3. DESIGN AND DEVELOPMENT OF EXPRESSION SYSTEMS FOR THE PRODUCTION OF RECOMBINANT STAPHYLOKINASE
Staphylokinase (SAK), an extracellular protein of several strains of *Staphylococcus aureus* is one of the most promising blood clot dissolving agents that has immense potential for the treatment of patients suffering from thromboembolic disorders and myocardial infarction (Collen *et al.*, 1992a; Collen *et al.*, 1993c). Emerging studies on thrombolytic potential of SAK has indicated that due to its high fibrin-specificity for Pg activation, better efficacy towards platelet-rich arterial clots and reduced allergic effects, it offers promise as a useful alternative to presently utilized thrombolytic agents.

SAK forms bimolecular complex with blood proteins, such as Pg and Pm and exerts its fibrinolytic effects through conversion of an active non-specific serine protease, Pm, to a highly specific proteolytic enzyme. SAK-Pm complex can recognize blood zymogen Pg as a substrate and convert it into Pm that is capable of degrading blood clots. In a plasma milieu, SAK is able to dissolve fibrin clots without any associated fibrinogen degradation (Collen *et al.*, 1993; Lijnen *et al.*, 1991). This fibrin-specificity of SAK is the result of inhibition of SAK-Pm complex formation by α2-antiplasmin complex at the fibrin surface, resulting in very localized Pg activation at the fibrin surface (Sakai *et al.*, 1989; Lijnen *et al.*, 1992; Silence *et al.*, 1993a, b). This unique property of SAK may be highly advantageous for therapeutic purposes. Recent clinical trials have shown that SAK is as effective as tPA in achieving early perfusion in myocardial infarction patients (Vanderschueren *et al.*, 1995). Its utility in thrombolytic treatment has now been established by several limited clinical trials (Collen *et al.*, 1993; Vanderschueren *et al.*, 1995), which indicates that SAK may be one of the ideal alternative thrombolytic agents of future.

SAK is a single chain 15.5 kDa protein, consisting of 136 amino-acid residues. It is produced in very low amount by its natural host, *S. aureus* (Lack, 1948). The purification of SAK from its natural host for detailed physical and biochemical studies as well as for evaluation of its thrombolytic potential has been elusive because of its low expression levels and concomitant secretion of potent exotoxins (Collen *et al.*, 1992a; Scorer *et al.*, 1993). Production of intact, biologically active SAK from bacterial expression system has been a challenge because of amino-terminal micro-heterogeneity, plasmid instability or
low-production yield. Considering its therapeutic applicability and clinical implications in thrombolytic therapy, attempts have been made in the past to develop different systems for large-scale production of the thrombolytic agents (Gerlach et al., 1988; Miele et al., 1999). To search for an ideal source of SAK production through recombinant routes, the gene encoding SAK was isolated from its natural host *Staphylococcus aureus* and cloned into various heterologous hosts, e.g., *E. coli*, bacillus and yeast (Sako et al., 1983). In case of bacillus, SAK appeared proteolytically degraded (Gerlach et al., 1988) and in yeast, it was found glycosylated that reduced the plasminogen-activation function of this protein (Miele et al., 1999). In *E. coli*, extracellular production of SAK resulted in low level of SAK production carrying amino-terminal degradation. To overcome these problems, SAK has been expressed using a strong expression signal, which required addition of exogenous inducers that make these systems highly expensive when utilized for large-scale production of this protein. Thus, these systems had one or another limitation. Therefore, to undertake research on structure-function of SAK, we attempted to develop a suitable recombinant system for generation of sufficient amount of native and genetically engineered SAK protein. This chapter deals with development of an efficient expression system for the intracellular production of SAK in a bacterial (*E. coli*) and in a unicellular eucaryotic host (*Pichia pastoris*).

