African Journal of Biotechnology Vol. 9(47), pp. 8110-8117, 22 November, 2010
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.263
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Engineering thermostable xylanase enzyme mutant from *Bacillus halodurans*

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Accepted 1 June, 2010

Endo-1,4-beta-xylanase is the main enzyme in xylan-backbone hydrolysis and has received attractable research interest due to its significant application in various industrial processes such as food, feed, waste treatment, fuel and chemical production, paper and pulp industries; but these applications require thermostable xylanase enzymes. Error-prone polymerase chain reaction (PCR) and site-directed mutagenesis were used to engineer new thermostable mutants of *Bacillus halodurans*. The results showed that the rate of mutagenesis in the error-prone PCR was at least 1%, resulting in more than 1000 mutated colonies. About one third of mutants lost the enzymes' activities and no sign of thermal stability improvement was observed in other mutants; less than 2% of mutants were active at temperature higher than 75°C and they lost their activities quickly at temperature higher than 80°C. In site-directed mutagenesis at position 274, threonine (T) amino acid changed to proline (P) but the extent of thermostability improvement in newly engineered mutants were not sufficient. Various screening, clustering, decision tree and generalized rule induction models used to search for patterns of thermostability and the frequency of glutamine was the most important feature in many bioinformatics models. The importance of these methods has been discussed here.

Key words: Bioinformatics, modeling, protein, thermostability.

INTRODUCTION

Cellulose (a polymer of β-D-glucose), hemicellulose (a complex mix of polymeric carbohydrates including xylan, xyloglucan, glucomannan, galactoglucomannan and arabinogalactan) and lignin (a complex polyphenolic compound) are collectively referred to as lignocellulose, which makes up the major polymeric constituent of plant cell walls and wood. Xylanolytic enzymes are hemicellulase enzymes that catalyze hydrolysis of xylan, usually associated with cellulose and lignin component. Several enzymes are involved in the hydrolysis of xylan polymers of which the most important are the endo-1,4-β xylanase (EC 3.2.1.8) (Sa-Pereira et al., 2003). In recent years, xylanases have received attractable research interest due to their signifi-cant application in various industrial processes such as food, feed, waste treatment, fuel and chemical production, paper and pulp industries (Chantasingh et al., 2006). For environmental reasons, xylanases are desirable in that they reduce the amount of chlorine and chlorine dioxide used for bleaching paper pulp. During the bleaching of kraft paper pulp, the lignin in wood chips is removed by sequential treatments with chlorine, chlorine dioxide and NaOH. The chlorine and chlorine dioxide create persistent organic chemicals that are toxic to organisms in the waterways close to paper plants and may present health risks to humans as well. Pre-treating paper pulp with xylanases can enhance the efficiency of the chemical extraction of lignin and so reduce the amounts of chlorine and chlorine dioxide required (Sudha et al., 2003). However, such applications

Abbreviations: PCR, Polymerase chain reaction; **GPCR,** G protein-coupled receptor; **LB,** Luria-Bertani ; **IPTG,** isopropyl-β-D-thiogalactopyranoside; **FPLC,** fast protein liquid chromatography; **DNS,** 3,5-dinitrosalicylic acid; **T,** threonine; P, proline; **IMAC,** immobilized metal affinity chromatography; **GRI,** generalized rule induction; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis; RBB, Ramazol brilliant blue.

require xylanase(s) with particular properties, the bio-bleaching of paper pulp requires a xylanase with better thermostability (Ompraba et al., 2007). Two approaches have been taken: screening organisms from various sources in nature for high xylanase activity and engineering known xylanases to improve their characteristics. Possibly, both approaches are necessary to obtain ideal xylanases as its thermost-ability is tried to be increased by error-prone polymerase chain reaction (PCR) and site directed mutagenesis (Ruller et al., 2007).

