African Journal of Biotechnology Vol. 11(19), pp. 4424-4433, 6 March, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.3439
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Construction of recombinant *Pichia* strain GS115-Ch-Glu expressing β-glucosidase and cyanide hydratase for cyanogenic glycosides detoxification

Chou-Fei Wu[1,2], Ai-Juan Feng[1,2], Xiao-Ming Xu[2], Si-Han Huang[2], Mao-Cheng Deng[2], Xin-Ning Zheng[3], Xiao-Ying Wu[1,2], Juan Peng[2], Jian-Ping Yuan[2]* and Jiang-Hai Wang[2]*

[1]School of Life Sciences, Sun Yat-Sen University, Guangzhou 510275, People's Republic of China.
[2]Guangdong Provincial Key Laboratory of Marine Resources and Coastal Engineering, School of Marine Sciences, Sun Yat-Sen University, Guangzhou 510006, People's Republic of China.
[3]R & D Center, Guangzhou Enenta Electrical Equipment Co., Ltd, Guangzhou 510635, People's Republic of China.

Accepted 18 January, 2012

A recombinant *Pichia* strain GS115-Ch-Glu expressing both β-glucosidase and cyanide hydratase was constructed to remove cyanogenic glycosides in edible plants. The β-glucosidase could hydrolyze cyanogenic glycosides into cyanide and its gene (*Glu*) was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR). A cyanide hydratase could catalyze cyanide to formamide and its gene (*Ch*) was obtained by C (PCR). Both *Glu* and *Ch* genes were integrated into the genome of *Pichia* strain GS115 by homologous recombination. The engineering strain GS115-Ch-Glu was confirmed by multiple analytical methods, and was inoculated to induce expression of the recombinant *Glu* and *Ch* genes. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showed that the fused proteins of interest had specifically been expressed as 31 and 52 KDa, which corresponded to the predicted molecular weights of the two enzymes. The enzyme preparation containing cyanide hydratase and β-glucosidase produced by strain GS115-Ch-Glu could completely decompose cyanogenic glycosides in flaxseed power into cyanide, and further catalyze the hydrolysis of over 80% cyanide.

Key words: Cyanogenic glycosides, recombinant strain, cyanide hydratase, β-glucosidase, detoxification.

INTRODUCTION

The cyanogenic glycosides (CGs) belong to secondary metabolites and widely exist in edible plants, such as cassava, flaxseed, almond and sorghum (Vetter, 2000). In the intestines of mammals, these compounds could liberate acutely toxic hydrocyanic acid (HCN) due to the effect of β-glucosidase and cause the toxicity of the mammalian respiratory system, nervous system and endocrine system (Wu et al., 2008). Therefore, the detoxification of CGs is very important for the safety of these edible plants. Some researchers have been devoted to the study of biosynthesis of CGs for the development of cyanogens free transgenic plants, and certain progresses have also been made (Nielsen et al., 2008; Ganjewala et al., 2010). So far, the popular detoxification of CGs has been tried by a diversity of conventional methods such as boiling, roasting, autoclaving, microwave and extrusion (Madhusudhan and Singh, 1985; Feng et al., 2003; Nambisan, 2011). In contrast with these conventional methods, the fermentation detoxification method has many advantages of high efficiency, energy saving, and safety, and gains an increasing attention for CGs detoxification (Bradbury, 2006). It has been reported that for cassava flour directly

*Corresponding author. E-mail: wangjh@gig.ac.cn, yuanjp@mail.sysu.edu.cn. Tel: +86-20-39332212, +86-20-39332213.

Abbreviations: RT-PCR, Reverse transcriptase-polymerase chain reaction; **PCR,** reverse transcriptase-polymerase chain reaction; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresi

mixed with water using the proportion of flour to water (1:1.25), after fermentation under the catalysis of endogenous β-glucosidase for 2 h in the sun, the total cyanide content was reduced 3-6 fold (Bradbury and Denton, 2010). However, the method only caused a limited disruption of plant cells with the restricted contact between CGs and endogenous β-glucosidase and thus resulted in residual cyanide in the product, which may as well be sufficient to produce toxic symptoms (Cardoso et al., 2005).

Sornyotha et al. (2010) proposed an efficient cell wall-degrading method, which could enhance endogenous β-glucosidase liberation from the parenchyma by 90%. Since the endogenous β-glucosidase would be denatured when the sample was dried at temperatures higher than about 80°C (Bradbury and Denton 2010), the fermentation methods using endogenous β-glucosidase were ineffective, unless exogenous β-glucosidase was added. Some researchers had successfully isolated some microorganisms such as lactic acid bacteria, yeast and moulds, which could produce effective exogenous β-glucosidase. These microorganisms were added to CGs-containing samples and completely degraded the CGs by fermentation (Lei et al., 1999; Vasconcellos et al., 2009). However, it had been reported that bioactive ingredient (lignan) in flaxseed could be destructed under acidic condition of the pH (Li et al., 2008; Yuan et al., 2008), which may occur due to the metabolism action of microorganisms. Thus, it is a good idea to detoxify CGs with single β-glucosidase, which could retain the beneficial nutrients at the same level as untreated flaxseed (Yamashita et al., 2007).

β-Glucosidases exist in all living organisms including archaea, bacteria, fungi, plants and animals (Park et al., 2005). These enzymes have been extensively studied due to its strong potential of utilization in the food and medical industries (Briante et al., 2004; Gu et al., 2009). For example, the β-glycosidase from *Thermus flavus* AT-62, which was overexpressed in *Escherichia coli* BL21 (DE3) with a pET21b(+) vector system, displayed a strong activity for lactose hydrolysis at 70°C and pH 7.0 (Kang et al., 2005). It has been reported that cytosolic β-glycosidase in mammals could efficiently hydrolyze several naturally occurring plant glycosides, including amygdalin and L-picein (LaMarco and Glew, 1986). Although, β-glucosidase of human liver had been successfully expressed (de Graaf et al., 2001), its application study on CGs detoxification is absent. Furthermore, cyanide hydratase which catalyzes the hydrolysis of cyanide to formamide (Dent et al., 2009) has been used in the bioremediation of cyanide-containing wastes. Thus, it has the potential to absorb HCN formed from CGs hydrolysis instead of directly releasing into air by vaporization in the fermentation methods mentioned above, which had caused an environmental pollution. The genes of cyanide hydratase from a variety of cyanide-tolerant microorganisms have been successfully cloned and expressed (Watanabe et al 1998, Basile et al., 2008). However, no report about its application on CGs detoxification has been published, as well as the coexpression of *Ch* with *Glu* gene in *Pichia*.

