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PAPER

Intein based bioprocess for production of a synthetic antimicrobial peptide: An alternative route to solid phase peptide synthesis

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Despite increasing demands within the healthcare market, the growth of peptide based therapeutic products is challenged by the need for sustainable processing strategies using cheaper and eco-friendly processes. To overcome this hindrance, this study aims to evaluate the potential of intein autocleavage based bioprocessing platform as an alternative to the solid phase peptide synthesis (SPPS) route for economical peptide manufacture. The robustness of the bioprocessing strategy was evaluated by designing a processing platform for manufacturing a 11-mer synthetic antimicrobial peptide. The peptide was successfully produced in its active form using the *SspDnaB* mini intein system and showed antimicrobial efficacy against *E. coli,* a common pathogen. The cleaved peptide and the cleavage reaction was further characterized by RP-HPLC, MALDI-TOF mass spectrometry and calorimetric methods. Process simulation studies were also conducted using the Super Pro Designer software to compare the overall process economics and environmental impact of the proposed bioprocessing strategy with the SPPS methodology. Operating costs for the intein based bioprocessing strategy was found to be at least two to three times cheaper compared to the SPPS method while being 5-8 times more environment friendly.

Introduction

The demand for protein and peptide based products both as therapeutics and medical diagnostics / imaging is on the rise in the current healthcare market.[1] More than 60 synthetic therapeutic peptides have reached the American, European and/or Japanese pharmaceutical markets, and it is expected that the market value of synthetic peptides would reach €11.5 billion by the end of 2013.[2] As drug candidates, peptides are more advantageous than chemical or protein-based drugs. For instance, peptides are known to possess better penetrability within tissues and lesser immunogenicity associated problems.[3] Compared to small molecule drugs, peptides demonstrate better selectivity and efficacy, coupled with reduced toxicity.[4, 5] However, the economics of the current pharmaceutical industry is plagued by concerns like cost-competition (especially following patent expiry) and economic pressures imposed by governments trying to develop low cost treatment regimens for the masses.[2]

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The industry also needs to address concerns related to sustainability and the need for greener processes for the bulk production of peptides.[6] At present, the most common strategy for producing synthetic peptides is the Solid Phase Peptide Synthesis (SPPS) strategy, first introduced by Merrifield in 1963.[7, 8]

Today the SPPS chemistry has been optimised to show wide versatility and the associated instrumentations have also been well developed to automate the synthesis process almost to its entirety.[9, 10] However, the growing need for sustainable greener processes puts chemical synthesis at a disadvantage due to the usage of large volumes of organic solvents and toxic chemicals that are environmentally damaging.[11] Due to their potential to increase levels of volatile organic compounds (VOCs) within the atmosphere, recent legislations have curbed the usage of organic solvents within the pharmaceutical industry[12] and mandated steps to be adopted to prevent release of VOCs. A bioprocessing alternative therefore seems imperative, but peptide biomanufacturing is challenged by proteolytic degradation and multiple downstream purification steps, which reduces product yield.[13] The recent advent of intein-based auto-cleavage strategy appears to be able to overcome these problems,[14-17] and merits further studies. The aim of this study is to systematically evaluate the feasibility of an intein based bioprocessing platform as an alternative to SPPS for economical and environmentally sustainable production of peptides.

To evaluate the intein bioprocessing platform, we chose a synthetic 11-mer antimicrobial peptide, known as P11-5, recently

designed by Qi et al.[18] Being an antimicrobial peptide, this model peptide is potentially toxic to the expressing host organism, *Escherichia coli* (*E.coli*), and presents a challenge for recombinant peptide production. P11-5 was expressed as an intein fusion protein and the peptide was recovered through the intein auto-cleavage reaction. The cleavage reaction was characterized by different methods including reverse phase high performance liquid chromatography (RP-HPLC), MALDI-TOF (matrix assisted laser desorption / ionization time of flight) mass spectroscopy and calorimetric studies. The P11-5 recovered from the bioprocessing route showed comparable activity against *E. coli* as the chemically synthesised peptide. To assess process feasibility with respect to economics and environmental sustainability, the intein bioprocessing flowsheet was simulated to produce the target peptide at pilot scale (400 L culture) using the Super Pro designer v8.5 software (Intelligen Inc., USA) and compared with the SPPS process which was also simulated using the same software to obtain the same product yield. Economic and environmental impact analyses of the two processes showed that the intein bioprocessing strategy was superior to the SPPS platform. This outcome opens the way for detailed process optimization studies of peptide production *via* intein cleavage bioprocessing, which shows tremendous potential in large scale peptide production applications.

Materials and methods

All chemicals were obtained from Sigma Aldrich unless specified otherwise.