Thermostable proteins maintain their activities and are stable at high temperatures. Identifying and under-standing the factors contributing to the stability of proteins from organisms living under extreme conditions has been a long standing problem. The first high resolution crystal structure of thermolysin was reported in 1974 (Matthews et al., 1974) and other authors commented on the stereochemical basis of thermostability of ferredoxins and hemoglobin A2 (McRee et al., 1990). Since these pioneering efforts, several investigators have focused on the problem of the molecular basis of protein thermost-ability. Several reasons have been attributed to the greater stability of the thermophilic proteins (Zhou et al., 2008). Among the most prominent ones are greater hydrophobicity (Miyazaki et al., 2006), better packing, deletion or shortening of loops (Liu and Wang, 2003), smaller and less numerous cavities, increased surface area buried upon oligomerization (Gribenko et al., 2009), amino acid substitutions within and outside the secondary structures (Sriprang et al., 2006), increased occurrence of proline residues (Arnorsdottir et al., 2009), decreased occurrence of thermolabile residues (Koutsioulis et al., 2008), increased helical content, increased polar surface area (Jayaraman et al., 2006), increased hydrogen bonding (Lee et al., 2004), salt bridges (Ge et al., 2008) and count of oxygen, as well as count of positively and negatively charged residues.

Several methods based on amino acid substitutions have been proposed for predicting the stability of proteins (Gromiha et al., 2002). Prediction accuracy using amino acid sequences is significantly lower than that using structural data (Parthiban et al., 2007). However, several attempts have been made to understand the role of amino acid sequences on thermophilic protein stability. In addition, the amino acid sequences of genomes have been used to study the stability of thermophilic proteins (Ralph et al., 2008). Intra-helical salt bridges reportedly are prevalent in thermophiles, and the amino acid composition on the protein surface might be an important factor in stability (Umemoto et al., 2007). Moreover, the proteomes of thermophilic proteins are enriched in hydro-phobic and charged amino acids at the expense of polar ones (Yang et al., 2005). Many studies have indicated that sequence-based prediction approaches, such as protein 3D structure prediction based on sequence alignment (Wang et al., 2009), protein folding rate prediction (Shen et al., 2009), protein structural class prediction (Chen and Shen, 2008), protein quaternary attribute prediction (Chou and Cai, 2003), identification of enzymes and their functional classes (Xiao et al., 2008), enzyme active site prediction (Chou and Cai, 2004), enzyme specificity prediction (Chou and Elrod, 1999), identification of membrane proteins and their type (Chou and Shen, 2007), protein subcellular location prediction (Chou and Shen, 2009), protein secondary structural contents prediction (Ding et al., 2009), signal peptide prediction (Chou and Shen, 2007), identification of proteases and their types (Chou and Shen, 2008), protein cleavage site prediction (Chou, 2009), and identification of G protein-coupled receptor (GPCR) and their types (Xiao et al., 2009), can timely provide very useful information and insights for both basic research and drug design and hence are widely welcome by science community.

Most protein evolution approaches proceed by the stepwise accumulation of mutations. As for any enzyme, attempts to improve or alter protein function through muta-genesis and directed evolution must balance diversity with mutational disruptiveness (Stephens et al., 2007). Today, there are three main avenues for enzyme improvement: site-directed mutagenesis, random mutagenesis and gene shuffling (Sriprang et al., 2006). Enzymes fold to a unique native structure which confers biological function, and most random mutations that destroy function do so by destabilizing this native structure. To engineer new thermostable mutants of xylanase enzyme from *Bacillus halodurans*, active at optimum temperature higher than 75°C, error-prone PCR and site-directed mutagenesis was applied on this enzyme.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions

B. halodurans sp. strain was used as a source of chromosomal DNA for xylanase gene cloning. *Escherichia* coli DH5α and *E. coli* BL21 CodonPlus®-RIL (Stratagene, La Jolla, CA) were used as the host for cloning vectors. pUC119 and pTrc99A were obtained from Amersham Biosciences (Buckinghamshire, England). The *E. coli* strains were cultivated in Luria-Bertani (LB) medium or 2x YT medium.

