Enhanced Production of Thermostable α-Amylase in Bacillus subtilis

Plasmid construction

The B6-1-5-amyL vector was specifically engineered to express thermostable α-amylase in *Bacillus subtilis* 168 *htr9* (Figure 1S) This dual-host design supports efficient cloning, selection, and expression across bacterial strains, making it highly effective for amylase production studies. This engineered vector system demonstrates the versatility and efficiency of dual-host design in biotechnological applications. The thermostable α-amylase expressed by this vector could potentially enhance industrial processes that require high-temperature enzymatic activity. Furthermore, the ability to clone, select, and express genes in different bacterial strains offers researchers greater flexibility in optimizing amylase production for various experimental and industrial needs.

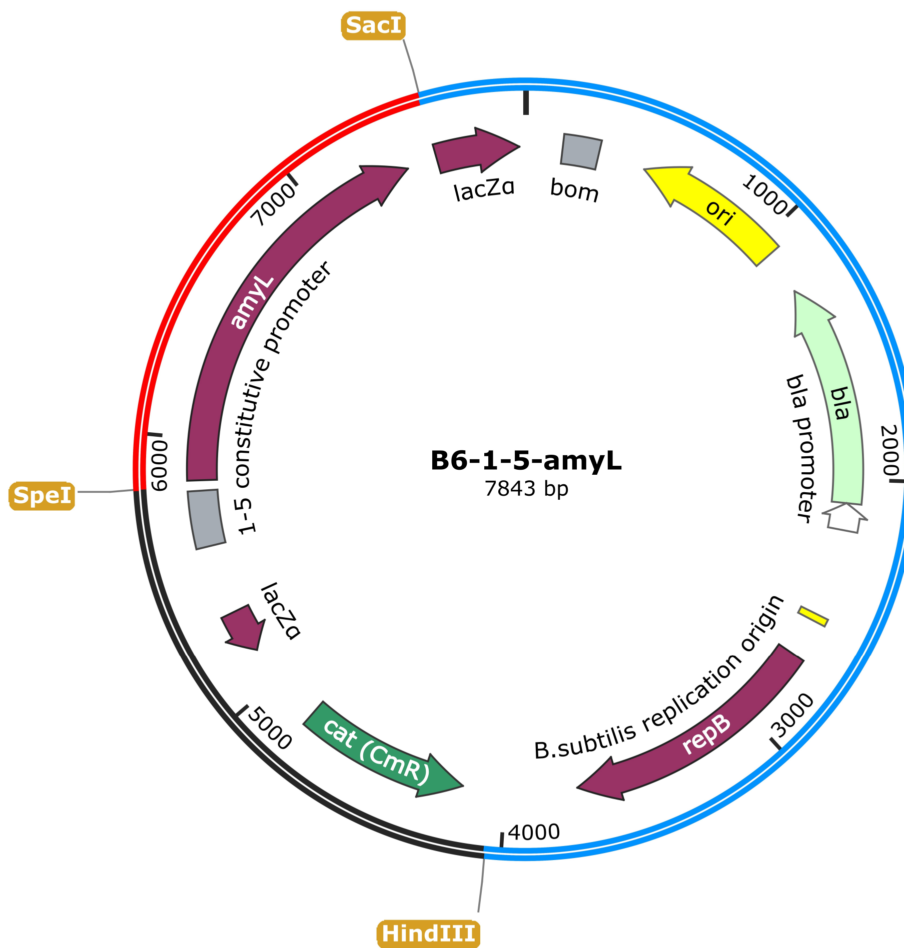

Figure 1S. B6-1-5-amyL shuttle expression vector map, illustrating the cloned *Bacillus licheniformis* 104.K α-amylase (*amyL*) gene under the control of a *1-5* constitutive promoter. The vector includes *chloramphenicol* and *ampicillin* resistance gene for selection, and origins of replication for both *E. coli* and *B. subtilis*. The construct was designed using SnapGene software.