In the present paper, we intend to develop a novel fermentation method for CGs detoxification using an enzyme preparation, which contains β-glucosidase and cyanide hydratase with a high degrading activity for CGs and strong adsorption ability for HCN, respectively. The aim of the present study was to construct a recombinant *Pichia* strain bearing β-glucosidase and cyanide hydratase genes. To study its effect of CGs detoxification, we presented a fermentation method to remove CGs from defatted flaxseed powder using the compound enzyme preparation overexpressed by this recombinant *Pichia* strain.

MATERIALS AND METHODS

Reagents and plasmid

Total RNA, extracted from a healthy human liver tissue, was purchased from Shanghai Genomics (China). Restriction nucleases, *Sna*B I, *Avr* II, *Sal* I and *Sac* I, were purchased from NEB (USA). First-strand cDNA synthesis kit, DNA purification kit, DNA gel extraction kit, T4 DNA ligase and Taq DNA polymerase were purchased from TaKaRa (Japan). E.Z.N.A® Bacterial DNA Kit was purchased from Omega (USA). Other reagents were of analytical purity. Deionized water was used for the preparation of all solutions. Expression plasmid pPIC9K containing ampicillin gene, kanamycin gene, HIS4 ORF, AOX promoter and MCS, was a gift from Dr. Guo-Jun Huang at School of Life Sciences and Food Engineering, Nanchang University (China).

Microorganism, medium and culture conditions

E. coli DH5α was used as a host strain for subcloning, and cultured at 37°C on the Luria-Bertani medium (5 g L^{-1} yeast extract, 10 g L^{-1} tryptone, and 10 g L^{-1} NaCl). *Pichia* GS115 was used as a host strain for gene expression, and cultured at 30°C on the buffered glycerol-complex (BMGY) medium (10 g L^{-1} yeast extract, 20 g L^{-1} tryptone, 13.4 g L^{-1} YNB, 0.04 g L^{-1} biotin, 10 g L^{-1} glycerine and 0.1 M potassium phosphate buffer with pH = 6.0) and the BMMY medium (10 g L^{-1} yeast extract, 20 g L^{-1} tryptone, 13.4 g L^{-1} YNB, 0.04 g L^{-1} biotin, 5 g L^{-1} methanol and 0.1 M potassium phosphate buffer with pH = 6.0). Wild-type strain *Bacillus* sp. CN-22 (CCTCC AB 2011127, China Center for Type Culture Collection) was isolated from cyanide-contaminated electroplating sludge.

Amplification of *Ch* and *Glu* genes

Two pairs of gene-specific primers based on the *Glu* gene sequence NM_020973 and *Ch* gene sequence EF198139 were used in this study. The sequences of the primers are depicted in Table 1. The forward primer has a *Sna*B I restriction site, and corresponds to a sequence of the end of the initiation codon. The reverse primer has an *Avr* II restriction site, and corresponds to a sequence of the end of the stop codon. The *Glu* gene encoding β-glucosidase was obtained by real-time polymerase chain reaction (RT-PCR) based on human liver total RNA. The *Ch* gene encoding cyanide hydratase was obtained by PCR based on *Bacillus* sp. CN-22 genome DNA. PCR and RT-PCR were performed according to the instructions

Table 1. Sequence of primers based on the *Glu* and *Ch* genes.

Name	Sequence	Note
GLUp1	5′-gctagacgTACGTAgctttccctgcaggatttggatg-3′	The forward primer of *Glu* gene
GLUp2	5′-cgactagcCCTAGGctacagatgtgcttcaaggccat-3′	The reverse primer of *Glu* gene
CHp1	5′-gctagacgTACGTAtccattgccgtgatcca-3′	The forward primer of *Ch* gene
CHp2	5′-cgactagcCCTAGGtcactccgtacgcgc-3′	The reverse primer of *Ch* gene

described in reference (Bleve et al., 2003).

PCR for *Ch* gene was carried out in a 20-µL reaction mixture containing 1 U Taq DNA polymerase. The PCR mixture was initially pre-heated at 95°C for 5 min for initial denaturation before 30 cycles of amplification, which consisted of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and elongation at 72°C for 1 min. Final extension of the reaction was carried out at 72°C for 10 min. RT-PCR for *Glu* gene consisted of two steps. The first step was the synthesis of the first strand of cDNA, which was carried out in a 20-µL reaction mixture containing 1 U reverse transcriptase for 30 min at 42°C. While the second step was the PCR amplification of *Glu* gene, which was carried out at the same reaction mixture and conditions of *Ch* gene amended with elongation for 90 s.

Construction of expression vector pPIC9K-Ch and pPIC9K-Glu

Plasmid DNA extraction from *E. coli*, DNA restriction, ligation, and *E. coli* transformation were carried out as described previously (Joseph and David 2001). Expression vector pPIC9K-Glu and pPIC9K-Ch were constructed, and illustrated in Figure 1. The amplified DNA fragments of *Ch* and *Glu* were digested with *Sna*B I and *Avr* II, and spliced into the corresponding cloning site in pPIC9K respectively, which was also digested with *Sna*B I and *Avr* II. The recombinant vectors were transformed into *Escherichia coli* DH5α. This bacterial strain was cultured at 37°C on the Luria-Bertani medium, supplemented with 50 µg ml^{-1} ampicillin. Positive recombinant clones were selected and their plasmids were extracted and sent to a commercial laboratory for sequencing to confirm the orientation and integrity of the target genes.