Cloning of the xylanase gene and DNA sequencing

The xylanase gene from *B. halodurans* was cloned by conventional DNA cloning techniques. The chromosomal DNA of this strain was digested with restriction enzymes and southern blot hybridization was carried out using a mixed oligonucleotide probe designed from the N-terminal amino acid sequence of purified xylanase. The DNA fragments containing the expected xylanase gene were inserted into a pUC119. After transformation of *E. coli* DH5α, a positive clone was selected by colony hybridization using the mixed oligonucleotide probe. The clone was subjected to restriction mapping and DNA sequencing. DNA sequencing was done by the Li-Cor Model 4000 Long Read IR DNA sequencing system (Li-Cor Biotechnology, Lincoln, NE) and the

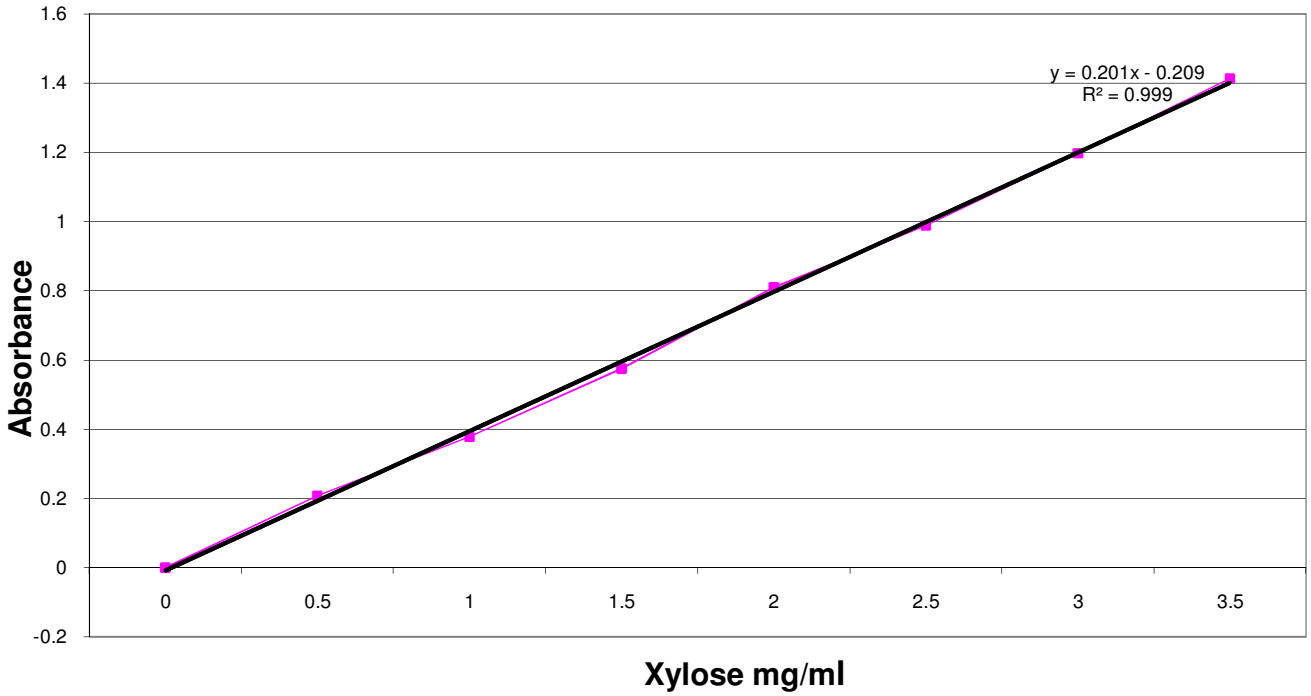

Figure 1. Standard curve for xylose showing the fitted curve for different concentration of xylose and their absorbance.

ABI Model 377 cycle sequencing system (Perkin-Elmer, Waltham, MA). The resulting sequences were analyzed using GENETX software package (Genetyx, Tokyo, Japan).