Cloning

Codon optimized nucleotide sequence for the P11-5 peptide (GKLFKKILKIL) was synthesized as a duplex by First Base, Singapore. The duplex had sticky ends to facilitate insertion into the pTWIN1 vector (NEB, USA) between the SapI and PstI restriction sites, and expression of the peptide at the C-terminal fragment of the fusion protein consisting of the CBD-Intein and the target sequence (Fig. 1). The plasmid obtained was transformed into *E. coli* BL21(DE3) (Stratagene, Singapore) by heat shock method. The transformed cells were grown overnight on an ampicillin (100 µg/ml) selective LB (Luria-Bertani) agar medium and inoculated into LB medium (50 ml) also containing the same antibiotic. The cells were then grown overnight (12 h) in a shaking incubator at 37 °C and 200 rpm.

P11-5 fusion protein expression and purification

Overnight *E. coli* culture as obtained above, was used to prepare 400 ml of 1% (v/v) bacterial suspension in LB (containing 100 µg/ml ampicillin) for P11-5 fusion protein expression. The bacterial cells were grown at 37 °C and 200 rpm until mid-log phase (OD_{600}= 0.4-0.5), followed by fusion protein induction using 0.1 mM isopropyl-1-thio-β-D-galactoside (IPTG). Protein expression post induction was carried out overnight at 15 °C, following which bacterial cells were harvested by centrifuging at 5,200 ×g for 30 min. The cell pellets obtained were washed with phosphate buffered saline (PBS) and then re-suspended in lysis buffer (0.5 M NaCl, 0.1 mM EDTA, 0.15% TWEEN 20 in 20 mM phosphate buffer, pH 8.5). 10 ml of this bacterial suspension was disrupted using a sonicator (Branson, USA) at 30% amplitude strength for 5 min with 5 s pulse on and 7 s pulse off. The insoluble protein fraction was removed by centrifugation at 10,000 ×g for 30 min at 4 ºC. The supernatant was diluted to a total protein concentration of 1 mg/ml and incubated with chitin beads (NEB, USA) pre-equilibrated in the lysis buffer (or the binding buffer, used synonymously henceforth), at levels equivalent to 1 ml bead for every 5 mg target protein. The fusion protein was incubated with the beads for 1 h at 4º C to allow binding. The bound proteins were recovered by centrifugation, and washed with the binding buffer followed by a cleavage buffer (20 mM phosphate, pH 6.5) wash at 4 ºC. The beads were then incubated in the cleavage buffer for 16 h at room temperature (25 °C) on a roller mixer.

Analytical methods

Total protein concentration was determined by Bradford assay. Verification of fusion protein expression was performed by SDS PAGE. The gels were stained using Coomassie Blue and scanned using a densitometric scanner (BioRad, USA); protein band intensities were quantitated using the GelPro 3 software (Media Cybernetics, Inc., USA).

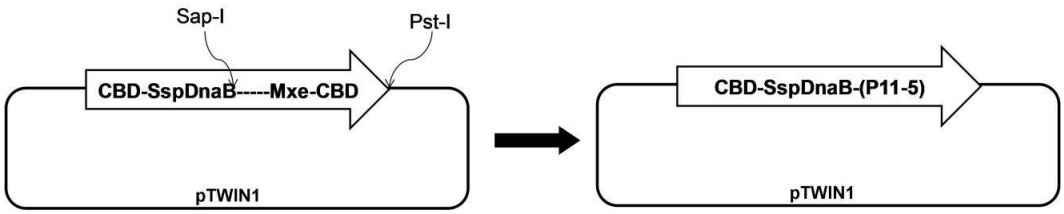
P11-5 characterization by reversed-phase high performance liquid chromatography

Progress of the intein cleavage reaction was monitored by reversed-phase high performance liquid chromatography (RP-HPLC) using a C18 Jupiter RP column (5 µm particle size, 300 Aº pore size, 150×4.6 mm; Phenomenex, USA) connected to a Shimadzu LC-20 AT HPLC system (Shimadzu Corporation, Japan). RP-HPLC was performed at a constant mobile phase flow rate of 0.5 ml/min and trifluoroacetic acid counterion (0.05 % v/v) was added to all HPLC buffers. The RP column was equilibrated in 10 % v/v acetonitrile–water gradient for 5 min, and a 10–100 % v/v acetonitrile–water gradient was employed over the next 30 min, followed by column stripping with acetonitrile for 5 min. Absorbance was measured at 214 nm using a UV detector (Shimadzu SPD-20A). Samples were filtered using a 0.2 µm filter prior to column injection. Data acquisition was performed using the Shimadzu LC solution software (LC Solutions; Shimadzu Pte. Ltd, Singapore). A standard curve was first generated by integrating the peak areas obtained from injecting varying quantities (0.1-1 µg) of chemically synthesised P11-5 (>90 % purity, GL Biochem, China). The amount of peptide released through the intein cleavage was then determined by injecting the known amount of the cleavage mixture supernatant to the RP column.

The molecular weight of the cleaved peptide was also verified using (MALDI-TOF) mass analyzer (ABI 4800, Applied Biosystems, USA).