### 3.1 Cloning of *sak* gene from *Staphylococcus aureus* into *E. coli*

The structural gene encoding SAK (*sak*) was retrieved from the genomic DNA of *S. aureus* (local isolate designated as SAK11) through PCR amplification using primer pair R KD5 and R KD7, designed on the basis of the known nucleotide sequence of *sak* gene (Sako and Tsuchida, 1983). PCR product was cloned in plasmid vector, pBS KS+ and the authenticity of the cloned gene was established through nucleotide sequencing (Fig. 3.1), which matched perfectly with the published *sak* gene sequence (Sako and Tsuchida, 1983).
**ATG CTC AAA AGA AGT TTA TTA TTT TTA ACT GTT TTA TTA TTC TCA**

**M L K R S L L F L T V L L L L F S**

**TTT TCT TCA ATT ACT AAT GAG GTA AGT GCA TCA AGT TCA TTC GAC AAA GGA**

**F S S I T N E V S A S S S F D K G**

**AAA TAT AAA AAA GGC GAT GAC GCG AGT TAT TTT GAA CCA ACA GGC CCG TAT**

**K Y K K G D D A S Y F E P T G P Y**

**TTG ATG GTA AAT GTG ACT GGA GTC TAT GGT GAA ACT AAA AAA TAT TGT TTA TCC**

**L M V N V T G V D S K G N E L L S**

**CCT CAT TAT GTG GAT GGC ACT ACA CTT ACA AAA GAA**

**P H Y V E F P I K P G T T L T K E**

**AAA ATT GAA TAC TAT GTC GAA TGG GCA TTA GAT GCG ACA GCA TAT AAA GAG**

**K I E Y Y Y V E W A L D A T A Y K E**

**TTT AGA GTA GCT GAA TTA GAT CCA AGC GCA AAG ATC GAA GTC ACT TAT TAT**

**F R V V E L D P S A K I E V T Y Y GAT AAG AAT AAG AAA AAG GAA GAA ACG AAG TTC TTT CCT ATA ACA GAA AAA**

**D K N K K K E E T K S F P I T E K**

**GGT TTT GTT GTG CCA GAT TTA TCA GAG CAT ATT AAA AAC CCT GGA TTC AAC**

**G F V V P D L S E H I K N P G F N**

**TTA ATT ACA AAG GTT GTT ATA GAA AAG AAA TAA**

**L I T K V V I E K K &**

---

**Fig. 3.1 Nucleotide and deduced amino acid sequence of sak gene;** DNA sequence of antisense strand of sak gene of the *Staphylococcus aureus*. Nucleotides in bold indicate the signal sequence for extracellular secretion.
3.1.1 Expression of sak gene in E. coli: production of recombinant SAK

For high level production of SAK, sak gene was cloned under IPTG inducible T7 promoter using pET9b vector. Cloning vector containing sak gene was linearized with NdeI enzyme. The NdeI digested pBSSAK was then ligated with the NdeI fragment of pET9b (Fig. 3.2) and transformants were selected on LB supplemented with ampicillin and kanamycin plates. Transformants were screened for the right orientation by digestion of integrated plasmid with EcoRI and the clones giving two fragments of almost equal size were selected and designated as pETpBSSAK. The construct pETpBSSAK, was then transformed into E. coli BL21DE3 and the clones were screened for the expression of SAK. To check overexpression, single colony carrying pETpBSSAK was inoculated in LB supplemented with ampicillin and kanamycin and induced with 0.2 mM IPTG when OD<sub>600</sub> was 0.3–0.5 and allowed to grow further for another 8-10 h. Expression of recombinant SAK protein was monitored by running cell lysate on 15% SDS-PAGE followed by coomassie brilliant blue staining. The E. coli cells expressing sak gene, exhibited a distinct thick protein band corresponding to size of the 15.5 kDa that constituted 20-25% of total cellular protein (Fig. 3.3). The Pg activation activity of SAK in cell lysate was found to be 7.0 IU/μl.