Expression and purification of recombinant xylanase

The xylanase gene was amplified by PCR using 5`-CGAATTCATGAAAGCTCAAGGAGGACCACCAA-3` and 5`-CAAGCTTAATGG TGA-TGGTGATGGTGATCAATAATTCTCCAGT AAGC-3' as the forward and reverse primers, respectively. These contain the *BspHI* and *HindIII* site (underlined). The amplified DNA fragment was digested with *BspHI* and *HindIII* and inserted into the sites of pGEM-T Easy, and the resulting plasmid was purified by Perfectrep Plasmid Mini (Eppendorf, Germany). The plasmid was cut with the enzymes again and then transformed into PTrc99a, transformed to DH5α and finally transformed to *E. coli* BL21 CodonPlus®-RIL (Stratagene). The transformants were grown at 37°C in LB plates containing amplicin (100 µg/ml) overnight, and 20 ml of culture was added to 500 ml of LB medium, left for 70 min (log phase bacteria) when the culture absorbance (at 600 OD) was near 1, 200 µl of 1mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce gene activity, left for 5 h and cells were harvested and sonicated, cell debris was removed by centrifugation and the resulting supernatant was subjected to fast protein liquid chroma-tography (FPLC) Bio-RAD (BioLogic DUOflow) with BIO-RAD-Bio Frac. The G column packed by using Pharmacia-LKB-Pump P-1 with safferos and enriched by $Ni(No_3)_2$ for 15 min was washed with water and the supernatant added to columns to separate xylanase-histidine.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and xylanase activity

SDS-PAGE was performed on a 12.5% polyacrylamide gel by the method of Laemmli (1970).

Xylanase enzyme activity by 3,5-dinitrosalicylic acid (DNS) method

Xylanase activity was determined using Ramazol brilliant blue (RBB)-xylan (Sigma Chemical Co., U.S.A.) as a substrate. 50 µl of RBB-xylan solution (10 mg/ml) was added to 100 µl of enzyme solution and 150 µl of 50 mM sodium acetate buffer (pH 4.5). After incubating for 30 min at 70°C, 200 µl of samples or xylan standards was added to a tube containing 1 ml of DNS reagent; 800 µl of deionized water was added and left in boiling water bath for 10 min, cooled down at room temperature and 1 ml of 40% potassium sodium tartarate and 1 ml of deionized water was added and the absorbance was read at 540 nm and standard curve drawn (Figure 1). One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µmole of the reducing sugar as xylose from xylan per min.

Error-prone PCR

To generate a library of xylanase variants, mutations were introduced using error-prone PCR. Besides a control PCR reaction, a range of mutagenic conditions was chosen for the first round of mutation. The control reaction contained 1.5 mM $MgCl_2$ and 0.1 mM of each nucleotide. The mutagenic conditions contained 10 mM $MgCl_2$, 1 mM of $MnCl_2$ and 1 mM of each nucleotide. They were subjected to a second round of mutagenesis, using again 10 and 1 mM of $MgCl_2$ and $MnCl_2$, respectively. Standard T3 and T7 primers (Integrated DNA Technologies, USA) were used for the PCR reactions. The amount of template DNA (10 ng) and the primers (200 ng) were kept constant in all PCR reactions in a total volume of 50 µl. Thermocycler settings (PCR Genius, Techne) were 95°C for 5 min, 94°C for 30 sec, 55°C for 30 sec and 72°C for 2 sec (30 cycles) and then kept at 72°C for 5 min. The PCR products were isolated from a 0.8% agarose gel and purified using a GFX PCR DNA and gel band purification kit (Amersham). The purified PCR products were BspH1- and HindIII-digested and subsequently ligated into DH5α vector using standard

molecular biology procedures as mentioned earlier. More than 200 white colonies grew on the plates and the transformation efficiency was checked by PCR. Finally, 6 colonies subcultured into LB and 3 of them sequenced showed at least 1% mutation rate in each sample. Then all colonies were subcultured into LB and grown overnight, plasmid was extracted, cut with BSPH1 and HindIII enzymes, cut from gel, purified and then ligated into PTrc99a and again transformed into DH5α, gene availability was checked by PCR and identical colonies on plates subcultured into 1.5 ml LB overnight. In the morning, 12.5 ml of LB was added to each tube, left on 37°C for 70 min, 1 µl of 1 M IPTG was added, left again for 5 h at 37°C, spun down (10 min at 4000 rpm, 4°C) and cells were put in freezer. The samples were suspended in 2 ml of buffer, sonicated and spun down and enzyme activity checked at 80°C for 30 min as mentioned earlier.