Study of the intein cleavage reaction by isothermal titration calorimeter

The intein cleavage reaction was monitored using an isothermal titration calorimeter (Nano-ITC, TA instruments, USA). 100 µl of the CBD-P11-5 fusion protein bound chitin bead suspension (in cleavage buffer) was introduced into the cell of the calorimeter. The reaction cell was maintained at 25 ºC and the heat flow measured overnight, without stirring to minimize background noise generated by chitin resins knocking against the walls of the



P11-5 = AAC GGTAAACTGTTCAAAAAAATCCTGAAAATCCTG TAATAATGA CTGCA

Fig. 1 Plasmid cloning strategy for expression of P11-5 fusion protein. The pTWIN1 plasmid was digested with Sap-I and Pst-I to remove the second intein system. The nucleotide sequence used for cloning the P11-5 gene is also shown. The underlined nucleotides encode for the peptide while those on the 5′- and 3′-ends were used for ligating the sequence to the plasmid post-digestion with restriction enzymes. TAATAA towards the 3' terminal are the stop codons.

calorimeter. The baseline subtracted signals were presented by plotting the average heat flow at 60 s intervals.

Antibacterial characterization of P11-5

The cleaved P11-5 was dried in a vacuum centrifuge at 30 °C and subjected to antibacterial assays using the diffusion method.[19] Lawns of exponential phase *Escherichia coli* (ATCC 8739) (0.5 McFarland) were prepared on 90 mm × 10 mm Petri plates containing 25 ml of Muller-Hinton agar using a sterile cotton swab. An aliquot of ~10 µg peptide derived from chemical synthesis or intein cleavage bioprocess were spotted in the pre-coated agar plates. Buffer without peptides were used as the negative control.

Process simulation studies for feasibility assessment

Process simulation studies were performed using the Super Pro v8.5 software (Intelligen Inc., USA) to compare the economic and environmental feasibility of the intein bioprocessing strategy at pilot scale (using a 400 L culture) to that of the widely used SPPS method. The objectives of the simulation studies were to: i) compare the operating costs for the two processes under consideration for assessment of process economics and ii) to obtain mass balances for both processes that can be used for environmental impact assessment. The simulations were run in design mode such that the sizing of the equipment was done by the software. Operating cost analysis were performed by the Super Pro based on factors commonly employed in bioprocessing plants.[20] The raw materials costs were obtained from suppliers' list prices for the largest quantity available (Tables S1 and S2 in supplementary notes). Both bioprocess and SPPS plants were assumed to run at 100% efficiency with continuous manufacturing throughout the year. Quality assurance / quality control costs were neglected for both plants under consideration at this stage of the simulation. The facility dependent costs were also ignored within the calculations presented here since it would heavily depend on the location of the plant. Moreover, due to the significant difference in the nature of wastes between the two conceptual plants (the SPPS process expected to produce more organic solvents as waste) waste disposal costs were also not considered in the operating cost analysis. The wastes were analyzed separately in the environmental analysis section. The simulation strategy for the two processes is presented below.

Bioprocess simulation

The bioprocess flowsheet used for simulation was in accordance to our P11-5 bioprocess, assuming general rules of thumb for recombinant protein expression, as discussed in a previous study[21] (shown in Fig. S1). The simulation started with the mixing and heat sterilization of the media components. Bacteria were then grown for 24 h at 15 ºC in a bioreactor. A 400 L culture was used for the simulation where it was assumed that the final cell density was 50 g dry cells / L of culture with the recombinant protein produced at 0.05 g/g of biomass. The fermentation broth was harvested and cooled to 4°C before further processing. The cell broth was first passed through a microfilter to recover the cells and then resuspended in lysis buffer (refer to P11-5 fusion protein expression section). Cells were then lysed using a high-pressure homogenizer (two passes) operated at 800 bar. Cell debris was separated from the soluble cell extract in a disk-stack centrifuge. The soluble cell extract was filtered by a dead-end filtration device to remove all cell debris and then concentrated by ultrafiltration. The lysate was fed to a reactor where it was mixed with chitin beads suspended in the binding buffer to obtain a final protein concentration of 1 mg/ml. Binding was carried out for 60 min in the reactor following which the supernatant was removed and the beads washed with binding buffer (200 L) to remove the remaining contaminants from the protein-bound chitin beads. The reactor was then fed with (100 L) of cleavage buffer and the cleavage reaction was allowed to continue for 16 h at 25°C. The intein cleavage efficiency was assumed to be 50% for this study. The supernatant containing the cleaved P11-5 peptide was collected from the reactor. To prepare for reuse, the chitin beads were regenerated with 0.3 M NaOH and washed with water before equilibration with the binding buffer. The chitin beads were considered as a consumable for the cleavage process, priced at $200 / L, with an estimated binding capacity of 5 mg/ml and recycled once every 5 batches. The cleavage reaction step marked the end of the simulation for the bioprocessing strategy. A final yield of 22.2 g per batch was obtained through the simulated process described above.

