Azura Amid *Editor*

Recombinant Enzymes - From Basic Science to Commercialization

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Chapter 11
Case Study: Recombinant Bromelain Cloning, Characterization and Upstream Processes

Azura Amid, Nurul Azira Ismail and Mohd Jamil Aizat Jamaluddin

Abstract This chapter presents the recombinant stem bromelain cloning procedure followed by its characterization and finally upstream processing. Most of the procedures had patents filed in Malaysia, Europe and the United State of America with application numbers PI 20095434, 10015711.4, and 12968766, respectively. Portions of the experimental procedures were also presented in our publication (Amid et al., Process Biochem 46:2232–2239, 2011). The entire cloning process, characterization and the upstream processing of the recombinant bromelain production are briefly explained.

11.1 Cloning of Recombinant Bromelain [1]

Detail procedures have been discussed by Amid and co-workers [1]. In most cases, experiments aimed at identifying suitable genetic material for use in the cloning procedure. In our case, active recombinant bromelain must be able to be expressed in simple organisms such as *E. coli* because *E. coli* requires simple fermentation media and can be harvested after a short period. Therefore, suitable genetic material should be full length mRNA encoding stem bromelain because mRNA contains only coded nucleic acids versus genomic DNA which contains coded and non-translated regions. In fact, each gene requires unique cloning procedures dependent upon the amount of available target gene information and the final aim of the cloning procedures. For stem bromelain, the detailed mRNA sequence was obtained from the National Centre for Biotechnology (www.ncbi.nlm.nih.gov) and our cloning aim to express stem bromelain protein in a prokaryote host. Therefore, the suitable cloning

A. Amid (✉) · M. J. A. Jamaluddin
Biomolecular and Bioprocess Engineering Research Unit, Department of Biotechnology Engineering, Faculty of Engineering, International Islamic University Malaysia, P.O. Box 10, 50728 Kuala Lumpur, Malaysia
e-mail: azuraamid@iium.edu.my

M. J. A. Jamaluddin
e-mail: jameeljamaluddin@hotmail.com

N. A. Ismail
Campus Puncak Alam, University Technology Mara, Shah Alam, Selangor, Malaysia

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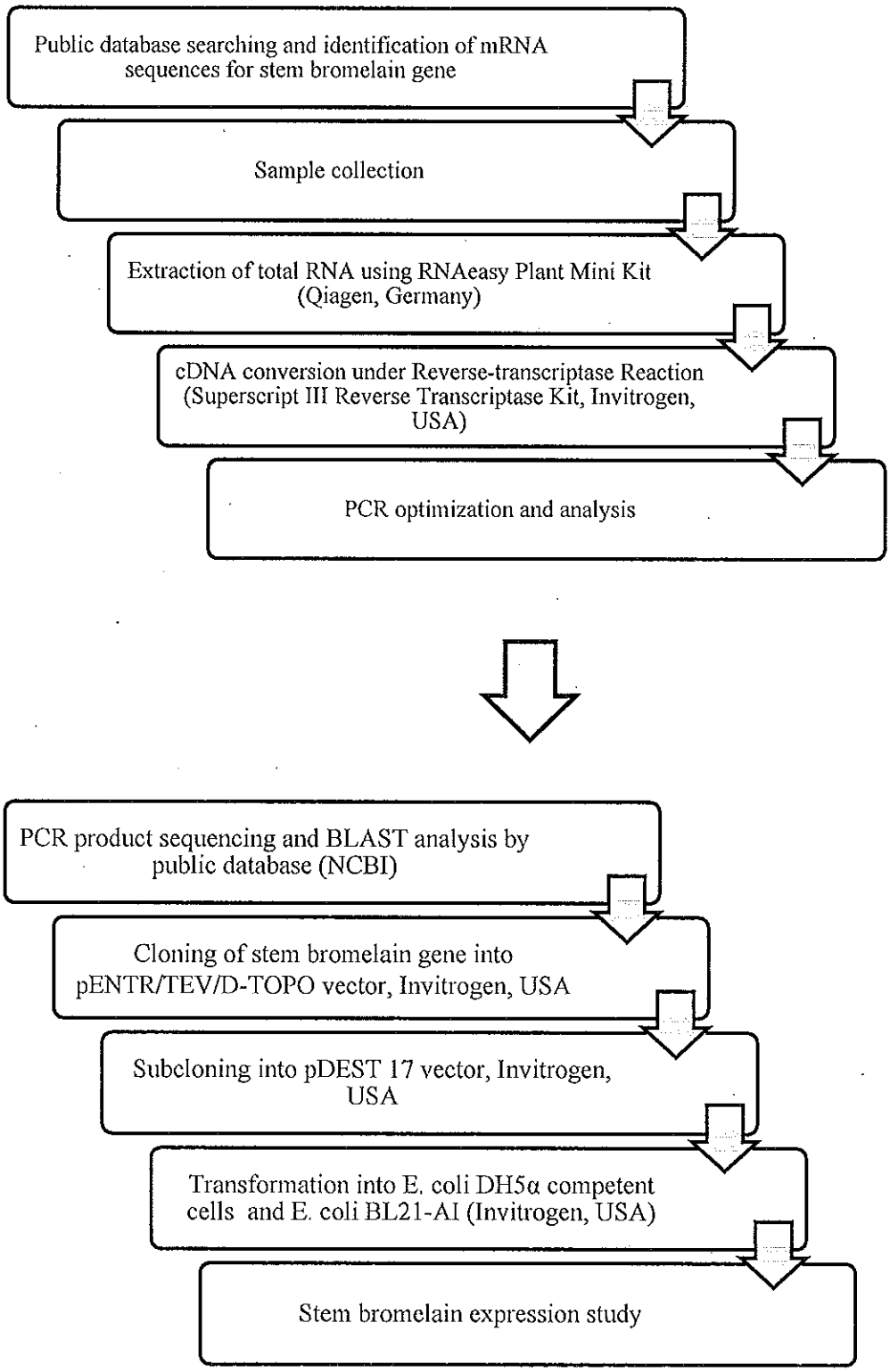

Fig. 11.1 Flow chart summarizing the entire cloning procedure

methods were PCR for target gene amplification, direct ligation into the cloning vector, transformation into a suitable host (*E. coli*) and finally, protein expression. Figure 11.1 summarizes the cloning procedures.