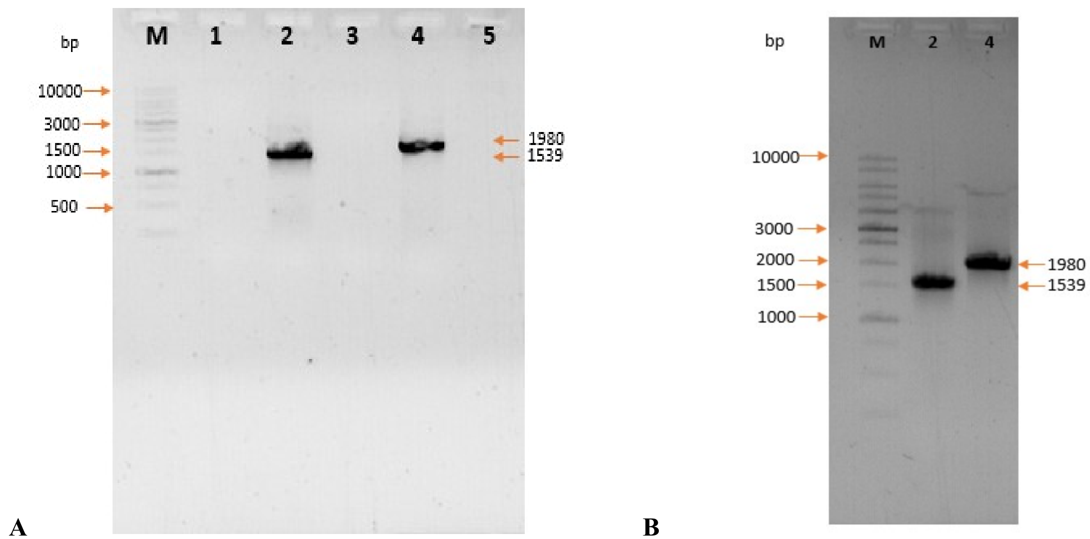

Figure 2S. (**A**) 0.8% agarose gel electrophoresis of PCR products amplified using specific primers targeting the α-amylase (*amyL*) gene (see manuscript Table 1). Genomic DNA from *B. licheniformis* 104.K and *B. velezensis* amyR strain were used as templates in all PCR reactions. Lane M: 1 kb DNA ladder (EVROGEN). Lane 1: PCR product with *B. amyloliquefaciens*-specific primer. Lane 2: PCR product with *B. licheniformis*-specific primer, showing a ~1,539 bp fragment. Lane 3: PCR product with *B. subtilis*-specific primer. Lane 4: PCR product with *B. velezensis*-specific primer, showing a ~1,980 bp fragment. Lane 5: PCR product with a second *B. velezensis*-specific primer. (**B**) 0.8% agarose gel electrophoresis of PCR products amplified using specific primers. Lane M: 1 kb DNA ladder (EVROGEN). Lane 2: PCR product with *B. licheniformis*-specific primer, showing a ~1,539 bp fragment. Lane 4: PCR product with *B. velezensis*-specific primer, showing a ~1,980 bp fragment.

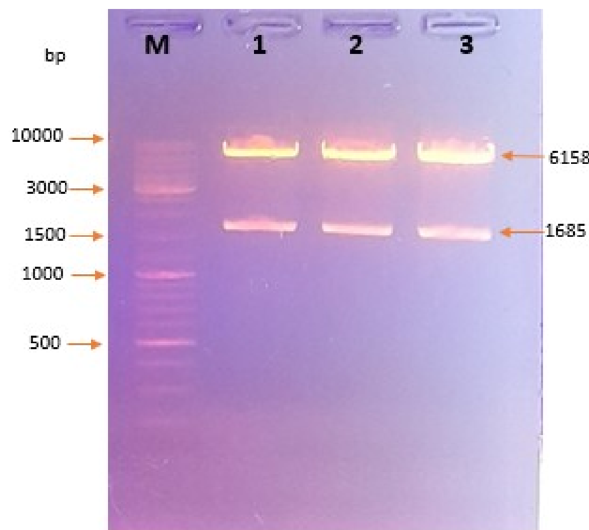

Figure 3S. Restriction digestion analysis of the B6-1-5-amyL vector construct. Plasmids extracted from three positive colonies (Lanes 1, 2, and 3) were digested with *SacI* and *SpeI* enzymes, yielding two fragments: a 1,685 bp *amyL* gene fragment and a 6,158 bp vector backbone. Lane M: GeneRuler 1 kb DNA ladder (Thermo Scientific).

Bacterial Strains

In this study on the expression of thermostable α-amylase, we utilized various mutant strains of *Bacillus subtilis* 168. These strains were designed by Alexandr V. Kachan and Anatoly N. Evtushenkov. We attempted to express recombinant amylase in the following mutant strains: *B. subtilis* 168 *htr9*, *B. subtilis* 168 *ehp241*, *B. subtilis* 168 *cst10*, *B. subtilis* 168 *dlt46* and *B. subtilis* 168 *esc11* (Table 1S).

Table 1S. Bacterial strains used for the cloning and expression of thermostable α-amylase enzyme.

Bacteria name	***Description of mutation***	***Source***
B. subtilis 168 htr9 (WT)	Deletion of *amyE* gene	This work
B. subtilis 168 htr9 ehp241	Deletion of *amyE* gene with insertion of hybrid *prsA* gene	This work
B. subtilis 168 htr9 cst10	Deletion of *amyE* gene with inactivation of *sccRS* operon	This work
B. subtilis 168 htr9 dlt46	*Deletion of amyE gene with inactivation of dltD operon*	This work
B. subtilis 168 htr9 esc11	*Deletion of amyE gene with promoter of cssRS operon replaced with strong promoter (Pspac)*	This work
E. coli TOP10	*F⁻ mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74deoR recA1 endA1 araΔ139 Δ(ara, leu)7697 galU galK λ⁻ rpsL(Str^R) nupG*	Invitrogen, USA