Site-directed mutagenesis

Stratagen kit (QuickChange II) was used to change threonine (T) to proline (P) at position 274 of mature protein and the recommended procedure by manufacturer was followed. Again, the products were transformed to DH5α and subsequently subcultured into LB, sequenced and again the activity of new xylanase mutants was checked as mentioned earlier.

RESULTS

Practicing the aforementioned procedures, an N-terminal hexa-histidine tag (HHHHHH) was added to the xylanase enzyme and it was over expressed in bacterial culture and then was isolated by immobilized metal affinity chroma-tography (IMAC) system through affinity partnership that exists between histidine residues in the enzyme and Ni(II) ions complexes (FPLC output is shown in Figure 2).

The rate of mutagenesis in the error-prone PCR was at least 1%, resulting in more than 1000 mutated colonies. Following the procedures mentioned, xylanase genes of 200 colonies were cut, transformed, expressed and extracted by FPLC. The activities of xylanase enzymes at different temperatures were determined. About one third of mutants lost the enzymes' activities and no sign of thermal stability improvement was observed in other mutants; less than 2% of mutants were active at temperature higher than 75°C and they lost their activities quickly at temperature higher than 80°C (Figure 3).

The results of sequencing showed that site directed mutagenesis was unable to improve thermostability in newly engineered mutants and expressed xylanase enzymes; this means having a proline at position 274 does not contribute to thermostability of *B. halodurans*. The above finding confirmed that it is vital to determine the features that contribute to the thermostability of xylanase proteins and so various screening techniques (anomaly detection and feature selection), clustering methods (K-Means, TwoStep cluster), decision tree models (Classification and Regression Tree, CHAID, Exhaustive CHAID, QUEST, C5.0) and generalized rule induction (association) (GRI) models were applied to search for patterns of thermostability and to find features that contribute to enzyme thermal stability. The results showed that arginine as the N-terminal amino acid was found solely in proteins working at temperatures higher than 70°C. Fifty-four protein features were shown to be important in feature selection modeling, and the number of peer groups with an anomaly index of 2.12 declined from 7 to 2 after being run using only important selected features; however, no changes were found in the numbers of groups when K-means and twostep clustering modeling was performed on datasets with/without feature selection filtering. The depth of the trees generated by various decision tree models varied from 14 (in the C5.0 model with 10-fold cross-validation and with feature selection of the dataset) to 4 (in CHAID models) branches. The performance evaluation of the decision tree models tested here showed that C5.0 was the best and the quest model was the worst. There were no significant difference in the percent of correctness, performance evaluation and mean correctness of various decision tree models when feature selected datasets were used, but the number of peer groups in clustering models was reduced significantly ($p < 0.05$) compared to datasets without feature selection. In all decision tree models, the frequency of glutamine was the most important feature for decision tree rule sets; moreover, in all GRI association rules (100 rules), the frequency of glutamine was used in antecedent to support the rules.

DISCUSSION

Xylan consists of 1,4-glycosidically linked-D-xylose with branches containing xylose and other pentoses, hexoses and uronic acids. Xylan can be degraded by either acid or enzymic catalysis. The enzymic process has the advan-tages of a highly efficient conversion rate, and the mild conditions required are non-corrosive and non-environ-mentally hazardous. Although, the complete breakdown of xylan requires the action of several different enzymes, the epolymerizing endo-1,4-,f xylanase (EC 3.2.1.8) is the key enzyme. Consequently, xylanases have possible applications in waste treatment, fuel and chemical production and paper manufacture. One of the main issues in industry usage of this enzyme is its purification and finding an easy and applicable way to extract it from other cell content. IMAC is a separation technology used in aqueous solvents systems. This coupled with the fact that IMAC systems are very robust, can be recycled many times and the eluents are water based that can lead to a cheaper, efficient and more environmentally benign separation technology. In this technique, a metal ion is attached by a chelating compound to a solid support. A short tag with affinity to the metal ion is attached to the target protein to allow binding of the protein to the immobilised metal and thus to achieve purification. In this study, a hexa-histidine tag