3.1.2 Development of a novel expression system for large-scale production of recombinant SAK

Although expression of sak gene under T7 promoter resulted in accumulation of large amounts of recombinant SAK (20-25% of total cellular proteins), requirement of IPTG for expression of recombinant protein makes the system highly expensive when utilized for the large-scale production of SAK. Additionally, use of IPTG for large-scale protein production of recombinant proteins is not usually preferred due to its undesirable effects on induction of other unwanted proteins. Therefore, a novel expression system that does not require any gratuitous inducer has been developed for SAK production. Such
Fig. 3.2 Strategy for expression of SAK under T7 promoter in *E. coli.*
Fig. 3.3 Intracellular expression of SAK in *E. coli*. Lane 1: Protein molecular weight marker; Lane 2: *E. coli* BL21DE3 pET; Lane 3: *E. coli* BL21DE3 pETpBSSAK; Lane 4: *E. coli* JM109 pOXYSAK; Lane 5: *E. coli* BL21DE3 pETpUCVSAG. The arrow indicates the overexpressed proteins.
system may find its utility for large-scale SAK production apart from providing a facile source of SAK protein for structure-function studies.

An expression system based on the regulatory sequence (OXY promoter) of a bacterial hemoglobin gene vgb (Vitreoscilla hemoglobin gene) was developed, which is regulated by availability of atmospheric oxygen and does not require any chemical or physical agent for protein induction. Integration of OXY promoter sequence with reporter genes CAT and xylE has shown that expression of reporter gene remains low during exponential growth phase under aerobic growth conditions. Activity of this promoter is hyperinduced when the cells reach stationary phase or are exposed to low oxygen conditions. Looking at these unique characteristics, the OXY promoter was selected for developing novel SAK expression system.

3.1.2.1 Construction of OXY promoter expression cassette

Based on nucleotide sequence of OXY promoter, a novel protein expression cassette was designed and constructed (Fig. 3.4) using four oligonucleotides (OXY1, OXY2, OXY3, and OXY4). It consists of binding sites for oxygen sensitive regulatory proteins such as FNR (Ferredoxin-NADP+ reductase) and CRP (Catabolite repressible promoter) at specific positions. The four oligos were mixed, annealed and klenow fill-in was done followed by PCR amplification using primers OXY 1 and OXY4. PCR product was digested with HindIII and BamHI and ligated with HindIII and BamHI digested pUC18. Ligation mix was transformed into E. coli JM109. Recombinant plasmid carrying OXY promoter sequence was designated as pOXY. Gene encoding SAK was PCR amplified from pETSAK plasmid using primers VSAKNT and VSAKCT. PCR product was digested with AflII and BamHI and ligated with AflII and BamHI digested pOXY vector (pOXYSAK) (Fig. 3.5). Ligated product was transformed into E. coli JM109 and insertion of sak gene at right position was confirmed by analyzing the restriction digestion pattern and DNA sequencing of the cloned gene. Expression of SAK under OXY promoter in E. coli was monitored by running the cell lysate on 15% SDS-PAGE followed by
GATCAAGCTTATCAGTTACACGAGGACGCTGGTTAAAAGTATTTGAGTTTTGATGTGTG
GATTAAGTTTGGAGGTCATAAGATTATAATATGATGATGCTTCACAATTCTGATATGGC
AAAACCATAATAATGAACCTAAGGAAGCCTCATGGGATCCGATC

**Fig. 3.4 Nucleotide sequence of OXY promoter;** CRP sequence is indicated by bold letters and FNR binding site is underlined.
Fig. 3.5 Cloning strategy for the expression of SAK under OXY promoter.
coomassie brilliant blue staining. Expression level of SAK under OXY promoter exhibited a pattern that was more or less similar to that of its expression (6.4 IU/μl) in *E. coli* BL21DE3 under T7 promoter (Fig. 3.3).

### 3.1.3 Enhanced production of recombinant SAK using recombinant bacterial hemoglobin

Expression of *sak* gene under OXY promoter resulted in the accumulation of substantial amounts of intracellular SAK that in turn, overburdened the recombinant *E. coli* cells and resulted in less cell productivity as compared to control cells. To enhance overall cell growth and biomass of the cell culture producing recombinant SAK, attempts were made to enhance the energy level of cells by engineering a novel hemoglobin gene known to increase the respiratory activity of some procaryotic and eucaryotic cells. (Magnolo *et al.*, 1991; DeModena *et al.*, 1993; Khosla and Bailey 1988; Chen *et al.*, 1994; Khosravi *et al.*, 1990; Khosla and Bailey, 1988; Kallio *et al.*, 1996).

