded to have a high aspartic acid, glycine and alanine contents (Hamaguchi *et al.*, 2000).

Collection of Peas Pods

Samples of pods from all peas varieties were collected as follows: 5 pods from each of the blank (B) and the two culture filtrates (S&L) were collected for the morphological and chemical analytical measurements.

Chemical analysis of the dried powdered beads
A: Season 2011-2012

In this season (2011-2012) only liquid state fermentation culture filtrate of keratinase enzymewas used. The test plant had been sprayed by this culture filtrate**,** which was referred to in table (4) as enzyme (Enz.). The comparison was carried out between the enzyme sprayed and the control (water- sprayedplants) plants. The enzyme has a positive effect on tested parameters of the treated plants, Table 4.

Table 4: Shows the chemical assay and contents of dried powdered beads for 2011/2012 season.

Parameter	GROUP	Mean	S.D	t-value	p-value
Moisture, %	**Enz.**	6.70	0.37	1.47	0.152
	Control	6.51	0.36		
Protein, %	**Enz.**	38.69	1.14	1.74	0.093
	Control	36.30	5.20		
Ash, %	**Enz.**	4.67	0.42	0.53	0.601
	Control	4.75	0.39		
Carbohdrate, %	**Enz.**	50.39	2.09	1.34	0.190
	Control	52.23	4.87		
Na, %	**Enz.**	0.58	0.41	2.95	0.006*
	Control	0.26	0.11		
K, %	**Enz.**	1.86	0.19	3.92	0.001*
	Control	1.62	0.13		
Ca, %	**Enz.**	0.02	0.00	6.77	0.000*
	Control	0.01	0.00		
Fat, %	**Enz.**	0.53	0.03	1.50	0.144
	Control	0.52	0.02		

S.D = Standard Deviation
* = There is a significant difference between Enz. &Control by using independent t-test at P< 0.05.

B: Season 2012-2013

The plants were sprayed by keratinase enzymefrom liquid state fermentation (L) and solid state fermentation (S) in season (2012-2013). The blank (B) had been sprayed by water, Table 5.

Table 5: Chemical analysis and contents of beads dried powdered for 2012/2013 season.

Parameter	Group	Minimum	Maximum	Mean	S.D	F-value	p-value
Moisture, %	**B**	6.30	6.60	6.44 [a]	0.09	4.27	0.021*
	L	6.25	6.61	6.45 [a]	0.14		
	S	6.38	6.78	6.57 [b]	0.16		
Protein, %	**B**	36.63	43.26	39.03 [a]	2.55	1.83	0.173
	L	37.13	43.00	39.55 [a]	1.97		
	S	35.00	40.23	38.11 [a]	1.64		
Ash, %	**B**	4.11	4.91	4.45 [a]	0.29	1.43	0.250
	L	4.11	5.33	4.52 [a]	0.49		
	S	4.10	4.60	4.31 [a]	0.20		
C.H. , %	**B**	45.41	53.00	50.09 [ab]	2.74	2.90	0.066
	L	46.45	52.12	49.39 [a]	2.08		
	S	48.56	54.11	51.34 [b]	1.85		
Na, %	**B**	0.29	1.92	0.59 [a]	0.51	1.31	0.280
	L	0.27	0.57	0.42 [a]	0.11		
	S	0.27	0.80	0.45 [a]	0.17		
K, %	**B**	1.60	1.92	1.79 [b]	0.11	2.53	0.092
	L	1.55	1.90	1.74 [ab]	0.11		
	S	1.62	1.88	1.70 [a]	0.09		
Ca, %	**B**	0.01	0.04	0.022[b]	0.01	3.63	0.035*
	L	0.01	0.02	0.016[a]	0.00		
	S	0.00	0.03	0.016 [a]	0.01		
Fat, %	**B**	0.50	0.54	0.518 [a]	0.01	4.68	0.015*
	L	0.51	0.55	0.531 [b]	0.01		
	S	0.51	0.54	0.521 [a]	0.01		

- **S**.D = Standard Deviation
- * = There is a significant difference between B&L&S by using One Way ANOVA at P< 0.05
- The same letter means that there is no significant difference between each two groups by using Duncanmultiple comparison test at p<0.05
- The different letters means that there is a significant difference between each two groups by using Duncanmultiple comparison test at p<0.05

Table 6: The morphological data based on average weight, length, width, number of beads for pods and weight of 100 beads for season (2012-2013).