Next, we needed to identify source material for total RNA isolation. Because stem bromelain was the target protein, pineapple stem was the most suitable plant material RNA source. In this study, pineapple stems were collected from the Malaysia Agricultural Research and Development Institute (MARDI), Jalan Kebun, Klang, Selangor, Malaysia.

After source material collection, total RNA must be extracted. There are multiple total RNA extraction techniques and many commercial extraction kits are available. RNA extraction kit selection is normally based on individual experience and other factors such as simplicity, availability and reproducibility. In this experiment, total RNA extraction was performed using the RNeasy Plant Mini Kit (QIAgen, Germany). However, all equipment must be RNAse free treated to ensure a sufficient RNA amount can be isolated.

11.1.1 Complementary DNA (cDNA) Synthesis

When using mRNA as a genetic source material, the cDNA synthesis step is required because mRNA does not have the stability for genetic manipulation. In this section, mRNA (total RNA containing mRNA, tRNA and rRNA) is used to synthesize cDNA using the SuperScript II Reverse Transcriptase. Again, the choice of kit is dependent on a scientist's experience and preference.

11.1.2 Bromelain Gene Amplification

There are multiple procedures for obtaining the correct DNA fragment for cloning purposes such as restriction enzyme digestion, mechanical restriction and polymerase chain reaction (PCR). However, each method has its advantages and disadvantages. Because the complete stem bromelain mRNA sequence was available in public databases, gene amplification by PCR techniques was simplified. Initially, proper primer pair design needs to be performed. Approximately 30 years ago, scientists designed primer pairs manually to ensure the stringency of primer binding because primer binding can affect the number of PCR products during amplification. General considerations are primer length, melting temperature, a unique template DNA sequence and avoidance of repetitive and single-base sequences [2]. Currently, there are many software programs that are useful for designing primers such as Prime3 version 4 (http://frodo.wi.mit.edu/), Autoprime (http://www.autoprime.de/AutoPrimeWeb), and Primer Premier (http://www.premierbiosoft.com/primerdesign/index.html). After primers design, primer synthesis is performed. Many scientific supply companies provide this service. Normally, primers can be received within three working days along with the necessary details. After the primers arrive, primer optimization can be performed. Our laboratory has a standard reagent mixture for PCR components and only annealing temperature optimization was performed. The following protocol is used in our laboratory to determine the optimal annealing temperature. The aim of these experiments is to obtain a single PCR product band. The PCR product

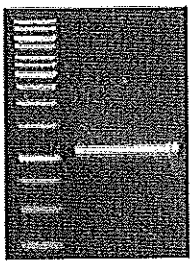

Fig. 11.2 A single band representing a desirable PCR product after annealing temperature optimization. *M* denotes the molecular weight marker

was analyzed using gel electrophoresis, Ethidium Bromide (EtBr) staining and was documented using a gel documentation system (Fig. 11.2).

11.1.3 Verifying the Correct DNA Fragment for the Target Gene

The next step after DNA fragment amplification is verifying that the amplified sequence is correct. Usually, Basic Alignment Search Tool (BLAST) is used for this purpose. This resource is provided by the NCBI public database. However, before proceeding to BLAST analyses, amplified DNA fragments are first purified followed by sequencing. Many scientific companies can provide sequencing services. Researchers provide the company with the primer details used during the PCR process. When the sequencing results arrive, BLAST analyses can be performed. BLAST results determine if the amplified DNA sequences are suitable for ligation into the desired cloning vector. The sequencing results were aligned with the complete stem bromelain mRNA sequence using the "nucleotide BLAST" program (http://blast.ncbi.nlm.nih.gov/).

11.1.4 Bromelain Gene Ligation into the Entry Vector

A fragment of DNA material cannot propagate itself to produce enough genetic material for subsequent generations. The DNA fragment requires a host for propagation. However, the DNA fragment cannot be inserted into a suitable host without the assistance of a vector. A vector is usually double stranded DNA equipped with an origin of replication (ORI), the initial site for DNA replication, together with other necessary tools such as unique restriction sites for ligation, selectable markers for the selection process and must be small enough for genetic manipulation. Vectors intended for different applications i.e., gene structural studies or protein expression. Some vectors have a broad range of hosts, referred to as shuttle vectors. This study used pENT/TEV/D-TOPO vector. This vector is equipped with a kanamycin resistance gene as a selectable marker, the pUC ori gene as the origin of replication as well as other specific features (from pENTR™ Directional TOPO® Cloning Kit User Guide 2012 http://tools.invitrogen.com/content/sfs/manuals/pentr_dtopo_man.pdf) (Fig. 11.3).