An attempt was made to express SAK in recombinant *E. coli* cells expressing VHB. The strategy followed is shown in Fig. 3.6. The pETSAK vector expressing SAK under the T7 promoter was digested with *EcoRI* and ligated with *EcoRI* digested pUCVGB vector expressing VHB under the native oxygen responsive promoter. Ligated product was transformed into *E. coli* JM109 and transformants were screened on LB plates containing kanamycin and ampicillin. All the clones carrying recombinant plasmid (pETpUCVSAK) could be recognized by red tinge imparted by VHB expression. Plasmids were isolated and transformed into *E. coli* BL21DE3 cells. Individual colonies were cultured in LB containing kanamycin and induced with IPTG when cells reached log phase (OD$_{600}$ = 0.3-0.5). SDS-PAGE (15%) analysis of cell lysate showed that SAK constitutes 20-25% of the total cell protein content (Fig. 3.3).

Growth profile of the cells at high and low aeration (200 rpm and 150 rpm respectively) indicates no major differences in the densities of cells co-expressing VHB and
Fig. 3.6 Cloning strategy for co-expression of SAK and VHb.
SAK in comparison to cells expressing only SAK. A significant increase in SAK expression (9.5 IU/µl) is observed upon VHb expression along with SAK (Table 3.1).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>High aeration (200 rpm)</th>
<th>Low aeration (150 rpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SAK</td>
<td>VHbSAK</td>
</tr>
<tr>
<td></td>
<td>OD₆₀₀ nm</td>
<td>IU/µl</td>
</tr>
<tr>
<td>3</td>
<td>1.0±0.05</td>
<td>3.4±0.10</td>
</tr>
<tr>
<td>10</td>
<td>1.7±0.06</td>
<td>6.9±0.13</td>
</tr>
<tr>
<td>20</td>
<td>2.1±0.04</td>
<td>3.9±0.09</td>
</tr>
</tbody>
</table>

Table 3.1 Comparative SAK activity of *E. coli* cells carrying pETpBSSAK and pETpUCVSASK at different time points. The results are mean±S.E.M of three determinations.

### 3.2 Purification and biochemical properties of recombinant SAK

#### 3.2.1 Purification of recombinant SAK

*E. coli* cells carrying recombinant plasmid for the expression of SAK were induced with 0.2 µM IPTG and harvested after 8-10 h of induction. Cells were sonicated and the cell lysate was used for two-step purification of SAK. Cell lysate was applied to SP-sepharose column and washed with 0.1 M NaCl, 0.01 M Phosphate buffer, pH 6.2 and eluted with a gradient of buffer from 0.1 M NaCl to 0.5 M NaCl during which SAK eluted at approximately 0.25 M NaCl. The pooled SAK fractions, identified with SDS-PAGE analysis, were adjusted to 2.5 M NaCl with solid NaCl and subjected to hydrophobic
interaction chromatography on Phenyl-sepharose column. After washing with equilibration buffer, SAK was eluted with 0.01 M phosphate buffer pH 6.2. Purified SAK migrates as a single band with an apparent molecular mass of 15.5 kDa on SDS-PAGE (Fig. 3.7a). Two-step purification results in recovery of nearly 40 mg protein per liter of culture.

### 3.2.2 Zymographic analysis

Biological activity of purified SAK was examined by zymography as mentioned in methods. Appearance of clear zone (Fig. 3.7b) corresponding to the bands obtained from SDS-PAGE of purified SAK clearly shows that SAK is functionally active.

### 3.2.3 Specific activity of recombinant SAK

Purified SAK was quantitated by using Bradford’s reagent and its specific Pg activator activity was determined. Specific activity of recombinant SAK is found to be comparable (1.6 IU/µg of protein) to the standard SAK preparation isolated from its natural host (a gift from Professor Patrick J. Gaffney, National Institute of Biological Standards and Control, South Mimms, Herts., U. K.).

