Abstract
A glucose repression-free expression system that is induced without added inducers has been constructed for the overexpression of a thermophilic and saccharifying a-amylase enzyme produced by a local *Bacillus licheniformis* MSG isolated from hot springs in South Gujarat. The structural gene encoding this a-amylase without its 5' negative regulatory sequence was cloned in an *Escherichia coli* expression vector. The recombinant clones showed variations in activity at different temperatures. DNA sequencing of two clones revealed Taq DNA polymerase-induced errors in the amylase sequences. The two clones shared about 99% sequence identity with a hyperthermostable *B. licheniformis* a-amylase from Iran. The clone that showed higher thermostability and specific activity was subsequently subcloned under the phosphate starvation-inducible *Bacillus subtilis* pst promoter in plasmid pHpSt. This plasmid was constructed by replacing the *Lactococcus lactis* p59 constitutive promoter upstream of the lacZ reporter gene in pHPS9 (*E. coli-B. subtilis* shuttle vector), with the pst promoter that was engineered with a modified and consensus ribosome binding site (RBS) sequence at its 3' end. The pst promoter showed thirteen fold higher expression of β-galactosidase than the p59 promoter in *B. subtilis* 1A748 under conditions of Pi that induces pst.

The subcloning of thermophilic a-amylase under the pst promoter resulted in three independent constructs differing in the spacing between the RBS and the start codon by 9 bp, 13 bp and 641 bp in plasmids pSTamy9, pSTamy13 and pSTamy641 respectively. Comparative expression of thermophilic amylase from these constructs in *E. coli* showed that the pSTamy9 construct provided about 1.6- and 3- fold higher expression of amylase than pSTamy13 and pSTamy641 respectively, thereby demonstrating the importance of the RBS spacing for optimal translation. Subsequent expression of pSTamy9 in *Bacillus subtilis* showed on an average ~ 4-5 folds secretory expression in an eight protease-deficient host, *B. subtilis* WB800 as compared to the non-protease deficient *B. subtilis* 1A297. In *B. subtilis* WB800, expression of thermophilic a-amylase from the pst promoter was induced 500-fold. This expression from the pst promoter was not repressed by 0.5% glucose, at which concentration the expression from the parent *B. licheniformis* MSG was completely repressed. Amylase expression from the pst promoter occurred when the Pi levels in the medium fell to < 0.1 mM. The expression of the cloned enzyme increased progressively with increasing concentration of glucose due to faster depletion of Pi resulting from an increased growth, and at 2% concentration, the specific activity of amylase increased 165-fold compared to that in the parent strain. The maximum volumetric production under this condition was 5670 U/ml which was ~ 590-times that from the parent culture in the presence of starch. The high level of expression also resulted in high molecular weight aggregates that were still active as observed on SDS-PAGE. The recombinant a-amylase showed a molecular weight of about 55, 000 which matched with that from the parent strain and was found to be highly thermostable and produced maltotriose, maltotetraose and maltopentaose from starch.