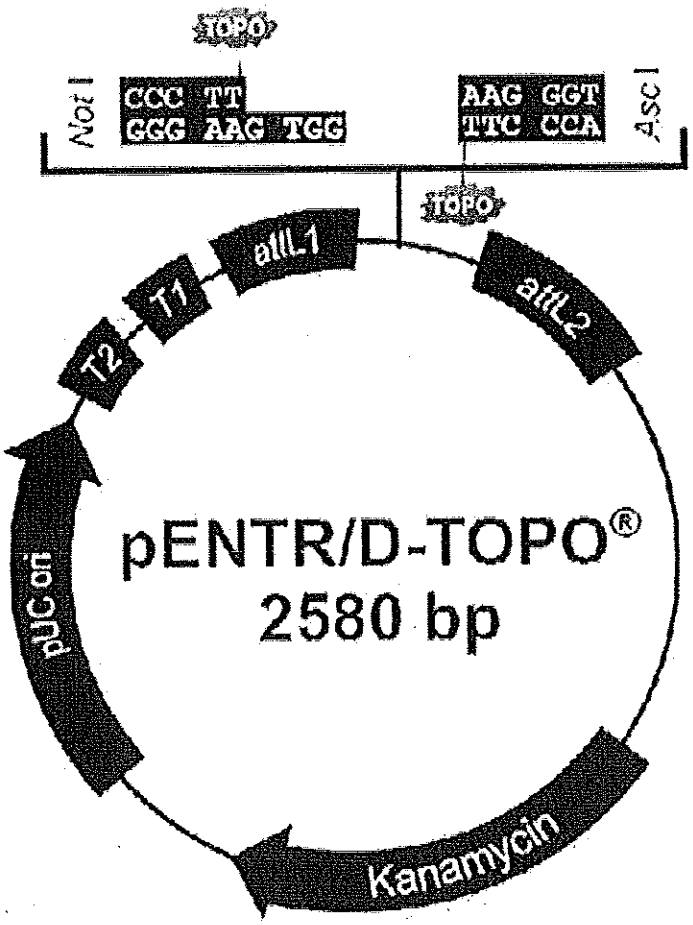

Fig. 11.3 Vector map for vector used to clone bromelain gene

11.1.5 Introduction into a Host Cell for Amplification

11.1.5.1 Transformation into *E. coli* Cells

After the candidate gene is cloned into a suitable vector, the new recombinant vector is transferred into a suitable host, in this case, *E. coli*. The simplest procedure is the heat shock method. During this procedure, the surface of the *E. coli* host, which has undergone competent cell preparation, is surrounded by the recombinant vector at ice cold temperatures. The lipid bilayers that structure the cell membrane are compressed at 4 °C. When the cell is rapidly transferred to a high temperature (42 °C), the lipid bilayers expand and form pores in the cellular membrane. This mechanism allows the recombinant plasmid to enter the cell. When the cell is again cooled to 4 °C the pores closed. The cell is now referred to as recombinant *E. coli* and harbors a recombinant vector. Once inside the host cells, the recombinant vector replicates and transfers to host daughter cells during cellular propagation.

11.1.6 Clone Selection

11.1.6.1 Colony PCR to Identify Positive Transformants

During transformation not all competent cells receive the recombinant vector. Colony PCR is a rapid method to identify positive transformants without having to extract the recombinant plasmid. The PCR procedure is similar to the previous reaction except the DNA template is a 1 μL sample from colonies grown on LB agar plates supplemented with 50 μg/mL kanamycin from the previous experiment. During DNA sampling, half of the colony is subcultured onto new LB containing kanamycin plates and the other half is mixed with 10 μl sterile distilled water. The results were obtained by analyzing the PCR product using agarose gel electrophoresis. Single bands similar in size to the bromelain gene need to be recovered to confirm the positive transformant.

11.1.6.2 Verification of Correct Insert Orientation in the Recombinant Plasmid by Restriction Enzyme Digestion

Identifying the correct orientation is very important to ensure the gene was transcribed and translated correctly. Without correct transcription and translation, no active protein will be expressed. Therefore, ensuring correct insert orientation is a useful method for avoiding repeated cloning procedures.

11.1.7 Subclone into Destination Vector by LR Recombinase Reaction

The recombinant plasmid must be subcloned into a destination vector after confirmation of the insert orientation. Destination vector selection is based on host type and final product. In this case, the pDEST 17 was chosen as the destination vector.

11.1.8 Pilot Expression Study

It is crucial to determine if the entire cloning process was successful. In this step, samples are divided into two groups: one group is induced with L-arabinose; one group is not (control). Supernatant is extracted from both samples and used in an enzyme activity assay. Figure 11.4 shows the results from the expression study using L-arabinose as an inducer.

Next, recombinant bromelain expressed by *E. coli* must be purified to be suitable for characterization experiments. Cell pellets were collected from an induced

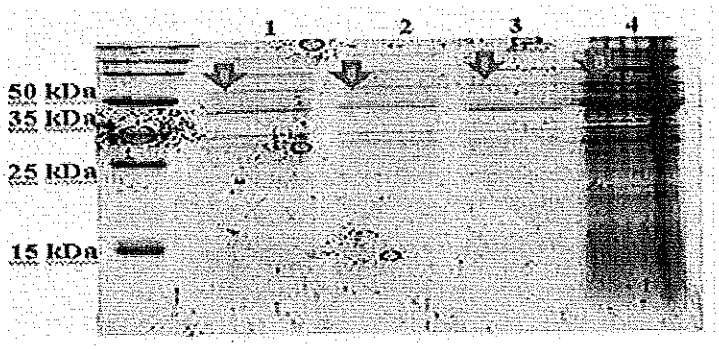

Fig. 11.4 SDS page analysis to determine protein expression after L-arabinose induction. Lanes *1* to *4* show the protein collected at hourly time points after L-arabinose induction. *Arrows* indicate the presence of recombinant bromelain in the cell lysate