### 3.2.4 Temperature stability of recombinant SAK

Thermostability of recombinant SAK was determined by incubating 0.05 mg/ml of purified SAK preparation at 25°C, 37°C and 65°C. Samples were taken out at different time points and checked for Pg activation property. Recombinant SAK appears to be quite stable at 25°C and 37°C (Table 3.2). It fully retains its Pg activation property even after 10 h incubation at 25°C. Upon incubation at 37°C nearly 20% reduction is observed. Pg activation activity of SAK reduces rapidly when incubated at 65°C.
Fig. 3.7a) **Purification of SAK.** Lane 1: Protein molecular weight marker; Lane 2: *E. coli* BL21DE3 pET; Lane 3: *E. coli* BL21DE3 pETpBSSAK; Lane 4: purified SAK.

b) **Zymography of purified SAK.** Lane 1: *E. coli* BL21DE3 pETpBSSAK; Lane 2: purified SAK. The arrow indicates the clearance zone on substrate plate.
Table 3.2 Thermostability of purified SAK. The results are mean±S.E.M of three determinations.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Percentage residual activity at different time points</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td>25°C</td>
<td>100±9</td>
</tr>
<tr>
<td>37°C</td>
<td>100±12</td>
</tr>
<tr>
<td>65°C</td>
<td>100±8</td>
</tr>
</tbody>
</table>

3.3 Expression of SAK in eucaryotic host, Pichia pastoris

Among eucaryotic host systems, Pichia pastoris is the most widely utilized production host for the expression of heterologous proteins. As compared to its counterpart, Saccharomyces cerevisiae, P. pastoris has many advantages for protein expression and secretion due to availability of strongly regulated promotors and ease to secrete protein in culture medium. Cloned gene can be stably integrated into the genome of P. pastoris. It is faster, easier and less expensive system to use than any other eucaryotic expression systems such as baculovirus and mammalian tissue culture and generally reaches to higher biomass, resulting in higher recovery of cloned product. From an expression system perspective, the most interesting of these is alcohol oxidase (AOX1), the first enzyme in the methanol utilization pathway. AOX in undetectable in cells cultured on carbon sources such as glucose, glycerol or ethanol, but constitutes up to 30% of the total soluble protein in methanol grown cells. It was anticipated that AOX synthesis would be regulated at the transcriptional level and that the promoter from this gene would be most...
useful for controlling the expression of foreign genes. It secretes very low level of native proteins, thus secretion of foreign protein becomes an effective purification step.

These advantages prompted us to select *P. pastoris* (strain GS115) as a unicellular eucaryotic host for the expression of SAK. Extracellular expression of SAK in *P. pastoris* resulted in highly attenuated Pg activator activity due to glycosylation of Asn$_{28}$ of recombinant SAK. An attempt was made for the intracellular expression of SAK in *P. pastoris*.

### 3.3.1 Cloning of sak gene in expression vector pPIC3

For the expression of SAK in *P. pastoris*, sak gene was cloned in expression vector pPIC3, which contains AOX1 promoter. An amplified product from the plasmid pBSSAK using primers, PSAKNT and PSAKCT was used as the source of sak gene. The PCR product was digested with BamHI and EcoRI and ligated with BamHI and EcoRI digested pPIC3 vector (Fig. 3.8). Ligation mix was transformed into *E. coli* JM109. Transformants were screened for insert by restriction enzyme digestion and further confirmed by nucleotide sequence analysis. Positive clone designated as pPIC3SAK, was used for further studies.

### 3.3.2 Integration of recombinant pPIC3SAK plasmid into the genome of *P. pastoris*

To achieve maximum stability of sak gene in *P. pastoris*, recombinant plasmid pPIC3SAK was integrated into genome of *P. pastoris* by homologous recombination between transformed DNA and region of homology within the genome (Fig. 3.8). Integration was targeted by digesting the plasmid pPIC3SAK with SacI prior to transformation. Linearized DNA was electrotransformed in the spheroplasts of *P. pastoris* strain GS115, as elaborated in the methods. Transformants were screened by histidine prototrophy (HIS+) on MD plates. Clones selected were further confirmed for the
Fig. 3.8 Strategy for cloning and integration of sak gene in *P. pastoris* genome.
integration of *sak* gene through PCR in which chromosomal DNA of clones were annealed to the primers specific to *sak* gene.