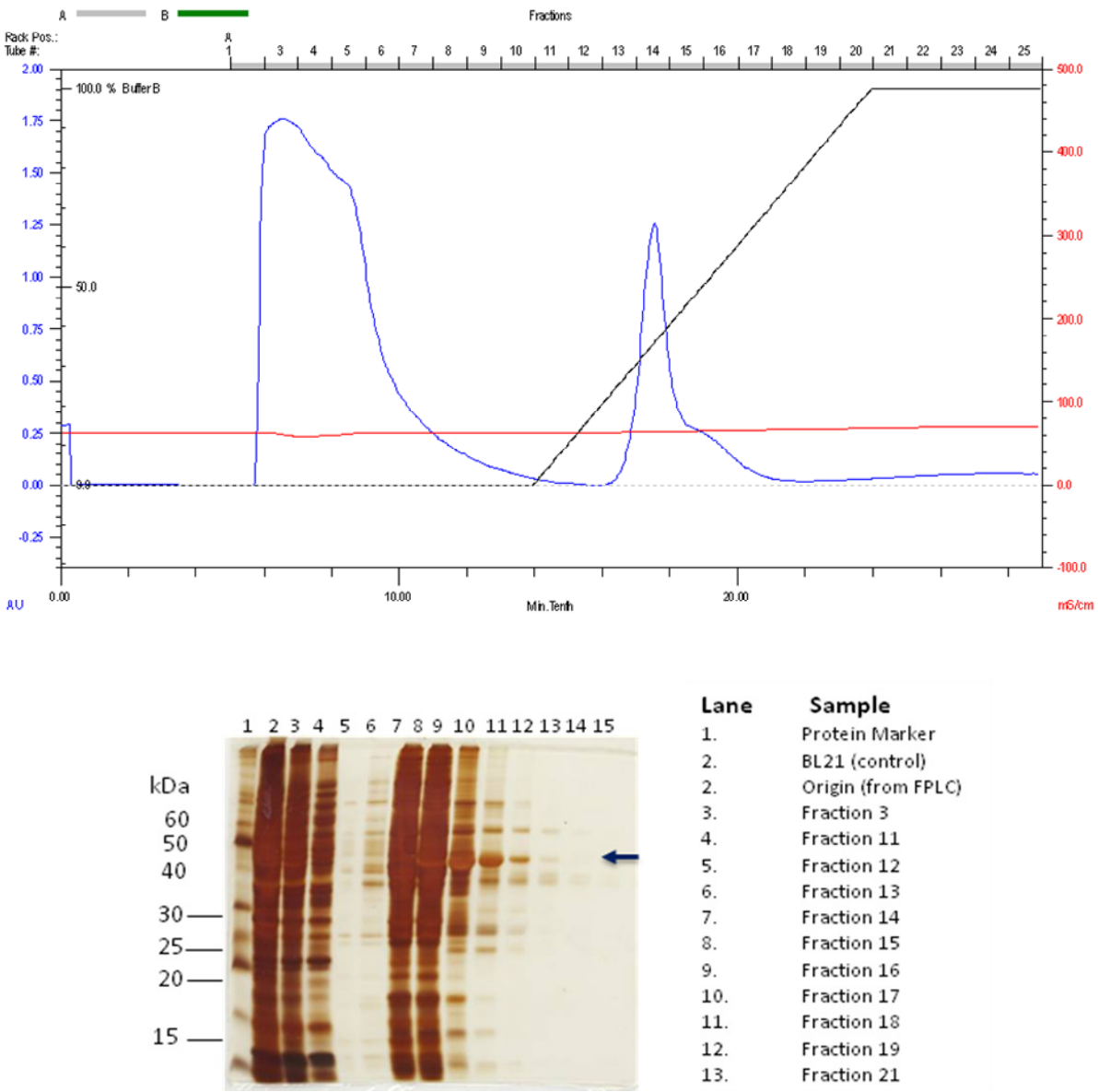

Figure 2. Output of FPLC separating his6 tags of xylanase enzymes and a gel run of different outputs.

has been added to xylanase enzyme and it has been over expressed in bacterial culture and was then isolated by IMAC system through affinity partnership that exists between histidine residues in the enzyme and Ni(II) ions complexes. The results showed that this method can be applied in large mass enzyme production and purification and paves a clear prospectus in industrial enzyme application.