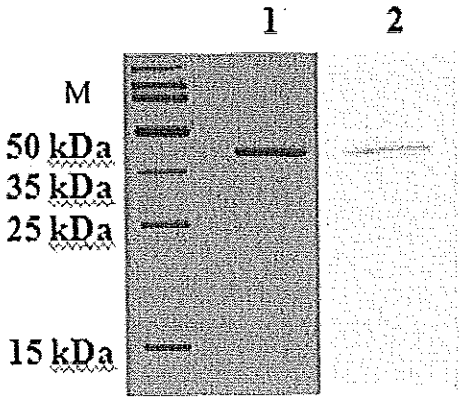

Fig. 11.5 SDS-PAGE and western blot results after protein purification under native conditions. *M* is the protein marker (10–150 kDa) followed by the protein sample by SDS-PAGE (*1*) and western blot (*2*)

culture and are then lysed to isolate the intracellular protein. The cells were lysed, washed and eluted using sodium dihydrogen phosphate (NaH_2PO_4), sodium chloride (NaCl) and imazadole at pH 8.0. Purification under native conditions indicates the target protein is soluble in the cytoplasm. Conversely, when the purified protein (native) does not appear as a band in the SDS-PAGE, the recombinant enzyme is within inclusion bodies, which is undesirable because the protein it is not in active form. The appearance of purified recombinant bromelain was confirmed using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis. Figure 11.5 shows the SDS-PAGE analysis results, clearly identifying recombinant bromelain as a single band after purification using a Ni-NTA column.

11.2 Recombinant Bromelain Characterization

11.2.1 Recombinant Bromelain Concentration

To determine the recombinant bromelain concentration in the purified samples, an enzyme-linked immunosorbent assay (ELISA) was performed. The ELISA method was used to accurately quantify the purified recombinant bromelain in the samples without the addition of other protein mixtures. Initially, a commercial bromelain

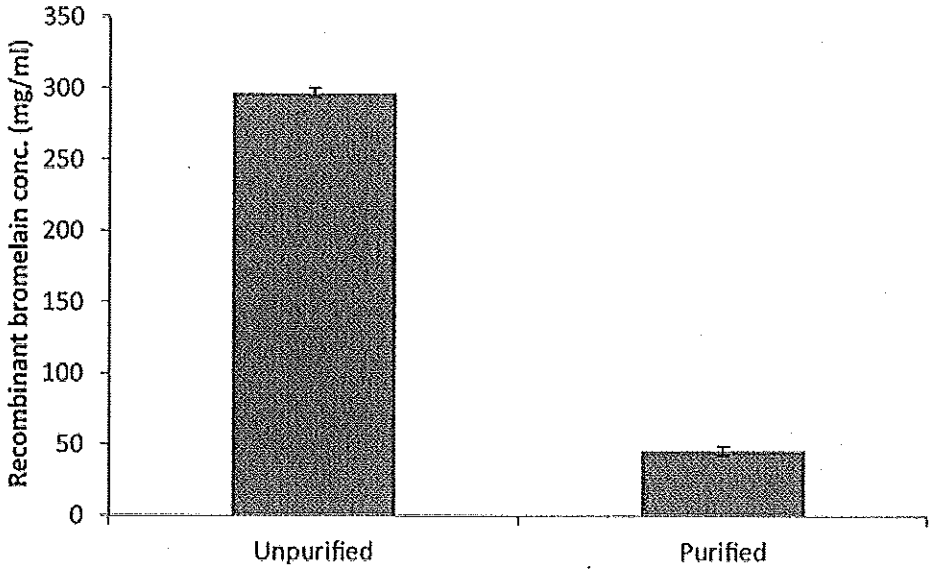

Fig. 11.6 Recombinant bromelain concentrations in unpurified and purified samples. Standard error is based on duplicate values

standard curve was constructed using ELISA. Figure 11.6 shows the recombinant bromelain concentration for two types of samples; unpurified and purified. Comparing both conditions, before and after purification, recombinant bromelain concentration in the unpurified sample is higher than the purified sample. After the purification process, the concentration was reduced because the majority *E. coli* proteins are removed during the purification process. Consequently, purification reduces the amount of recombinant protein in the sample [3]. Therefore, specific recombinant bromelain quantification can be accurately obtained using ELISA.

11.2.2 Recombinant Bromelain Activity

Recombinant bromelain activity was determined using titrametric and spectrophotometric methods. The optimal recombinant bromelain activity temperature and pH were measured using a continuous spectrophotometric procedure at 340 nm. In the titrimetric assay of recombinant bromelain activity, gelatin was used as a substrate and the proteolytic activity of recombinant bromelain was measured using the Gelatin Digestion Unit (GDU). GDU is used to determine the rate at which bromelain catalyzes the degradation of the protein gelatin (substrate) at specific temperatures and pH. Gelatin is a purified structural protein derived from animal tissues that is high in collagen such as tendons and cartilage. The rate at which gelatin (substrate) is degraded or the rate that the products of this digestion (amino acids) are produced can be related to bromelain activity. One unit of bromelain will hydrolyze 1.0 mg of amino nitrogen (Alanine, Lysine, Glycine, Tyrosine) from gelatin in 20 min at pH 4.5 and 45 °C. Figure 11.7 shows the enzyme activity of recombinant bromelain compared with commercial bromelain.

