Summary and Conclusion
Bacillus species have been widely used for commercial production of thermostable amylases but the synthesis of α-amylase in B. licheniformis and B. subtilis is repressed 10-15 fold in the presence of glucose and this phenomenon of catabolite repression (CR) is an important concern in large-scale industrial productions. Catabolite repression of α-amylase in B. licheniformis is mediated by the binding of the \(\text{trans}\) regulatory CcpA dimer to a \(\text{cis}\) cre operator sequence lying immediately upstream, but partially overlapping the structural gene. Although mutagenesis of \(\text{cis}\) regulatory sequences, solid state fermentation and cloning of amylases have been used to improve yield, the increase in fold production of amylase has not been significant.

An industrially competent, thermophilic, maltose-producing and cyclodextrin-hydrolyzing α-amylase with optimum activity at 94 °C and a wide pH range of 5-9.5 is produced by B. licheniformis MSG that was isolated from hot springs in South Gujarat. The structural gene encoding this B. licheniformis α-amylase (BLA) was amplified without its 5' negative regulatory sequence using Taq DNA polymerase and the PCR product that hybridized with an amylase-specific probe was then cloned into an E. coli expression vector. The recombinant cloned amylases showed variations in activity, thermostability and restriction profiles and this was attributed to Taq-induced errors in their sequences. DNA sequencing of three clones revealed that two were α-amylases that had three amino acid mismatches between themselves and shared 99.4 % identity with each other, while the third was not an amylase. The two amylases were found to show ~ 99 % identity with a hyperthermostable B. licheniformis α-amylase from Iran (GenBank Ac. No. AF438149). A clone that showed higher thermostability and specific activity was used in subsequent experiments for over expression. The GenBank accession number of this thermophilic α-amylase is GQ262779.

The strong, tightly regulated phosphate starvation-inducible \(\text{pst}\) promoter of Bacillus subtilis that is not repressed by glucose was chosen to over express B. licheniformis MSG α-amylase since this enzyme has a longer shelf-life in media or buffers having limited phosphate. The \(\text{pst}\) promoter was amplified such that it carried a primer-generated,
modified, strong ribosome binding site (RBS) at its 3' end. It was then used to replace the original \textit{Lactococcus lactis} \textit{p59} promoter in pHPS9 (\textit{E. coli- B. subtilis} shuttle vector) to generate a new expression vector, pHPst. A comparative analysis of promoter strengths in a \textit{Bacillus} background using β-galactosidase as the reporter gene demonstrated that the \textit{pst} promoter was induced about 1180-fold in medium containing initial phosphate (Pi) concentration of 0.42 mM Pi, and provided about 13-fold higher expression than the strong, constitutive \textit{p59} promoter.

The subcloning of the amylase gene downstream the \textit{pst} promoter was carried out in two steps to attain an RBS spacing of about 9 bp between the RBS and the start codon of amylase for optimal translation resulting in the construct pSTamy9. During the process two other constructs, pSTamy13 and pSTamy641 in which the RBS spacings were 13 bp and 641 bp respectively were also generated. The pSTamy9 construct produced about 1.6- and 3-fold higher expression of amylase in \textit{E. coli} compared to pSTamy13 and pSTamy641, respectively, thereby demonstrating the significance of the RBS spacing for high-level production.

The pSTamy9 construct was thus used to over express thermophilic α-amylase in \textit{B. subtilis} WB800 which is deficient in eight extracellular proteases, since even the robust \textit{B. licheniformis} α-amylase is considerably degraded by proteases in \textit{B. subtilis}. The yield of thermophilic α-amylase (BLA.MSG) from the protease-deficient \textit{B. subtilis} WB800 was on an average about 4-5 folds higher than that produced by \textit{B. subtilis} 1A297. Determination of inorganic phosphate (Pi) levels in the medium during growth revealed that expression of amylase from the \textit{pst} promoter and the alkaline phosphatase, a natural reporter for phosphate starvation, occurred when the Pi level fell below 0.1 mM.

The expression of the cloned thermophilic α-amylase from the \textit{pst} promoter in \textit{B. subtilis} WB800 (pSTamy9) in submerged culture conditions increased with increasing concentration of glucose (0.2 % to 2 %), whereas the expression of the enzyme from the parental culture was completely repressed by 0.5 % glucose. This clearly demonstrated that catabolite repression of the cloned enzyme was abolished. Further, estimation of Pi level showed that the enhancement of amylase expression by glucose occurred due to faster depletion of Pi resulting from increased growth. An overall ~165-fold increase in α-
amylase specific activity was achieved through this expression system compared to the parental *B. licheniformis* strain under starch induction. In terms of volumetric productivity, about 5670 U/ml of amylase was produced by the recombinant culture in the presence of 2% glucose after 40 h, while the parent culture yielded only 9.6 U/ml under starch induction after a similar time period. This represents about 590-fold more amylase than that produced by the parent strain.

The approximate molecular mass of the recombinant α-amylase was observed to be ~55 kDa on SDS-PAGE which matched with the molecular weight of the enzyme from the parental culture, suggesting that the *B. licheniformis* α-amylase signal peptide was efficiently processed in *B. subtilis*. Further, the enzyme was found to be extremely thermostable and retained activity even after boiling for 10 minutes in the presence of SDS. As observed on SDS-PAGE, the overexpressed enzyme tended to form active high molecular weight aggregates which were difficult to solubilize. Also the crude enzyme seen as an activity stained band on SDS-PAGE was not stained with silver nitrate or Coomassie blue. The recombinant α-amylase hydrolyzed starch to produce maltotriose (G3), maltotetraose (G4) and maltopentaose (G5) with traces of maltose (G2) but did not hydrolyze cyclodextrin, while the enzyme sample from the parental culture hydrolyzed starch to give G3 in higher amounts, G2, G4 and G5, and also hydrolyzed cyclodextrin to give maltose.

Thus, in the present study, a strong, glucose-activated expression system was developed for the over production of a thermophilic, maltogenic amylase that produces linear oligosaccharides of 3–5 glucose residues. It is anticipated that the high levels of amylase production as well as the potential industrial applications of this enzyme would make it attractive for commercialization in the local industries.