Construction of engineering strain of GS115-Ch-Glu

The construction of GS115-Ch-Glu consists of two steps. First, pPIC9K-Ch was linearized with *Sal* I, and then transformed to competent cells of *Pichia* GS115. Subsequently, His^+Mut^+ transformants were screened out and its phenotype was determined by the plating method of KCN. Secondly, pPIC9K-Glu was linearized with *Sac* I, and then transformed to competent *Pichia* cells of GS115-Ch. Subsequently, positive clones were selected on the plate containing esculin. Finally, the integration of target genes into the engineering strain genome was confirmed via PCR and sequencing (Figure 2).

The preparation and transformation of competent cells were performed according to the instructions described by Salamin et al. (2010). Expression vector linearization and His^+Mut^+ screening were performed according to the user manual of plasmid pPIC9K. Plating methods of KCN and esculin were carried out as previously described (Watanabe et al., 1998; Perry et al., 2007).

Expression of recombinant proteins

A single recombinant strain GS115-Ch-Glu was inoculated into a 10 ml BMGY medium in a 100 ml Erlenmeyer flask. The culture was grown at 30°C, and vigorously shaken at 200 rpm until the optical density at 600 nm (OD_{600}) up to 5.0. The cells were harvested by centrifugation at 3,000 × *g* and 4°C for 5 min, and resuspended in sterile water. Then, the cells was harvested and resuspended in the BMMY medium until $OD_{600} \approx 1.0$. Methanol was added every 12 h to a final concentration of 0.5% (v/v) in order to induce expression of the recombinant *Glu* and *Ch* genes. The culture supernatant was periodically taken after induction for 0, 6, 12, 24, 36, 48 and 60 h for Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Wu et al., 2009).

Preparation of defatted flaxseed powder

Flaxseed, purchased in June 2010, is indigenous to the Lanxi County of Heilongjiang Province, China. The flaxseed was dried at 70°C, and ground into powder with a disintegrator. The flaxseed powder was defatted with CH_2Cl_2 for 12 h in a Soxhlet extractor, and dried in a thermostatic oven at 70°C. After the above processes, one sample of defatted flaxseed powder was obtained for this study.

Enzyme preparation

According to the result of SDS-PAGE analysis, the recombinant *Pichia* cells were induced for 48 h at the optimal induction conditions. Then, the culture supernatant was collected and enzyme preparation in the supernatant was precipitated with the TCA method (Cui et al., 2011). The pellet was stored at -20°C after lyophilization for detoxifying CGs in defatted flaxseed powder.

Effect of enzyme preparation on CG detoxification

Twenty-five grams (25 g) defatted flaxseed power in a sealed 250 ml Erlenmeyer flask was mixed thoroughly with 6.25 g water, 1.24 g enzyme preparation, 50 mg $MgCl_2$ and 50 mg $MnCl_2$. After fermentation for 48 h under static condition at 50°C, sample 1 was collected. In the control group, 2.5 g freshly ground flaxseed, which could provide adequate endogenous β-glucosidase (Yamashita et al., 2007), was used to decompose CGs in the defatted flaxseed power instead of enzyme preparation. After fermentation for 48 h at the same conditions, control sample was collected. In order to find out whether the enzyme preparation decompose CGs into cyanide completely or not, a part of the collected sample 1 (5 g) was mixed with 0.5 g freshly ground flaxseed. After fermentation for another 48 h at the same conditions, sample 2 was collected.

The total cyanide content of samples was measured colorimetrically using barbituric acid and pyridine as described previously (Afkhami et al., 2007). The total cyanide in the sampling bottles was collected by absorption with a sodium hydroxide solution. The solution containing CN^- was diluted with distilled water and thermostatted at 30°C, then 0.50% (w/v) chloramine-T was added and the stopwatch was started just after the addition. An