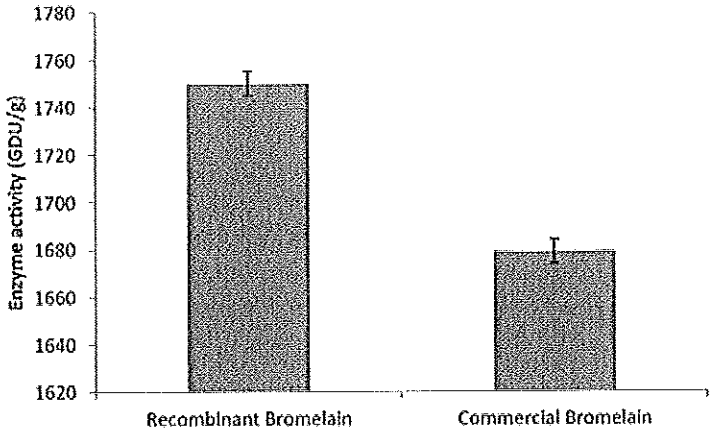

Fig. 11.7 Gelatin digestion unit of recombinant and commercial bromelain. Standard error is based on duplicate values

Table 11.1 Comparison of activity between recombinant and commercial bromelain

Samples	Recombinant bromelain	Commercial bromelain
Enzyme activity (U/mL)	48	33
Amount of total protein (mg/mL)	39	39
Specific activity (U/mg)	1.231	0.846

The digestion activity of recombinant bromelain against gelatin was 1750 GDU/g at pH 4.5 and 45 °C, whereas commercial bromelain produced 1680 GDU/g. The activity of recombinant bromelain was higher and closely related to the commercial bromelain (Fig. 11.7). This may be due to the higher purity of recombinant bromelain compared with commercial bromelain.

11.2.3 Recombinant Bromelain Specific Activity

The specific activity of bromelain was calculated based on the activity of the enzyme divided by amount of protein in the samples. Specific activity is normally expressed as units/mg and is an important measurement of enzyme purity and quality. Specific activity is a constant value across different batches of pure enzyme production. The specific activity of recombinant bromelain increases as the protein is purified (Table 11.1). The higher activity displayed by purified recombinant bromelain indicates an efficient purification strategy with 41-fold purification. The higher purity of the enzyme and higher specific activity are amenable to pharmaceutical and therapeutic applications.

11.2.4 Effect of Temperature on Recombinant Bromelain Activity

Bromelain activity at different temperatures ranging from 15 to 65 °C is shown in Fig. 11.8. The activity of recombinant bromelain is increases from 15 °C until it reaches maximal activity at 45 °C where the activity is 3.794 U/mg. Bromelain

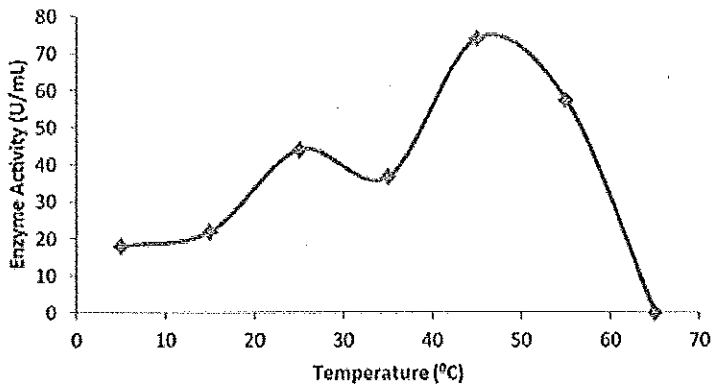

Fig. 11.8 Enzyme activity of purified recombinant bromelain at different temperatures

activity decreases at 55 °C and continues decreasing until the activity is abolished to 0 U/mg at 65 °C. Using the LNPE substrate, the purified recombinant bromelain demonstrated the highest hydrolytic activity at 45 °C under routine assay conditions. The activity of recombinant bromelain was moderate between 15 to 35 °C and the enzyme was devoid of detectable activity at 65 °C.

Based on the optimum temperature for recombinant bromelain activity, recombinant bromelain can be used for most industrial applications, particularly the baking and dairy industries [4]. Because the recombinant bromelain retains its activity at higher temperatures, it also may be used as a meat tenderizer in the food processing industry. Additionally, recombinant bromelain could be used as a biological detergent together with papain [5] and lipase in industrial purposes [6].

11.2.5 Effect of pH on Recombinant Bromelain Activity

Figure 11.9 shows recombinant bromelain activity versus pH ranging from pH 1 to 10. The purified recombinant bromelain demonstrated optimal activity at pH 4.6 for LNPE hydrolysis. The recombinant enzyme retains partial activity below pH 4.6 but fails to demonstrate significant activity beyond pH 6. Pillai and co-workers and Voegeli and co-workers demonstrated that pH 4.6 was the optimal condition for bromelain activity [7, 8] The maximum bromelain activity occurred at pH 4.6 following the theory mentioned in the bromelain assay description [9]. The maximal activity was achieved at 2.254 U/mg followed by the lowest point at pH 5, 0.135 U/mg. After reaching maximal activity, recombinant bromelain activity decreased to 0 U/mg activity. Recombinant bromelain activity ceases at pH 6–10. These results indicate that recombinant bromelain is active in acidic conditions rather than alkaline conditions. Recombinant bromelain is also inactive at neutral pH. This coincides with the majority of research results demonstrating that most proteases are active in acidic conditions excepting alkaline proteases such as keratinase [10]. The higher activity achieved in acidic conditions may be beneficial for

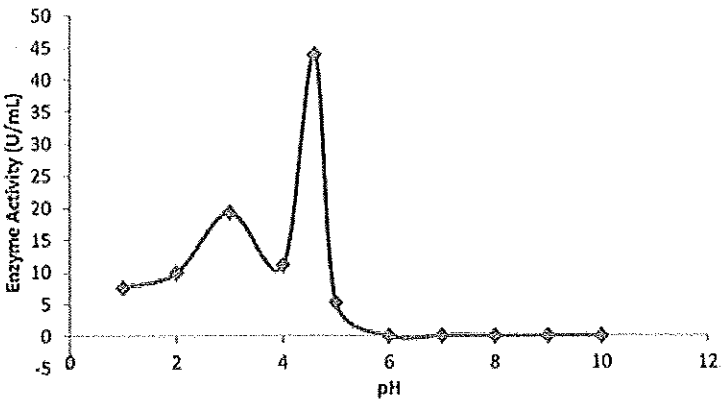

Fig. 11.9 Enzyme activity of purified recombinant bromelain at different pH levels

the use of recombinant bromelain in certain therapeutic applications. Recombinant bromelain could be used as a digestive enzyme in the acidic conditions of the stomach. Recombinant bromelain would also be active throughout the gastrointestinal tract [5, 11]. Moreover, recombinant bromelain could be used in cosmetic applications because would remain active in acidic environments, which is appropriate for the majority of the human body and skin [7, 8].