### 3.3.3 Selection of recombinant clones for SAK expression

Recombinant clones were further evaluated for SAK production. Single colony was inoculated in BMGY medium and allowed to grow at 30°C for approximately two days till the OD$_{600}$ reached to 1.0. Cells were harvested and pellet was resuspended in 50 ml of BMMY medium for induction and culture was again shifted to 30°C with vigorous shaking. Induction condition was maintained by adding 0.5% methanol after every 24 h. Samples were taken out at regular intervals and cell lysate was assayed for SAK activity up to 120 h after induction. Clonal variation was observed when screened 18 clones with respect to SAK production (Table 3.3). Clone number 8, designated GS115(SAK8), was finally selected based on its high productivity when induced.

<table>
<thead>
<tr>
<th>Clone number</th>
<th>SAK activity IU/ml</th>
<th>Clone number</th>
<th>SAK activity IU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>71±3</td>
<td>10</td>
<td>40±3</td>
</tr>
<tr>
<td>2</td>
<td>65±6</td>
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</tr>
<tr>
<td>9</td>
<td>65±5</td>
<td>18</td>
<td>52±4</td>
</tr>
</tbody>
</table>

**Table 3.3 Intracellular SAK activity in various *sak* positive *P. pastoris* clones.**

The results are mean±S.E.M of three determinations.
3.3.4 Pattern of recombinant SAK production in P. pastoris

Intracellular protein sample was obtained by cell lysis of induced culture of GS115(SAK8). Although Pg activation activity is observed in cell lysate but the level of SAK expression remains very low and no distinct protein band corresponding to SAK is observed on 15% SDS-PAGE analysis of cell lysate (Fig. 3.9a). Expression of SAK in P. pastoris was confirmed by Western blot analysis of cell lysate using polyclonal antibodies against SAK (Fig. 3.9b).

Discussion

Identification of fibrin-specific thrombolytic activity of SAK in human plasma and its potency similar to widely utilized thrombolytic agent, SK, in recent clinical trials has indicated that SAK may be an ideal candidate for developing new clot-dissolving agent of future. Structure-function studies of SAK require large amounts of purified protein and also a system that can be easily utilized for generating desired variants of SAK. We initiated with the aim to develop a system, which would produce intact, biologically active SAK. Gene encoding SAK was cloned and expressed under the control of its natural promoter and translation signal in B. subtilis or E. coli, resulting in accumulation of the gene product in periplasmic space or in the culture medium (Sako and Tsuchida, 1983; Behnke and Gerlach et al., 1987; Collen et al., 1992a). However, the processing, translocation and stability of sak gene product in these expression systems were sub-optimal, resulting in low yields. To overcome these shortcomings of previous expression systems, attempts were made to look for the feasibility of various systems to produce sufficiently large amounts of biologically active SAK.

Three natural variants of SAK encoding gene (sakØC, sak42D, sakSTAR) have been characterized which differ at four nucleotide positions within the SAK coding region. Three of these differences represent amino acid exchanges corresponding to positions 34,
Fig. 3.9a) Intracellular expression of SAK in *P. pastoris*. Lane 1: Protein molecular weight marker; Lane 2: *P. pastoris*; Lane 3: *P. pastoris* carrying integrated *sak* gene; Lane 4: *E. coli* BL21DE3 pETpBSSAK. b) Western blot analysis of SAK expressed in *P. pastoris*. Lane 1: Overloaded *P. pastoris*; Lane 2: Overloaded *P. pastoris* carrying integrated *sak* gene; Lane 3: purified SAK. The arrow indicates the position of SAK.
36 and 43 of polypeptide chain. Wild-type SAK has sequence Gly\textsubscript{34}, Arg\textsubscript{36}, Arg\textsubscript{43} (sak\textsubscript{42D}), Ser\textsubscript{34}, Gly\textsubscript{36}, His\textsubscript{43} (sak\textsubscript{STAR}) or Gly\textsubscript{34}, Gly\textsubscript{36}, His\textsubscript{43} (sak\textsubscript{OC}). The sak gene isolated from the local isolate was identical to that present on S. aureus phage S\textsubscript{OC} (Sako and Tsuchida, 1983).