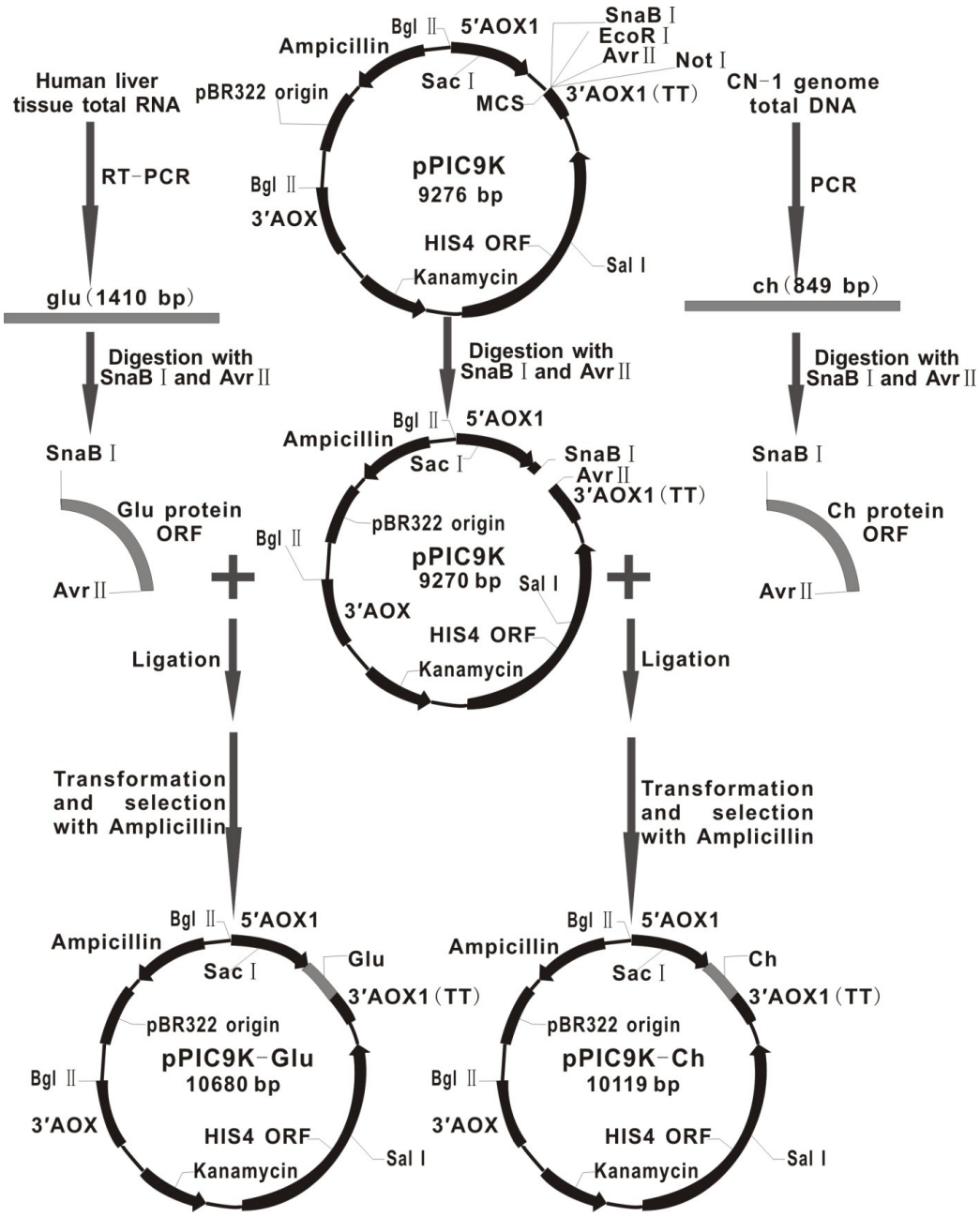

Figure 1. Schematic diagram illustrating the construction of expression vectors of pPIC9K-Glu and pPIC9K-Ch.

immediately barbituric acid-pyridine solution was added. A portion of the solution was transferred into a quartz cell to measure the increasing ratio in absorbance at 578 nm. The degradability of CGs and the absorption rate of HCN were calculated on the basis of the following equations, respectively:

$$Y_1\ (\%) = X_1 / X_2 \quad (1)$$

$$Y_2\ (\%) = (X - X_1) / X \quad (2)$$

Where, Y_1, Y_2, X, X_1 and X_2 represent the degradability of CGs, the absorption rate of HCN, and the content of HCN in the control, sample 1 and sample 2, respectively.

RESULTS

Identification of the expression vector pPIC9K-Ch and pPIC9K-Glu

The expression vectors pPIC9K-Ch and pPIC9K-Glu were identified by PCR and sequencing. PCR analysis of pPIC9K-Ch and pPIC9K-Glu are shown in Figure 3a and b. It can be seen in Figure 3a and b that an 850 bp DNA fragment was obtained by PCR from five-sixths clones, and a 1,400 bp DNA fragment was obtained from

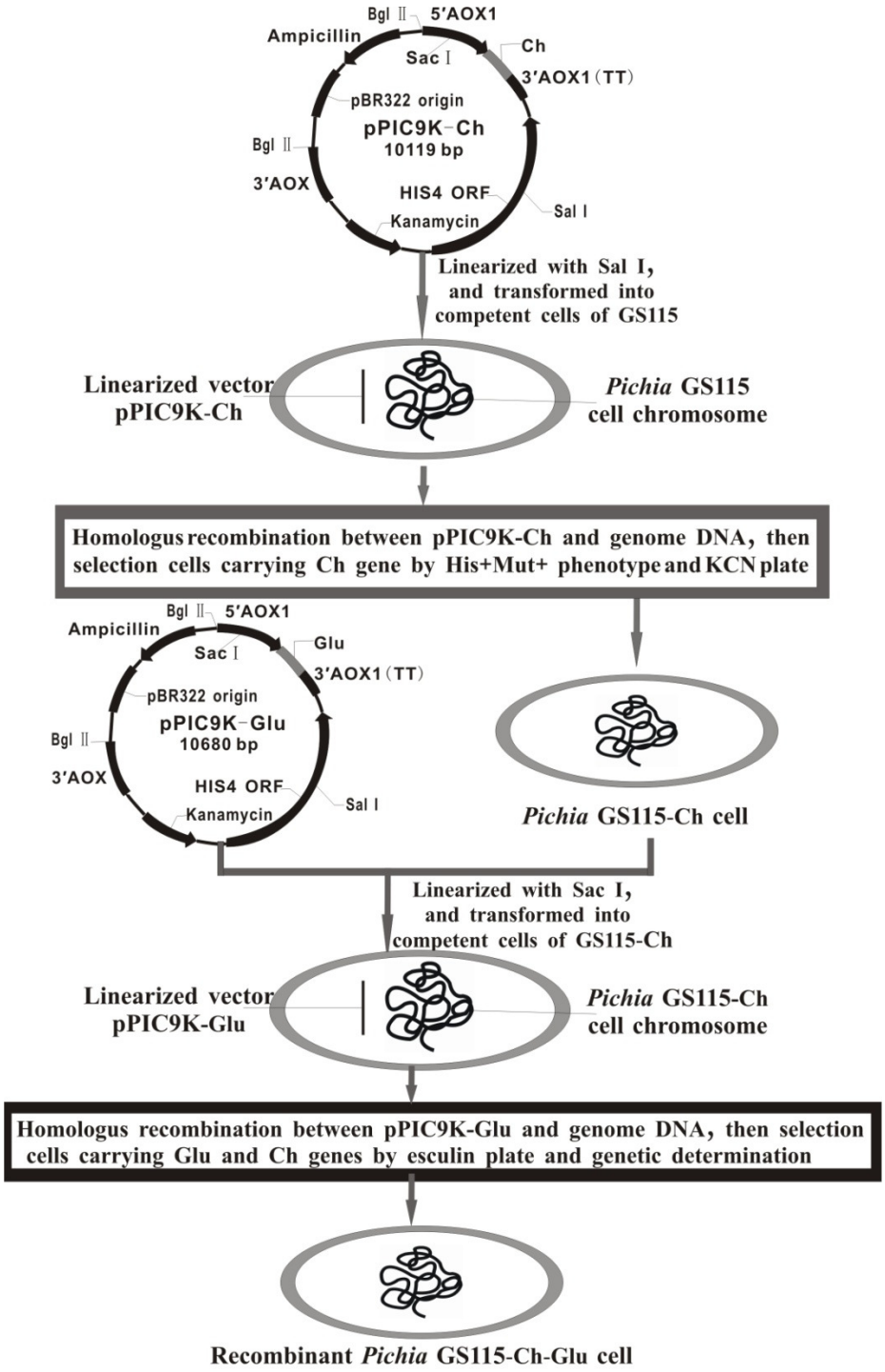

Figure 2. Schematic diagram showing the construction of recombinant *Pichia* GS115-Ch-Glu.