Solid phase peptide synthesis simulation

Simulation for the SPPS was carried out following the Fmoc chemistry reported previously.[22] Peptide polymerization started with the C-terminal amino acid bound to a resin bead, upon which the chain elongation reaction towards the peptide N-terminus was performed using appropriately protected amino acids. Standard instrumentation for the procedure, where the

simulation started with 50 mg equivalent amino acid for the primary peptide chain length elongation was assumed to occur in a single reactor, was considered for the simulation.[9] The Fmoc Wang resin was first wetted in 6 ml DMF (dry distilled) for 60 min. Inert atmosphere and pressure within the reaction vessel was maintained using nitrogen gas. De-protection of the resin bound amino acid was carried out using 20% piperidine in DMF for 30 min and then washed with 50 ml of dry DMF. In a separate reaction vial, one equivalent of the subsequent Fmoc-OH (amino acid) was mixed with 1 equivalent of HBTU and activated by 0.3 M N-methyl morpholine (NMM) for 5 min. The resin-bound de-protected amino acid in the reactor, was coupled to the latter activated amino acid. The coupling reaction was carried out for 60 min assuming 95% coupling efficiency. Washing was performed with DMF to remove any further unreacted amino acids. Continued reaction involving de-protection, activation and coupling, as described earlier, was performed for all remaining amino acids. The final peptide produced was left in its de-protected form still bound to the resin. The resins were filtered using a dead-end filter and then dried at 30 ºC for 24 h. Finally, cleavage of the Wang resin comprising the protecting groups and the polymer support was performed in 90% TFA and 5% anisole and water in a vessel for 60 min under stirring conditions. The TFA was evaporated and the peptide was precipitated and washed using diethyl ether in another vessel. The peptide was then dissolved in 10% acetic acid and fed to a freeze drier. Approximately 100 mg of the final peptide could be obtained through the above-mentioned process. The final polishing step was not included in the simulation to allow direct comparison with the simulation exercise conducted for the bioprocessing route. The lab-scale SPPS process was scaled-up using the Super Pro software to achieve batch yields equivalent to that obtained for the simulated intein bioprocess, i.e. 22.2 g per batch.

Environmental impact assessment

The objective of this part of the study was to compare the environmental impact of the intein bioprocessing strategy relative to the SPPS methodology. Diverse environmental impact assessment methodologies have been published in literature[23-25] including Life Cycle Assessment (LCA) strategies.[26, 27] LCA studies demand in-depth analysis of the raw material extraction methods, unit operations involved within the manufacturing methodology, subsequent use of the product and associated recycling and / or disposal of wastes. Such detailed assessments would be outside the scope of the present work and hence a simpler methodology based on a previously reported study[28] was adopted here. Briefly, Mass Indices (MIs) (in Kg/g of product) for all the input and output components of the concerned process were calculated from the material balance data, which was obtained from Super Pro Designer. For input or output materials, the MIs indicate the amount of a particular component that is consumed or produced to obtain a unit amount of the target product. In the next step, Environmental Impact (EI) of the individual components were determined by multiplying its MI with the corresponding Environmental factors (EFs) as discussed in detail by Heinzle E. et al.[28] The EI parameter helps to connect the mass consumed or formed to the corresponding environmental relevance of a compound. The sum of all the EIs (input or output) would thus yield the EI of the whole process, providing a rough estimate for the environmental burden for generating a unit amount of the target product produced by a particular processing strategy. EI was therefore used as a parameter for evaluating the environmental feasibility of the two alternate processing strategies for P11-5.

Results and discussion

Construct design and cloning

The pTWIN1 vector consists of two different inteins (*SspDnaB* and *Mxe gyrA*) in a single open reading frame (Fig. 1). The multiple cloning site for the vector is located between these two inteins. The N-terminal fragment consists of the *SspDnaB* intein (obtained from *Synechocystis sp.*) while the C-terminal fragment consists of the *Mxe gyrA* intein (obtained from *Mycobacterium xenopi*). From a bioprocessing perspective, *SspDnaB* is more advantageous compared to the *Mxe* intein due to its capability to undergo auto-cleavage which is induced by changing environmental conditions like temperature and pH. Furthermore, being a C-terminal intein, it can be used to express the target peptide without the need to introduce any additional methionine residue at the peptide N-terminus. In this study, the pTWIN-1 vector was digested with Pst-I and Sap-I restriction enzymes, followed by ligation with the desired DNA duplex sequence encoding the P11-5 sequence, which have the corresponding sticky ends. This cloning strategy allowed the expression of a fusion protein consisting of the CBD-Intein and P11-5 sequences from the N- to C- termini (Fig. 1). The success of the cloning strategy was verified by DNA sequencing and colony PCR following transformation of *E. coli* with the modified plasmid (data not shown).