Thermostable enzymes are of wide industrial and biotechnical interest because they are more stable and thus generally better suited for harsh processing conditions (Wakarchuk et al., 1994). The concept of thermostability is, however, not very clear, and thermostability is a relative term. Enzymatic activity is known to increase with increasing temperature up to the temperature at which inactivation starts to occur (Paloheimo et al., 2007). Thermostability is usually defined as the retention of activity after heating at a chosen temperature for a prolonged period. The most appropriate way to express thermostability is to measure the half-life of the enzyme activity at elevated temperatures (Yang et al., 2007). Thermostable enzymes are produced both by thermophilic and mesophilic organisms. Although thermophilic microorganisms are a potential source for thermostable enzymes, the majority of industrial thermostable enzymes originate from mesophilic organisms (Yang et al., 2005). The successful discrimination of thermophilic proteins from mesophilic ones is an important problem, and it would help greatly in designing stable proteins. Several investigations have been conducted in an effort

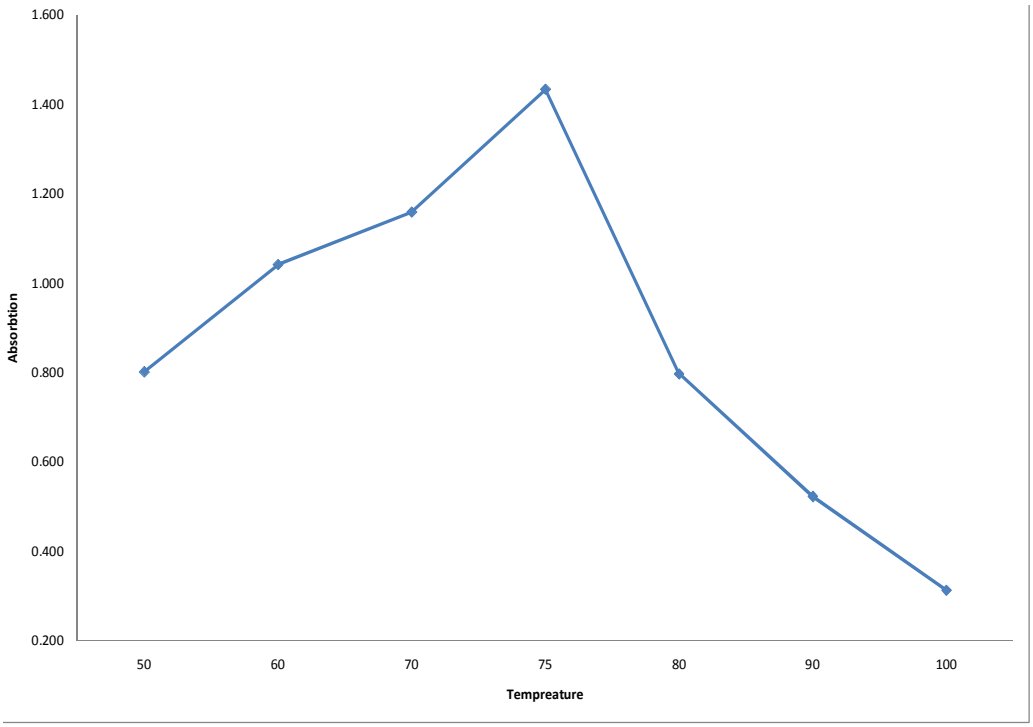

Figure 3. Xylanase activity of mutants of *B. halodurans* created by error-prone PCR.