Parameter	Group	Minimum	Maximum	Mean	S.D	F-value	p-value
Weightof 5 pods, gm	**B**	8.11	10.41	9.17 [a]	0.76	12.446	0.000*
	S	9.19	11.00	10.14 [b]	0.66		
	L	9.50	12.70	10.70 [b]	1.08		
Length of 5 pods, Cm	**B**	42.00	51.00	46.87 [a]	3.37	32.104	0.000*
	S	46.00	58.00	53.02 [b]	3.43		
	L	52.70	58.20	55.39 [c]	2.00		
Width of 5 pods, Cm	**B**	9.90	11.20	10.41 [a]	0.42	29.202	0.000*
	S	10.60	11.50	11.11 [b]	0.28		
	L	10.90	11.80	11.29 [b]	0.28		
Number of beads in 5 pods	**B**	20.00	30.00	26.33 [a]	3.31	17.502	0.000*
	S	25.00	35.00	30.27 [b]	3.45		
	L	29.00	37.00	33.00 [c]	2.45		
Weight of 100 beads, gm	**B**	21.74	26.58	23.59 [a]	2.03	9.35	0.000*
	S	24.48	28.46	26.18 [b]	1.53		
	L	23.52	27.14	25.38 [b]	1.39		

S.D = Standard Deviation
* = There is a significant difference between B&L&S by using One Way ANOVA at P< 0.05

Morphological data collection for season 2012-2013

Table 6 illustrates the morphological data of pods and 100 beads for season (2012-2013). The obtained results showed that an average weight, length, width of pods as well as number of beads for pods and weightof 100 beads are better for both (L&S) enzyme treated samples compared with the blank (B). While, the (L) treated was preferable than the (S) treated except for weight of 100 beads

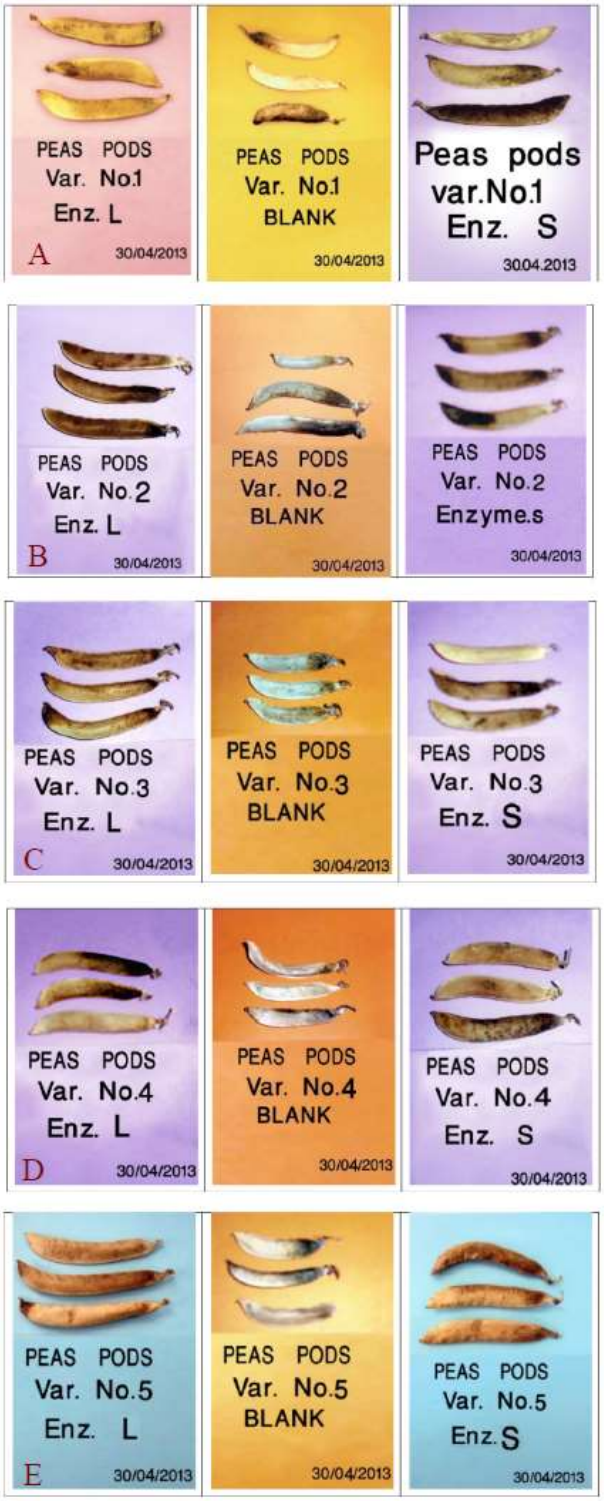

Fig. 4 (A-E): Morphological appearance of peas pods. A. Variety No. 1; B. Variety No. 2; C. Variety No. 3; D. Variety No. 4; E. Variety No. 5

It is obviously from the Figures (4a-e) that both pods treated by keratinase enzymes (L&S) are larger than the blank pods.

Fig. 4 (a-e): Illustrate the morphological appearance based on length, width and width of peas beads for season (2012-2013).

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