Fusion protein expression and purification

Fig. 2 confirms the successful over-expression of the CBD-P11-5 fusion protein (lane 2). The molecular weight of the intein tag is 27 kDa, while that of the P11-5 peptide is 1.3 kDa, to give a fusion protein with a molecular weight of ~28.3 kDa. It is also apparent that the majority of the recombinantly expressed protein remains in the soluble fraction of the bacterial cell lysate (Fig. 2,

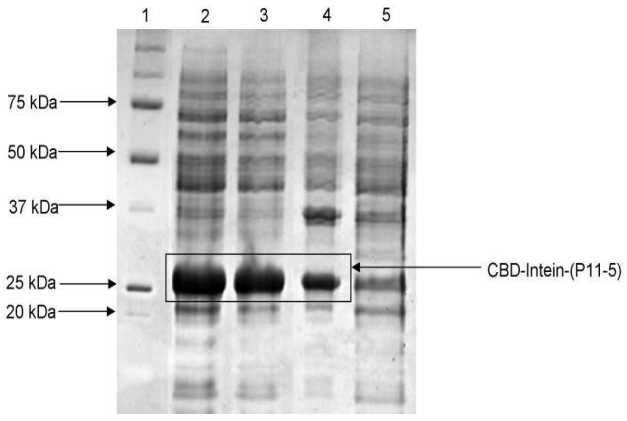


Fig. 2 SDS PAGE gel demonstrating the CBD-P11-5 fusion protein expression profile in *E. coli* (BL21DE3). Lane 1: Marker; lane 2: Induced whole cell proteins; lanes 3 and 4: Soluble and insoluble fractions of the expressed proteins; lane 5: Uninduced whole cell proteins.

lanes 3 and 4). Post-induction temperature for the protein expression was maintained at 15 ºC to minimize insoluble expression of the protein and allow better control of the *SspDnaB* intein autocleavage for improved recovery of the target peptide. All the protein bioprocessing operations up to the peptide cleavage step were therefore performed at 4 ºC. The expressed fusion protein was purified from the soluble protein extract by incubating the cell lysate with chitin beads (pre-equilibrated in the binding buffer), where a binding capacity of ~ 5 mg/ml of resins was observed, which agreed well with that reported in an earlier study.[21] Complete binding was observed within 30 min of incubation with the chitin resins (data not shown).

Fusion protein cleavage

Following binding, the chitin bound CBD-P11-5 was washed with the cleavage buffer and then incubated at room temperature (25 °C) for cleavage. The identification of the cleaved peptide was verified by RP-HPLC (Fig. 3A), where chemically synthesized P11-5 (> 90% pure) was also analyzed as the standard. The cleaved peptide eluted from the RP column at the same retention time as the chemically synthesized peptide. The identity of the cleaved product was further verified by analyzing the cleavage-mixture supernatant using MALDI-TOF mass spectrometry. Fig. 3B shows that a base peak indicating a product molecular weight 1.3 kDa, which agrees with the molecular weight of P11-5, dominates the spectra. Trace levels of contaminants present within the post-cleavage mixture can be removed by the subsequent polishing steps in the final purification process.

The intein cleavage reaction was, however, found to be relatively slow, where no detectable P11-5 could be observed in the RP-HPLC until after 12 h of incubation in the cleavage buffer (Fig. 4A). No significant increase in the cleavage yields could be observed beyond 16±1 h of incubation. A slow cleavage kinetics is well known for the inteins,[29] but the abrupt appearance of the cleaved peptide following 12 h incubation within the cleavage mixture supernatant (as shown in Fig. 4A) was interesting and merits further studies. Since all types of reactions possess their own thermodynamic signatures, we set out to perform calorimetric studies for the resin bound CBD-P11-5 cleavage reaction to monitor the reaction progress. The fusion protein bound chitin beads were washed with the cleavage buffer, and injected into a Nano-ITC. The corresponding thermodynamic profile of the cleavage reaction, presented in Fig. 4B, indicated an exothermic process proceeding through two different phases: first after 8 h and second after 12 h of incubation (Fig. 4B). The exact intein cleavage reaction mechanism remains debatable; however, there are evidences that the reaction proceeds through intermediates involving proton transfer between the intein's histidine and C-terminal asparagine (Asn) residues to complete an Asn cyclization step culminating in the final autocleavage of the peptide.[30, 31] Therefore, the first hump in Fig. 4B (~8 h) probably represents the energy barrier associated with the formation of the proton transfer transition states as described in recent literature. The time-point for the second hump (~ 12 h) matches with the point of P11-5 release within the cleavage reaction mixture supernatant (Fig. 4A). It would thus be reasonable to assume that the second peak in Fig. 4B corresponds to the final cleavage of P11-5 from the resin-bound intein fusion protein thereby