two-thirds clones. The sequencing result indicated that these DNA fragments had 99 and 100% identities with the sequences of *Pseudomonas aeruginosa* cyanide hydratase gene (EF198139) and *Homo sapiens* glucosidase gene (NM_020973) respectively. The complete sequence for cyanide hydratase gene of Bacillus sp. CN-22 was deposited in GenBank under accession numbers JN896988. The ORF for *Ch* and *Glu* genes were also right. Thus, the *Ch* and *Glu* genes were correctly spliced into the vector of expression plasmid pPIC9K

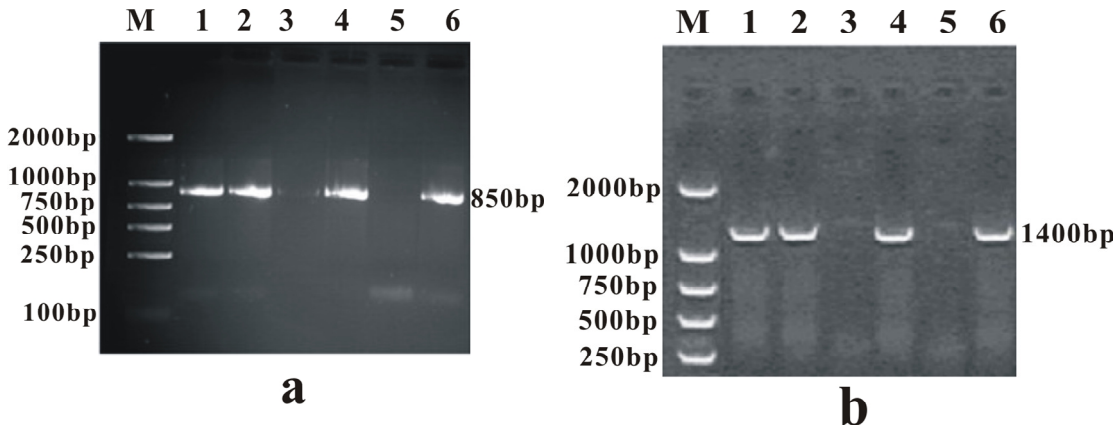

Figure 3. Identification of pPIC9K-Ch (a) and pPIC9K-Glu (b) expression plasmid by PCR. Lane M, DL2000 Marker; lanes 1-6, PCR products.

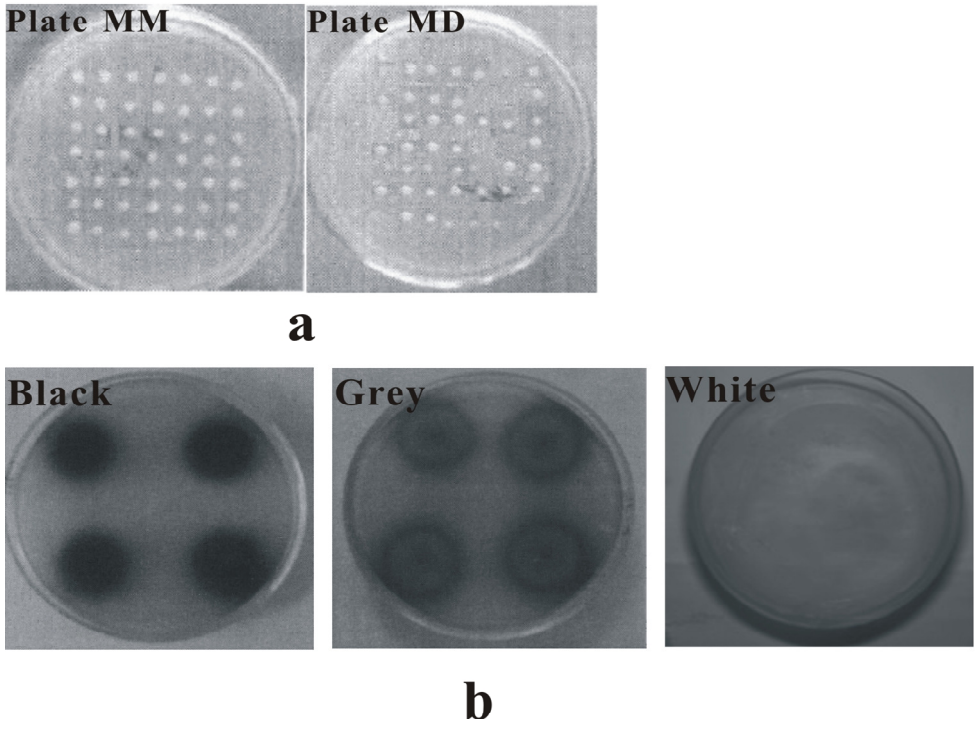

Figure 4. Screening of His^+Mut^+ phenotype (a) and β-glucosidase-producing strain (b).

Identification of engineering strain GS115-Ch-Glu

Screening of His^+Mut^+ phenotype

Expression vector pPIC9K has *Pichia* wild-type gene coding for histidinol dehydrogenase, which is used to complement *Pichia* GS115. Therefore, the GS115-Ch transformants, obtained by transforming linearized plasmid pPIC9K-Ch into competent GS115 cells, could grow normally both in plates MM and MD for pPIC9K inserting into genome DNA of *Pichia* GS115, which could not grow in a medium without histidine. The Mut^+ phenotype strain could grow naturally in media both MM and MD. As shown in Figure 4a, there were forty nine clones with natural growth in plate MD, of which twenty three clones can grow normally in plate MM. Therefore,

Table 2. The result of screening of the tolerating KCN strain.