11.3 Recombinant Bromelain Upstream Processes

To ensure high recombinant bromelain expression and productivity, the most suitable media had to be identified. During cloning and bench scale experiments, Luria Bertani agar and broth were utilized; however, the amount of recombinant bromelain produced was not sufficient for the commercial scale. Therefore, the upstream processing team investigated the most suitable media formulation for recombinant bromelain production. The team then optimized the fermentation process using the selected media. The entire experimental design is described below in Fig. 11.10.

11.3.1 Identifying a Suitable Media Formulation

Nine media formulations were tested to observe their effect on the growth of recombinant *E. coli* harboring the bromelain gene and recombinant bromelain expression. The chosen media were 2X YT, 5XLB, Studier Autoinduction, TY, LB (Miller), GE, Teriffic Broth (TB), Super Broth (SB) and M9 Minimal Media. Specific activity and cell dry weight were measured for analysis.

Figure 11.11 shows that the media formulation named Studier Autoinduction performed the best formulation by producing highest recombinant *E. coli* biomass and specific recombinant bromelain activity. Because Studier Autoinduction media

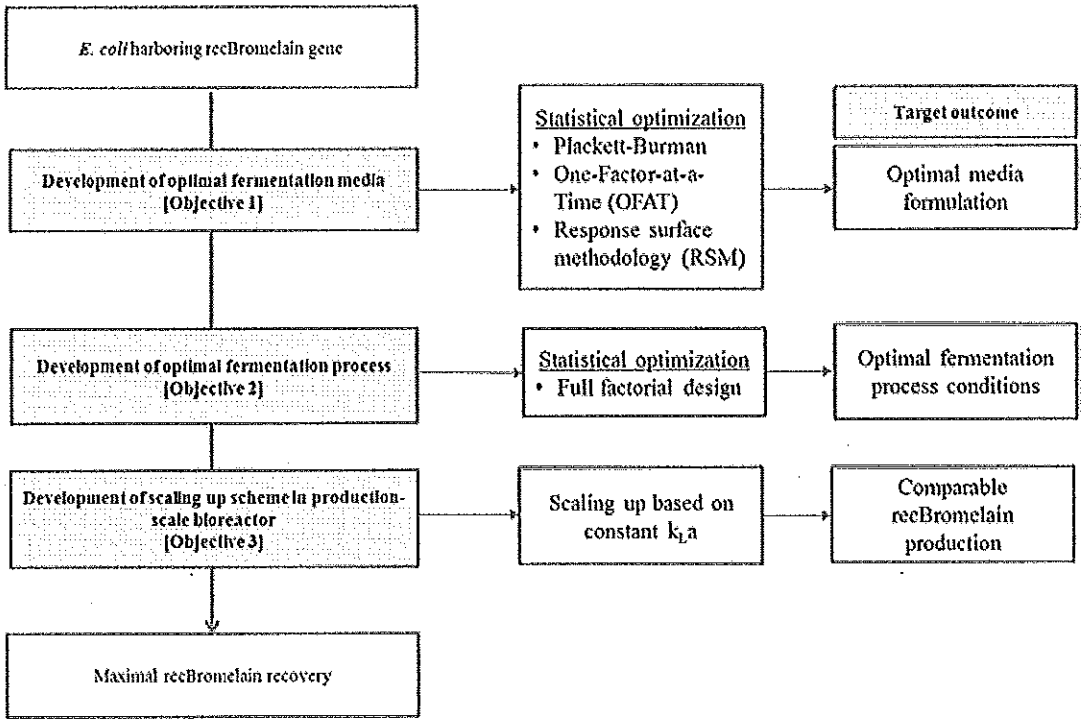

Fig. 11.10 Process flow diagram for the experimental design to determine the optimal media formulation and fermentation conditions for large scale recombinant bromelain production