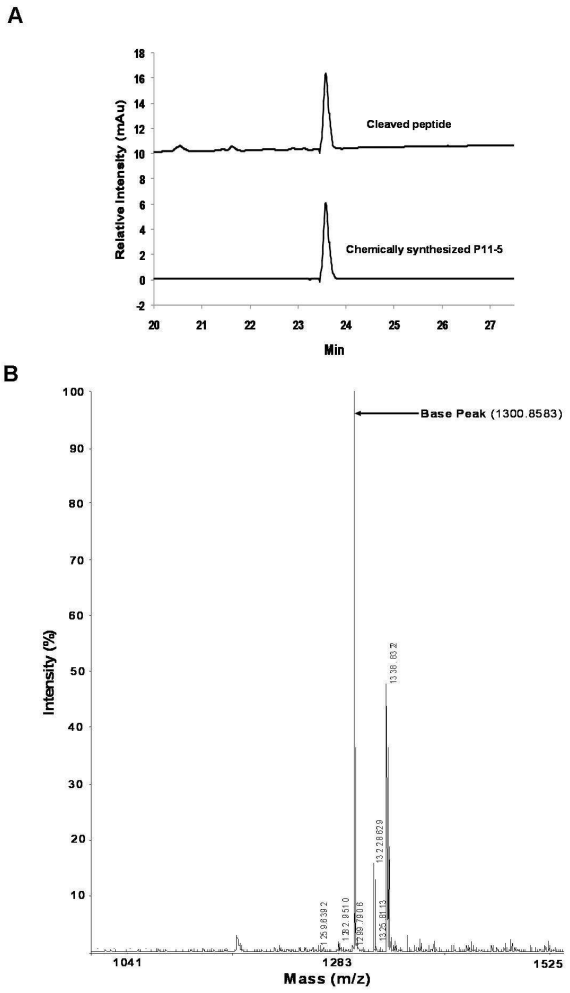


Fig. 3 (A) RP-HPLC traces of the chemically synthesized P11-5 and that obtained from the intein cleavage strategy. (B) MALDI-TOF analysis of the P11-5 cleavage reaction mixture supernatant. The molecular mass of the base peak of the spectrum corresponds to the theoretical P11-5 molecular mass of 1.3 kDa.

corroborating the RP-HPLC profile in Fig. 4A. To the best of our knowledge, this is the first reported thermodynamic profiling of a resin-bound intein cleavage reaction. The cleavage reaction is presumed to be highly dependent on the peptide sequence and hence conformation. Considering the importance of an efficient cleavage step on the overall process economics,[21] the use of calorimetry to monitor the completion of the cleavage reaction can serve as a reliable process analytical technology (PAT) tool to study the progress of subsequent cleavage operations involving manufacturing of other peptide molecules.

Bioactivity of P11-5

As P11-5 is an antimicrobial peptide, the biological activity of the peptide was determined by studying the growth inhibition behaviour of P11-5 towards *E. coli,* a common Gram-negative pathogen. Fig. 5 clearly shows that the *E. coli* growth inhibition zones were comparable for the P11-5 derived from the bioprocessing and chemical synthesis routes. This result merits detailed study of the intein based bioprocessing strategy as an alternative to the existing chemical peptide synthesis strategy. In

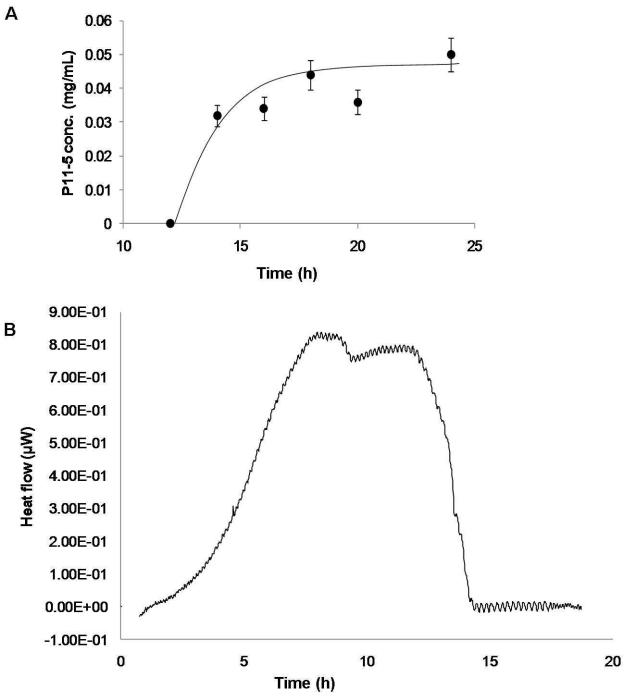


Fig.4 Intein cleavage characterization for P11-5. (A) Concentrations of P11-5 in the cleavage reaction supernatant over time as determined by RP-HPLC. The line is drawn to guide the eye; (B) Heat flow measured, using the ITC, from the resin bound CBD-Intein-P11-5 fusion protein incubated in cleavage buffer at 25 °C.

the next sections, the two production routes, i.e. bioprocessing versus chemical synthesis were compared in terms of process economics and their environmental impact.

Economic assessment of intein bioprocess versus SPPS strategy for large scale peptide manufacturing

Fig. 6 summarizes the overall process flowsheets for the intein bioprocessing and the SPPS routes for P11-5 production. In the bioprocessing strategy (Fig. 6A), the fusion protein over-expressed during the fermentation step was recovered from the bacterial cells *via* the primary recovery section, which comprised cell harvesting, homogenization and recovery of the soluble proteins by centrifugation. The purification and cleavage sections involved protein incubation with the chitin resins, followed by P11-5 peptide release through the intein auto-cleavage step. In the case of SPPS, following peptide synthesis (Fig. 6B), the target product was cleaved from the bound resins, separated through precipitation using an organic solvent and then freeze dried.