Strain	Concentration (mg L^{-1})
PCN1	800
PCN2	800
PCN3	1,100
PCN4	900
PCN5	700
PCN6	900
PCN7	1,000

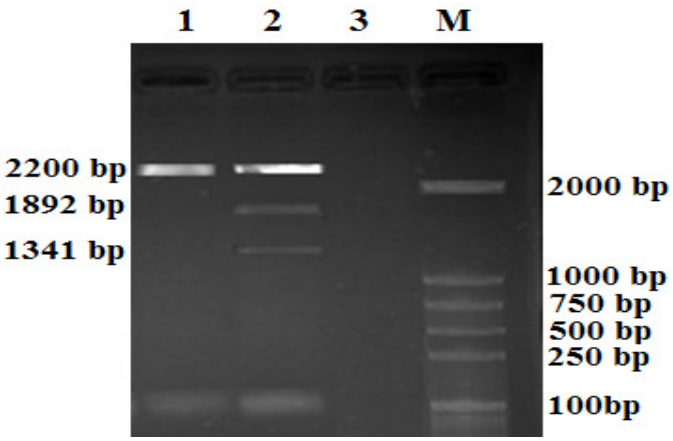

Figure 5. PCR analysis of recombinant *Pichia* GS115-Ch-Glu. Lane M, DL2000 Marker; lanes 1, 2 and 3 correspond to GS115 PCR product, GS115-Ch-Glu PCR product and negative control, respectively.

we successfully screened twenty-three clones with His^+Mut^+ phenotype.

Screening of the tolerating KCN strain

Seven *Pichia* engineering strains with 700 mg L^{-1} KCN tolerance were obtained by exposing recombinant GS115-Ch cells to increase KCN concentrations. Subsequently, the highest KCN tolerating concentration of these strains was studied. As shown in Table 2, strain PCN-3 owns the highest KCN tolerance with 1,100 mg L^{-1}. In this concentration, the OD_{600} of this strain could be up to 1.0 growing at 30℃ for 48 h, and others could not grow naturally. Thus, strain PCN-3 was used as a host cell of linearized pPIC9K-Glu plasmid for next construction.

Screening of β-glucosidase-producing strain

The enzyme activity of different β-glucosidase-producing cell suspensions of twenty *Pichia* engineering strains was studied on the plate containing esculin. The results suggest that one strain was black in the rear on the solid medium, implying that this strain owns a high activity of β-glucosidase; three strains were grey, indicating a low activity; and the other sixteen strains were white, indicating no activity. Obviously, three types of engineering strains with different enzyme activity were identified by the color reactions as illustrated in Figure 4b.

Genetic analysis of recombinant *Pichia* GS115-Ch-Glu

The PCR result for identifying recombinant strain *Pichia* GS115-Ch-Glu is shown in Figure 5. Based on the genome DNA extracted from recombinant strain GS115-Ch-Glu, three DNA fragments were obtained by PCR. The 1,341 bp fragment is composed of *Ch* gene (849 bp) and signal peptide sequence (492 bp) of pPIC9K. The 1,892 bp fragment is composed of *Glu* gene (1,410 bp) and a signal peptide sequence of pPIC9K. The 2,200 bp fragment is the product of *AOX*1 gene in strain GS115. On the same PCR condition, only 2,200 bp fragment could be amplified based on the GS115 genome, and no

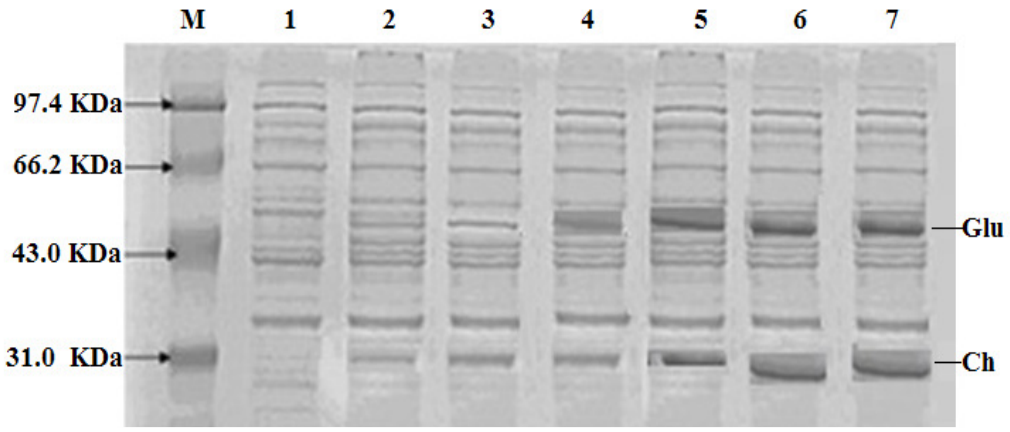

Figure 6. SDS-PAGE analysis of the expression of recombinant proteins induced by methanol at the ultimate concentration of 0.5%. Lane M, Protein marker; lanes 1 to 7 represent 0, 6, 12, 24, 36, 48 and 60 h, respectively.

Table 3. The total content of HCN in control sample, sample 1 and sample 2.

Name	Cyanide released (μg HCN g^{-1})
Control sample	393
Sample 1	78
Sample 2	76

Experiments were repeated at least three times, and the data were expressed as mean values with standard error of the mean (SEM) of three replicates.

fragment could be obtained in the negative control with sterilized ddH_2O as a template. Furthermore, the 1,341 and 1,892 bp DNA fragments were recycled, purified and sequenced. The sequencing result indicated that the *Ch* and *Glu* genes were correctly inserted into the *Pichia* genome DNA, and had no mutation in the construction of recombinant *Pichia* GS115-Ch-Glu.