Intracellular expression of SAK under T7 promoter resulted in accumulation of sufficiently large quantities of SAK inside the cells that constitute nearly 20-25% of total cellular protein. Despite the high expression level of SAK in E. coli, almost 100% of SAK remained in soluble form. Purified SAK appeared as a single 15.5 kDa band without any degradation product. Zymographic analysis and Pg activation capability of purified SAK clearly indicated full retention of biological activity comparable to that of native SAK. In addition, purified SAK was quite stable at ambient temperature.

To omit the regular use of IPTG for protein induction, an alternative SAK expression system was generated with an objective to use this system for large-scale SAK production at high cell density culture. During the fermentation process for large-scale production of recombinant proteins, oxygen level of the growth condition rapidly falls below 5% of atmospheric oxygen. Reduced oxygen level in growth conditions automatically induces the OXY promoter resulting in high level expression of gene cloned under this promoter. Expression level of SAK under OXY promoter was comparable with SAK expression level under T7 promoter. Therefore, expression of SAK under OXY promoter could provide a comparatively less expensive system for SAK production.

Ability of VHb to increase bacterial growth and to increase production level of protein when expressed in heterologous host was exploited in order to attain high cell density during recombinant SAK expression in E. coli. Although there was no significant difference in cell densities upon co-expression of SAK and VHb, the level of SAK production was markedly increased (~30%) in comparison to E. coli expressing SAK in absence of VHb that was evident from Pg activation activity of cell lysate (Table 3.1). When oxygen supply was limited to cells co-expressing SAK and VHb, there was ~40% increase in Pg activation activity of SAK without any significant elevation in final cell density (Table 3.1). These observations indicated that co-expression of VHb facilitated
SAK accumulation in *E. coli* cells under limited as well as adequate oxygen supply conditions, the effect being more pronounced in the former.

*P. pastoris* has been utilized widely to produce large amounts of recombinant proteins including SK and has been shown to be the alternative system for high cell density fermentation. *P. pastoris* is a faster, easier and less expensive system to use when compared with other eucaryotic expression systems and it generally reaches to higher biomass resulting in higher recovery of the cloned product. Unlike *E. coli*, *P. pastoris* expression system is free from endotoxins, therefore, feasibility of *P. pastoris* to produce SAK was explored. Expression of SAK in *P. pastoris* was merely detectable on SDS-PAGE analysis, but its low level expression was confirmed by Western blot analysis and also by analyzing Pg activation activity of the yeast cell lysate. Low expression level of SAK might be attributed to premature transcriptional termination of integrated sak gene in *P. pastoris* due to its high AT content. Similar effect was observed when Scorer *et al.*, (1993) made an attempt to express HIV-1 gp120 in *P. pastoris*.

As the SAK expression under AOX promoter in *P. pastoris* was not sufficient for the purification of SAK on large-scale for structure-function studies, *E. coli* SAK expression system under T7 promoter was selected for further studies.

The intracellular expression of sak gene reported here allowed us to produce intact SAK in *E. coli*. As a result, high amount of soluble SAK was obtained which was amenable to a simple and straightforward purification scheme resulting in recovery of nearly 40 mg of SAK per liter of culture. Purified SAK fully retained its functional activity and was quite stable at ambient temperature. Expression of SAK under OXY promoter excluded the need for expensive inducer IPTG making the system economical for large-scale production. Co-expression of VHb facilitated the expression of SAK in *E. coli* and might be exploited further for its large-scale production. *P. pastoris* is one of the most economical eucaryotic system for large-scale production of recombinant proteins but this system failed to overexpress SAK. Therefore, *E. coli* system was selected for expression and purification of SAK and its variants for structure-function characterization.