Since the final polishing step involving RP-HPLC was common for both the peptide processing strategies, this step was not included in the simulation study. Hence the intein bioprocessing strategy was simulated up to the purification and cleavage steps, while for the SPPS strategy, simulation was performed up to the freeze drying step. It is only reasonable to consider the intein based peptide bioprocessing platform as a potential alternative if it showed better process economics than SPPS. To determine the feasibility of the bioprocess strategy against the SPPS, we chose to adopt a conservative approach such that economic comparisons

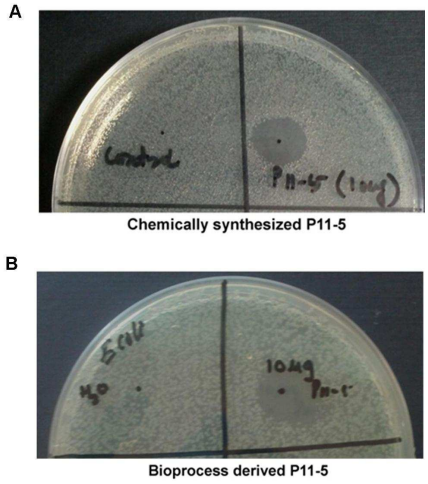


Fig. 5 Activity comparison for the chemically synthesized P11-5 (A) and bioprocess derived P11-5 (B).

could be made at scenarios involving the best plausible productivities for the latter process. Hence for simulation of the SPPS process, we assumed a 1:1 peptide coupling reaction at 95% efficiency irrespective of the peptide chain length, which can be considered practically optimistic.[11, 32] For the bioprocess route, 50% intein cleavage yield was assumed, based on our experimental results (Table 1). The SPPS yields were maintained to be similar to that obtained from each batch of our pilot scale bioprocess so as to normalize productivities (Fig. 7).

Based on the assumptions specified above, the operating costs computed under different scenarios of production are shown in Table 2, where it was observed that the operating costs for P11-5 production using our simulated bioprocess was cheaper compared to the chemical synthesis strategy. An important criterion that reduced the bioprocess operating costs is the relatively cheaper raw materials used for the bioproduction of the target peptide, compared to those for the SPPS strategy. The intein-based auto cleavage strategy is also relatively more economical compared to other enzymatic cleavage methods which require the use of expensive enzymes coupled with resource intensive downstream purification processes,[13, 33] thereby improving the overall process economics. The intein-based process is also advantaged over the SPPS route for production of longer peptides because the production costs are expected to reduce with the increase in peptide chain length for the bioprocessing strategy while the reverse would be true for the SPPS strategy,[11, 21] (Fig. 8). The results of our simulation studies show that the intein bioprocessing strategy is more economically feasible compared to

Table 1 Lab scale expression yields of CBD-P11-5.

Processing step		Yield (mg / L of culture)	Yield (%)
Fermentation		82 ± 10	-
Cleavage			
	Expected	3.8 ± 0.4	100±10
	Actual	1.7 ± 0.3	45±8

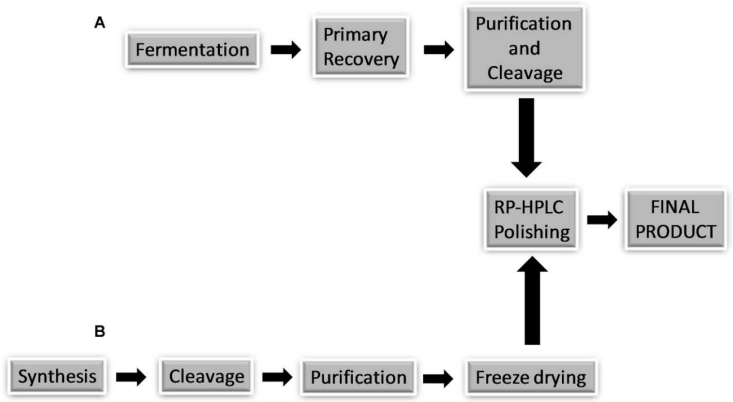


Fig. 6 Schematic representation of the (A) intein bioprocessing and (B) SPPS strategies.

Table 2 Operating cost comparison for the bioprocess and SPPS route (calculations based on 22.2 g batch yield for both processes).

Bioprocess		SPPS	
Cleavage Efficiency (%)	Opex ($/mg)	Coupling Efficiency (%)	Opex ($/mg)
10	4.26	80	23.07
20	2.13	85	12.58
30	1.42	90	7.10
40	1.06	95	4.13
50	0.85	100	2.40

the SPPS under the assumed conditions.