SDS-PAGE analysis of the expression of recombinant proteins

The recombinant *Pichia* GS115-Ch-Glu was induced by methanol at the ultimate concentration of 0.5%, and then supernatants at 0, 6, 12, 24, 36, 48 and 60 h were analyzed by SDS-PAGE. SDS-PAGE analysis through a 12% resolving gel showed the appearance of two novel bands at approximately 31 and 52 KDa after induction for 6 h, this sizes of which correspond to the predicted molecular weight of cyanide hydratase and β-glucosidase. SDS analysis also showed that the recombinant proteins were expressed to the highest level at 48 h. From 48 to 60 h after induction, the expression of recombinant proteins only increased little. Therefore, the optimal induction time for recombinant *Pichia* GS115-Ch-Glu was 48 h (Figure 6).

Effect of enzyme preparation on CGs detoxification

The total cyanide content of samples was measured colorimetrically using barbituric acid and pyridine. Table 3 shows that control sample, sample 1 and sample 2 released 393, 78 and 76 μg g^{-1} of HCN. The degradability of CGs and the absorption rate of HCN were 102.6 and 80.2% by the numerical calculation. The reason of CGs degradability exceeding 100% may be that more HCN had been absorbed by cyanide hydratase. Therefore, we can draw a conclusion that the CGs in the defatted flaxseed power could be decomposed into cyanide completely under the experimental conditions, and the absorption rate of cyanide reached over 80%.

DISCUSSION

In the present study, we successfully constructed a recombinant *Pichia* strain GS115-Ch-Glu expressing β-glucosidase and cyanide hydratase for the first time.

The results of KCN and esculin plate assay showed that the recombinant strain had a strong activity of both enzymes. Moreover, by the assay of Quantity One Software, we detected that the yield of β-glucosidase and cyanide hydratase expressed in a shaken flask amounted to 12.5 and 8.9% of the total protein in the supernatant, respectively. Thus, the recombinant strain GS115-Ch-Glu had potential practical values in CGs detoxification.

Human *Glu* and *Bacillus* sp. CN-22 *Ch* genes were correctly inserted into the vector of expression plasmid pPIC9K, a *Pichia* vector for secreted expression. The recombinant plasmids pPIC9K-Glu and pPIC9K-Ch were transformed into *Pichia* GS115, and the *Glu* and *Ch* genes were integrated into genome of GS115 cells by homologous recombination, respectively. Although, both genes have been successfully expressed in prokaryotic expression system (de Graaf et al., 2001; Basile et al., 2008), the expression of both β-glucosidase and cyanide hydratase in *Pichia* GS115 with pPIC9K vector system has not been previously reported. *Pichia* GS115 with pPIC9K vector system owns advantage of secreted expression of the desired gene using the α-factor secretion signal (Cregg et al., 2009). Therefore, enzyme preparation containing β-glucosidase and cyanide hydratase could be obtained by condensing supernatant with ease. In addition, *Pichia* has been endowed with the capacity for easy posttranslational modification of recombinant protein, and its intracellular environment is more suitable for correct folding of human β-glucosidase (Cregg et al., 2009). Moreover, cytosolic β-glycosidase, being present in the cytosol of liver of mammals has been shown to hydrolyze several non-physiological glycosides and not to hydrolyze any known physiological β-glycoside (LaMarco and Glew, 1986). Thus, it was presumed that human cytosolic β-glycosidase can hydrolyze CGs in edible plants. The experiment for CGs detoxification indicated that the CGs in the defatted flaxseed power could be decomposed into cyanide completely (Table 3), which is in accordance with the hypothesis mentioned previously. Consequently, β-glucosidase, expressed by *Pichia* is an effective enzyme for CGs detoxification.

Based on the known Ch gene sequences announced by NCBI web, the gene coding cyanide hydratase was obtained from wild-type strain *Bacillus* sp. CN-22 by a molecular biological technique. The recombinant strain GS115-Ch-Glu bearing this gene could also grow in the maximum concentration of KCN up to 1,100 mg L^{-1} (Table 2), indicating that the cyanide hydratase expressed in *Pichia* had strong activity for the biotransformation of cyanide into atoxic product too. In the experiment for CGs detoxification of flaxseed, we found that over 80% of cyanide was converted to formamide by the enzyme preparation containing 8.9% cyanide hydratase. In comparison with HCN being evaporated by heat, this approach is energy saving and provides environmental protection, which can meet the requirement of recently advocated low-carbon economy. Although, formamide might be less toxic than HCN, no study of formamide degradation has been reported yet. In the future, we hope to clone and express related genes for production of formamide-degrading enzymes in strain the *Bacillus* sp. CN-22 that has been successfully isolated in our laboratory, so as to remove residual formamide completely.

In conclusion, a recombinant *Pichia* strain GS115-Ch-Glu expressing both human cytosolic β-glucosidase and *Bacillus* sp. CN-22 cyanide hydratase was successfully constructed for the first time. This study shows that the enzyme preparation produced by this recombinant strain could completely decompose cyanogenic glycosides in flaxseed power into cyanide, and further catalyze the hydrolysis of over 80% cyanide. In addition, the strain GS115-Ch-Glu constructed in this study could be applied to treat cyanide-bearing effluent due to the cyanide hydratase it produces.

ACKNOWLEDGEMENTS

This work was supported by the Fundamental Research Funds for the Central Universities (No. 10lgzd07) and the R & D project (No. 2010-001) from Guangzhou Enenta Electrical Equipment Co., Ltd.

REFERENCES

Afkhami A, Sarlak N, Zarei AR (2007). Simultaneous kinetic spectrophotometric determination of cyanide and thiocyanate using the partial least squares (PLS) regression. Talanta, 71: 893-899.

Basile LJ, Willson RC, Sewell BT, Benedik MJ (2008). Genome mining of cyanide-nitrilases from filamentous fungi. Appl. Microbiol. Biotechnol. 80: 427-435.

Bleve G, Rizzotti L, Dellaglio F, Torriani S (2003). Development of reverse transcription (RT)-PCR and real-time RT-PCR assays for rapid detection and quantification of viable yeasts and molds contaminating yogurts and pasteurized food products. Appl. Environ. Microbiol. 69: 4116-4122.

Bradbury JH (2006). Simple wetting method to reduce cyanogen content of cassava flour. J. Food Compos. Anal. 19:388-393.