effort to understand the features that influence the stability of thermophilic proteins (Jiang et al., 2006). An increase in the Gibbs free energy change of hydration (Gromiha et al., 1999) and increases in the number of salt bridges and side chain-side chain interactions (Kumar et al., 2000), aromatic clusters (Saelensminde et al., 2009), contacts between the residues of hydrogen bonds (Saraboji et al., 2005), ion pairs (Maugini et al., 2009), electrostatic interaction of charged residues (Yao et al., 2002), amino acid coupling patterns, main-chain hydrophobic free energy and hydrophobic residues in thermophilic proteins have been show to enhance protein stability (Saraboji et al., 2005). The amino acid sequences of genomes have also been helpful in understanding the stability of thermo-philic proteins (Liang et al., 2005). The amino acid com-position on the protein surface might be an important factor that affects stability, as a specific trend was seen in the amino acid compositions in response to the require-ment of stability at elevated environmental temperature (Dominy et al., 2002). The proteomes of thermophilic proteins are enriched in hydrophobic and charged amino acids at the expense of polar ones (Brouns et al., 2005).

To date, various methods have been employed to engineer new thermostable mutants and here, two well known methods have been practiced. The results of this study confirmed the previous ones (Ruller et al., 2008) that the chance of getting any new favorite mutant by error-prone PCR is very low (less than 2% in this study) due to the nature of this method. There is no controlling tool to manage the rate and the extent of mutagenesis in this method (Stephens et al., 2007) and a lot of time and money should be set aside to examine mutants; as in this study, more than 200 colonies were checked and the output was not satisfactory. It should be also pointed out that some mutants lost their activity even at the optimum temperature showing that changes have been made at enzyme active site adding to the expenses of checking these mutants.

As mentioned earlier (see introduction), site directed mutagenesis has been used to alter nucleotides at a very specific location of gene and hence provide a better tool to specifically and on purpose change the gene function. According to previously published works (Viikari et al., 2007), it was decided to change one amino acid (T) to another one (P) at position 274 of *B. halodurans*

and it was hoped that this change may contribute to this enzyme thermal stability as has already been reported for other xylanases. The findings showed that this change may not be the same as previous studies as no significant changes in thermal stability were traced here. This finding opened up new windows to determine the most important features that contribute to protein thermostability; here different modelling techniques to study more than 70 features of some meso- and thermo-stable enzymes was applied in an attempt to understand their ability to with-stand higher temperatures. Different screening, clustering and decision tree modelling on two datasets: one with and one without feature selection filtering were used. The findings of this part will be prepared and presented as another paper but findings were very promising and give us a suitable tool to determine the features that contribute to thermostability and to induce new features in thermo-sensitive enzymes.

Although the results of feature selection modelling showed that 47 features had a value equal to 1, the frequency of glutamine ranked as the most important feature, and it was used in decision tree models to create the main subgroups and branches. The number of peer groups with anomalies decreased from seven (without feature selection) to two (with feature selection) groups, showing the positive effects of feature selection filtering on removing outliers. The number of clusters generated by K-means modelling did not change between the models with and without feature selection, although the number of records in the clusters changed. In the twostep model, the number of clusters decreased from six (without feature selection) to just two (with feature selection) groups.

The depth of trees generated by the various decision tree models varied from 14 (in the C5.0 model with 10-fold cross-validation and with the feature selection dataset) to 4 (in the CHAID models) branches. The best performance evaluation in the decision tree models tested was found in the C5.0 model and the worst was found in the quest model. No significant differences in the percent of correctness, performance evaluation and mean correct-ness of various decision tree models were found when feature selected datasets were used, but when feature selection datasets used the number of peer-groups in clustering, models reduced significantly. The results showed that amino acid composition can be used to discriminate between protein groups and most of the bioinformatics' algorithms can be used to discriminate between meso-philic and thermophilic proteins with accuracy in the range of 88 – 96%.

ACKNOWLEDGEMENT

The author greatly appreciates and acknowledges the support of Bioinformatics Research Groups, Green Research Center, Qom University.

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