Environmental feasibility of the intein bioprocess versus SPPS strategy

It was expected that the bioprocessing strategy would be more eco-friendly compared to the SPPS involving large volumes of

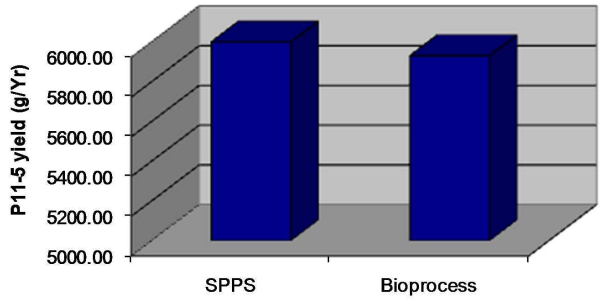


Fig. 7 Annual P11-5 production rate using SPPS and the bioprocessing route.

organic solvents and toxic chemicals. Our aim in this section was to obtain quantitative estimates of the environmental benefits that can be reaped upon adopting a bioprocessing strategy compared to the SPPS method. To achieve this aim, the EI of the two processes as discussed in the environmental impact assessment section, was compared. The EI is an effective parameter for estimating the environmental relevance of a particular process since it takes into account both the quantities of the material consumed and/or released along with its associated toxicities. Hence despite the fact that the MI of the bioprocessing strategy is significantly larger than the SPPS method (shown in Fig. S2, in supplementary notes) owing to the large volumes of water consumption, the EI signatures of the two processes more accurately assesses the true environmental impacts (Fig. 9). As seen in Fig. 9, the non-toxic raw materials involved in the intein bioprocess hardly imposes any environmental burden, but there is a significant increase in the EI at the output stage owing to the large volumes of biowastes generated through the process. Even then, the environmental impact of the bioprocessing strategy is dwarfed by those incurred by the SPPS methodology (Fig. 9). For the SPPS strategy, significant contribution to the EI was attributed to the use of large volumes of organic solvents and hazardous materials throughout the process. Based on our estimation under the assumed conditions, the bioprocessing strategy for large scale production of P11-5 would be approximately 5-8 times more environment friendly than the SPPS methodology. As productivities are expected to decrease

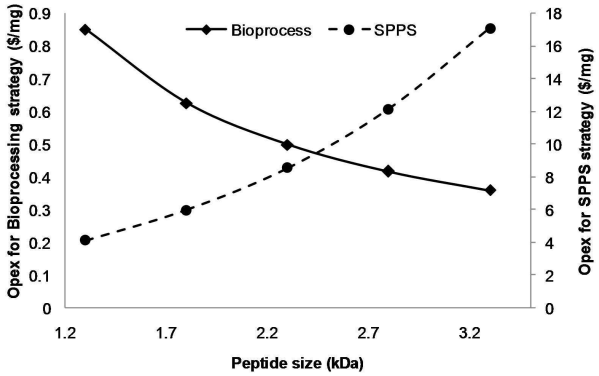


Fig. 8 Effect of peptide size on operating costs using the Bioprocessing and the SPPS routes

more with increase in peptide chain length for the SPPS strategy, compared to the bioprocessing route, the gap in the environmental benefits is expected to also increase

correspondingly because of the widening differences within the MIs of the toxic chemicals used in the former strategy. Based on the results of the simulation studies, it can thus be concluded that the intein-based bioprocessing alternative would be both economically and environmentally more sustainable compared to the SPPS route.

Conclusion

This study reports the production of an artificial 11-mer peptide, P11-5, using a bioprocessing strategy and establish its potential sustainability over the SPPS strategy using process simulation studies. An intein-based bioprocessing strategy was adopted to leverage on the ease of peptide expression and subsequent peptide release rendered by the intein technology. The bioprocess-derived P11-5 was found to be biologically active and retain its physicochemical characteristics, as determined through RP-HPLC and MALDI-TOF studies. The successful bioproduction of a synthetic peptide toxic to the expression host itself demonstrates the potential of the intein autocleavage strategy for establishing large scale bio-manufacturing platforms for other peptides. Owing to its wide versatility, the SPPS strategy would remain an indispensible part of the pharmaceutical industry at least during the drug design and development stages, where large number of candidates needs to be screened. However, this study shows that the difficulty in scaling the SPPS methodology can be overcome by successful implementation of intein-based peptide bioprocessing platforms, thereby opening the way for an economical supply of the peptides at large scale.

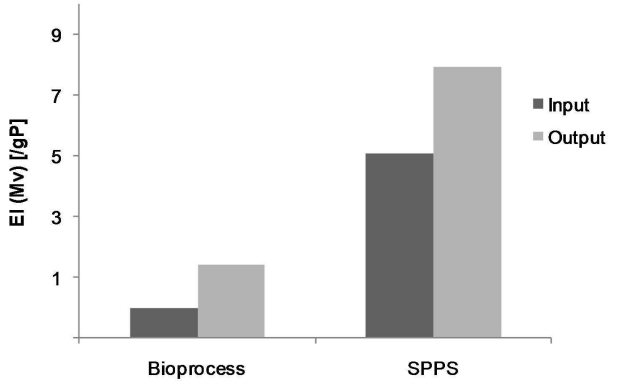


Fig. 9 Environmental index (EI) for the production of P11-5 using the simulated bioprocess and SPPS strategies.

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