Bradbury JH, Denton IC (2010). Simple method to reduce the cyanogen content of gari made from cassava. Food Chem. 123:840-845.

Briante R, Patumi M, Febbraio F, Nucci R (2004). Production of highly purified hydroxytyrosol from *Olea europaea* leaf extract biotransformed by hyperthermophilic β-glycosidase. J. Biotechnol. 111: 67-77.

Cardoso AP, Mirione E, Ernesto M, Massaza F, Cliff J, Haque MR, Bradbury JH (2005). Processing of cassava roots to remove cyanogens. J. Food Compos. Anal. 18: 451-460.

Cregg JM, Tolstorukov I, Kusari A, Sunga J, Madden K, Chappell T (2009). Expression in the yeast *Pichia pastoris*. Method Enzymol. 463: 169-189.

Cui YF, Yang Z, Zhu BH, Xiao LW, Tong PJ (2011). Application of trichloroacetic acid-acetone precipitation method for protein extraction in bone tissue. Acta Acad. Med. Sin. 33: 210-213.

de Graaf M, van Veen IC, van der Meulen-Muileman IH, Gerritsen WR, Pinedo HM, Haisma HJ (2001). Cloning and characterization of human liver cytosolic beta-glycosidase. Biochem. J. 356:907-910.

Dent KC, Weber BW, Benedik MJ, Sewell BT (2009). The cyanide hydratase from *Neurospora crassa* forms a helix which has a dimeric repeat. Appl. Microbiol. Biotechnol. 82: 271-278.

Feng DY, Shen YG, Chavez ER (2003). Effectiveness of different processing methods in reducing hydrogen cyanide content of

Flaxseed. .J. Sci. Food Agric. 83: 836-841.
Ganjewala D, Kumar S, Devi SA, Ambika K (2010). Advances in cyanogenic glycosides biosynthesis and analyses in plants: A review. Acta Biol. Szegediensis, 54: 1-14.
Gu NY, Kim JL, Kim HJ, You DJ, Kim HW, Jeon SJ (2009). Gene cloning and enzymatic properties of hyperthermostable β-glycosidase from *Thermus thermophilus* HJ6. J. Biosci. Bioeng. 107: 21-26.
Joseph S, David WR (2001). Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, New York, pp. 26-105.
Kang SK, Cho KK, Ahn JK, Kang SH, Lee SH, Lee HG, Choi YJ (2005). Cloning, expression, and enzyme characterization of thermostable β-glycosidase from *Thermus flavus* AT-62. Enzyme Microb. Technol. 37: 655-662.
LaMarco KL, Glew RH (1986). Hydrolysis of a naturally occurring β-glucoside by a broad-specificity beta-glucosidase from liver. Biochem. J. 237: 469-476.
Lei V, Amoa-Awua WKA, Brimer L (1999). Degradation of cyanogenic glycosides by *Lactobacillus plantarum* strains from spontaneous cassava fermentation and other microorganisms. Int. J. Food Microbiol. 53: 169-184.
Li X, Yuan JP, Xu SP, Wang JH, Liu X (2008). Separation and determination of secoisolariciresinol diglucoside oligomers and their hydrolysates in the flaxseed extract by high-performance liquid chromatography. J. Chromatogr. A 1185: 223-232.
Madhusudhan KT, Singh N (1985). Effect of detoxification treatment on the physicochemical properties of linseed proteins. J. Agric. Food Chem. 33: 1219-1222.
Nambisan B (2011). Strategies for elimination of cyanogens from cassava for reducing toxicity and improving food safety. Food Chem. Toxicol. 49: 690-693.
Nielsen KA, Tattersall DB, Jones PR, Møller BL (2008). Metabolon formation in dhurrin biosynthesis. Phytochemistry, 69: 88-98.
Park NY, Baek NI, Cha J, Lee SB, Auhe JH, Parka CS (2005). Production of a new sucrose derivative by transglycosylation of recombinant *Sulfolobus shibatae* β-glycosidase. Carbohyd. Res. 340: 1089-1096.
Perry JD, Morris KA, James AL, Oliver M, Gould FK (2007). Evaluation of novel chromogenic substrates for the detection of bacterial beta-glucosidase. J. Appl. Microbiol. 102: 410-415.
Salamin K, Sriranganadane D, Léchenne B, Jousson O, Monod M (2010). Secretion of an endogenous subtilisin by *Pichia pastoris* strains GS115 and KM71. Appl. Environ. Microbiol. 76:4269-4276.
Sornyotha S, Kyu KL, Ratanakhanokchai K (2010). An efficient treatment for detoxification process of cassava starch by plant cell wall-degrading enzymes. J. Biosci. Bioeng. 109: 9-14.
Vasconcellos SP, Cereda MP, Cagnon JR, Foglio MA, Rodrigues RA, Manfio GP, Oliveira VM (2009). In vitro degradation of linamarin by microorganisms isolated from cassava wastewater treatment lagoons. Braz. J. Microbiol. 40:879-883.
Vetter J (2000). Plant cyanogenic glycosides. Toxicon, 38: 11-36.
Watanabe A, Yano K, Ikebukuro K, Karube I (1998). Cloning and expression of a gene encoding cyanidase from *Pseudomonas stutzeri* AK61. Appl. Microbiol. Biotechnol. 50: 93-97.
Wu CF, Xu Y, Tao Y, Yang JY (2009). Establishment of hypoglycemic agent screening method based on human glucokinase. Biomed. Environ. Sci. 22: 62-69.
Wu M, Li D, Wang LJ, Zhou YG, Brooks MS, Chen XD, Mao ZH (2008). Extrusion detoxification technique on flaxseed by uniform design optimization. Sep. Purif. Technol. 61: 51-59.
Yamashita T, Sano T, Hashimoto T, Kanazawa K (2007). Development of a method to remove cyanogen glycosides from flaxseed meal. Int. J. Food Sci. Technol. 42: 70-75.
Yuan JP, Li X, Xu SP, Wang JH, Liu X (2008). Hydrolysis kinetics of secoisolariciresinol diglucoside oligomers from flaxseed. J. Agric. Food Chem. 56: 10041-10047.