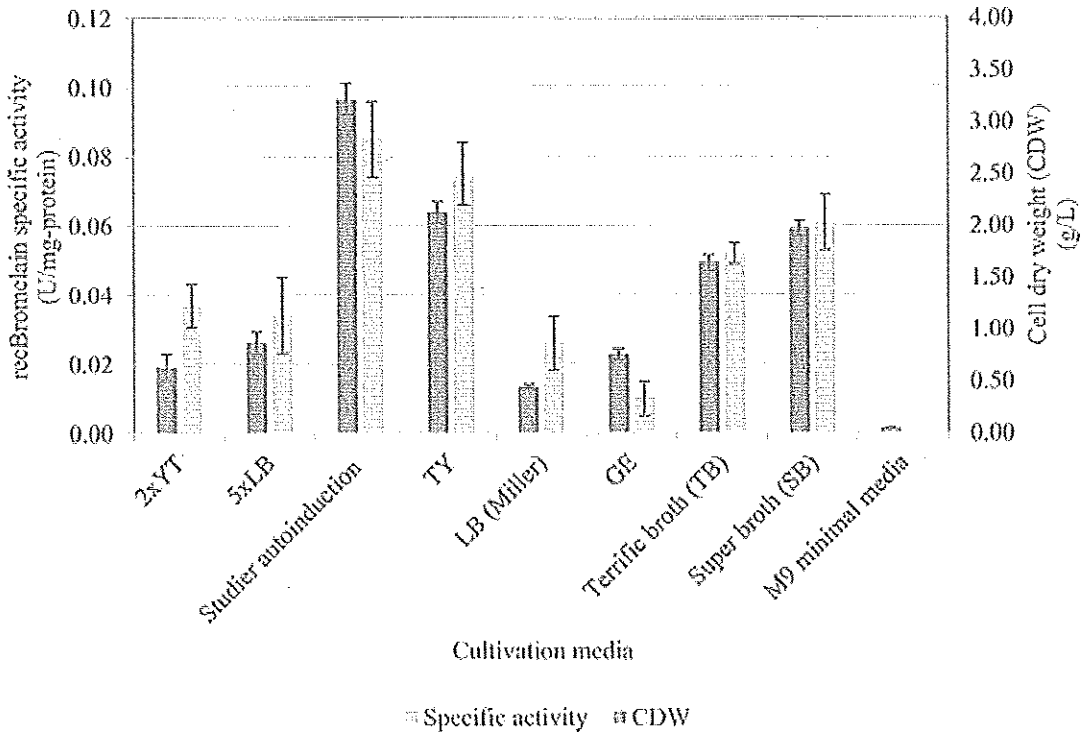

Fig. 11.11 Preliminary media screening. Comparison of recBromelain specific activities (right bar) and cell dry weights (left bar) as a function of culture media

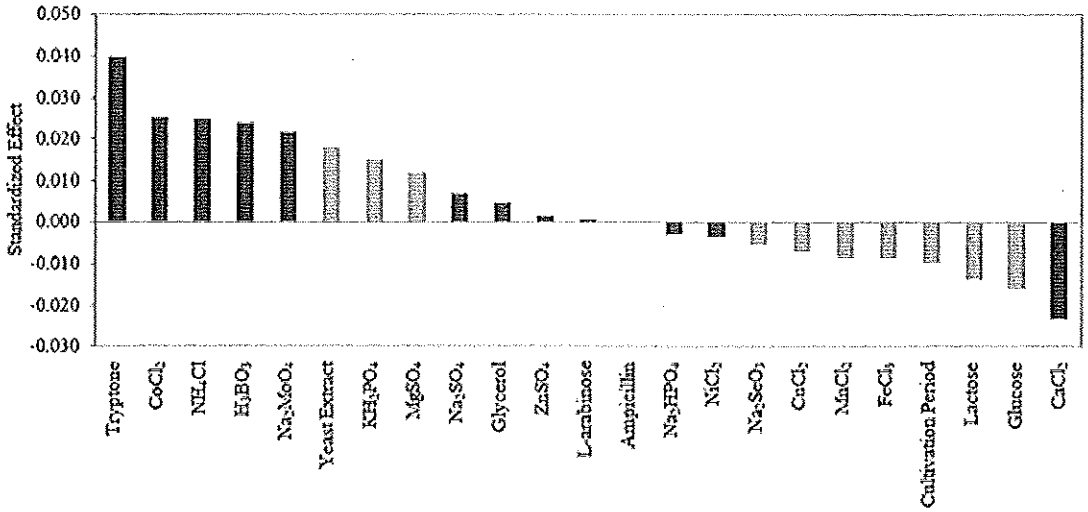

Fig. 11.12 The standardized effect of all parameters from Plackett-Burman screening. The columns shaded in dark grey represents highly significant parameters where $p<0.0001$; light grey represent significant parameters where $p<0.05$; and medium-dark grey represents insignificant parameters where $p>0.05$

has 22 components, the most significant media component had to be identified to minimize tedious experimental design. A Plackett-Burman experimental design was chosen to identify the most significant media component for examination in the subsequent media optimization experiment.

All tested media components are listed in Fig. 11.12. A positive effect is represented by a positive value and a negative effect is represented by a negative value. Figure 11.12 shows that several media components had significant effects, but three were chosen for subsequent experiments: tryptone, NH_4Cl and $CoCl_2$. To characterize the suitable range of each parameter, an OFAT experimental design was employed. The results indicated that 4–14% (w/v) tryptone, 100–260 mM NH_4Cl and 0.4–1.2 μM $CoCl_2$ were suitable ranges for use in media optimization experiments.

Table 11.2 shows the results from the media formulation optimization which indicated that the highest specific activity, 0.672 ± 0.31 U/mg, was obtained when 14% tryptone, 100 mM NH_4Cl and 0.4 μM $CoCl_2$ were added and the remaining components were fixed. Optimal media formulation alone does not ensure high recombinant bromelain production. Therefore, the team proceeded to optimize the fermentation process in a small scale bioreactor.

11.3.2 Optimizing the Fermentation Process Condition

The parameters obtained in the small scale fermentation process were used to scale up the fermentation process to obtain high recombinant bromelain production. Four parameters were tested during the fermentation process optimization: dissolved

Table 11.2 Results and the experimental design to determine the optimal media formulation for high recombinant bromelain specific activity

Run	X_1	X_2	X_3	Response	
	Tryptone % (w/v)	NH_4Cl (mM)	$CoCl_2$ (uM)	recBromelain specific activity (U/mg-protein)	
				Experimental	Predicted
1	4	100	0.4	0.137 ± 0.079	0.185
2	14	100	0.4	0.672 ± 0.305	0.640
3	4	260	0.4	0.096 ± 0.059	0.102
4	14	260	0.4	0.476 ± 0.456	0.462
5	4	100	1.2	0.040 ± 0.037	0.040
6	14	100	1.2	0.270 ± 0.148	0.325
7	4	260	1.2	0.123 ± 0.010	0.123
8	14	260	1.2	0.490 ± 0.495	0.504
9	4	180	0.8	0.240 ± 0.144	0.168
10	14	180	0.8	0.640 ± 0.392	0.608
11	9	100	0.8	0.137 ± 0.014	0.084
12	9	260	0.8	0.102 ± 0.039	0.090
13	9	180	0.4	0.160 ± 0.026	0.185
14	9	180	1.2	0.116 ± 0.013	0.109
15	9	180	0.8	0.221 ± 0.011	0.144
16	9	180	0.8	0.084 ± 0.012	0.144
17	9	180	0.8	0.090 ± 0.062	0.144
18	9	180	0.8	0.102 ± 0.021	0.144
19	9	180	0.8	0.116 ± 0.035	0.144
20	9	180	0.8	0.230 ± 0.015	0.144

oxygen (15–35% saturation), aeration (1–4 vvm), pH (6.6–7.4) and temperature reduction (20–30 °C).

The contour graph in Fig. 11.13 indicates that the optimal process conditions for 2 L bioreactor (1 L working volume) fermentations are: dissolved oxygen, 35%; pH, 7.4; temperature reduction to 30 °C; aeration, 1 vvm. The optimized media formulation and fermentation process were used in the scale up trial.

11.3.3 Scaling up Fermentation Volume for Recombinant Bromelain Production

Two scale up methods were tested for recombinant bromelain production in 30 L bioreactors (18 L working volume) with constant tip speeds and kLa ranges.

Table 11.3 summarizes our observations while scaling up the fermentation volume for recombinant bromelain production. Based on the results obtained, the constant kLa range method is suitable for scale up because the biomass only reduced

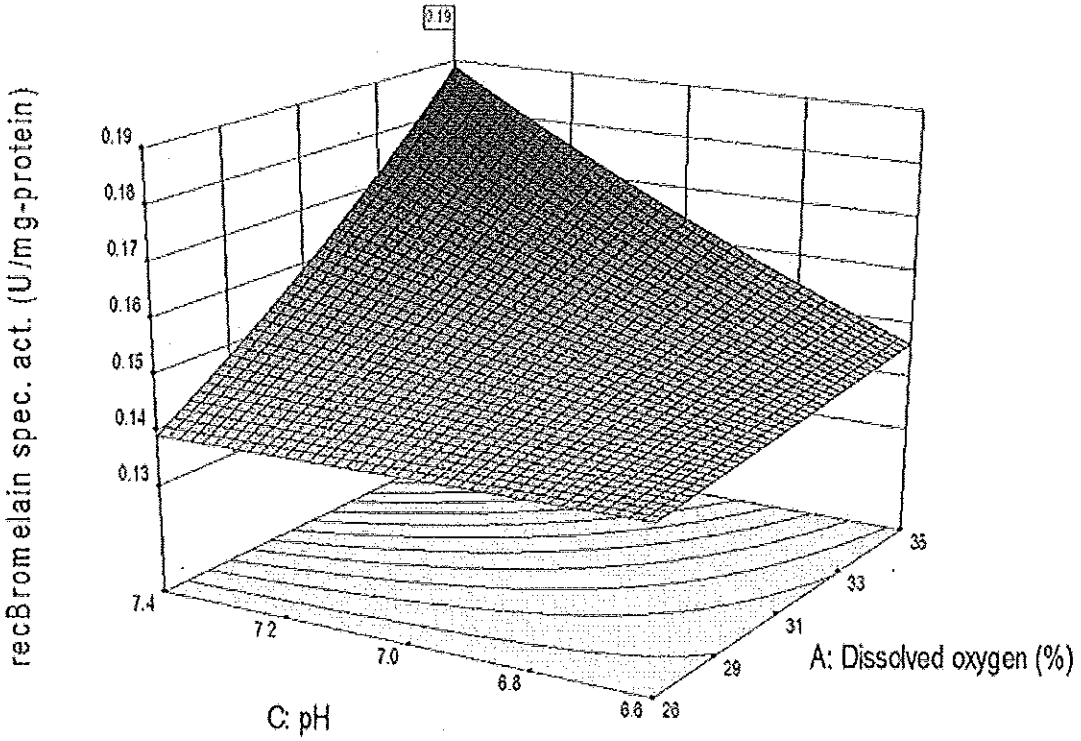

Fig. 11.13 The generated 3-D plot with respect to dissolved oxygen and pH, derived from statistical model equation

Table 11.3 Methods for scaling up fermentation volume for recombinant bromelain production

	1-L production	18-L production	
		Constant tip speed	Constant K_La range
Cascade setup (rpm)	300-1000	173-575 (calculated)	196-356 (calculated)
Inoculum		10 (~OD_{600} 9-11	
Fixed set points (optimized)	Aeration:	1 vvm	
	Temperature :	37°C (3 hours) and 30 °C (8 hours)	
	Dissolved oxygen :	35 %	
	pH :	7.4	
Wet cell weight (g/L)	37.6	33.3 (↓11.4%)	35.0 (↓6.9%)
Specific activity (U/mg-protein)	0.186	0.114 (↓38.7%)	0.174 (↓6.5%)

6.9% compared with the constant tip speed method (11.4% reduction). These results are also suitable for the specific activity of recombinant bromelain, where the constant kLa range method produced a 6.5% reduction compared with 38.7% using the constant tip speed method. Therefore, the constant kLa range method was chosen for scaling up the fermentation volume for recombinant bromelain production.

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