1. Plasmid Construction

All primers are listed in Supplementary Table S1, and all constructed plasmid are listed in Table 1.

For marker-free gene deletion, a series of plasmids were constructed. The vector pD-sacB-*ldh* was constructed for *ldh* deletion. The upstream and downstream fragments (*ldh*-F and *ldh*-B, forward and b*ack*ward fragments) were amplified from the *C. glutamicum* genome using primers D-*ldh*-FU/L and D-*ldh*-BU/L respectively. The two fragments were fused and amplified by fusion PCR with primers D-*ldh*-FU / D-*ldh*-BL. The fused fragment (*ldh*-FB) was digested with *Sal*I and *Hind*III, and then ligated into the same digested sites of pD-sacB to create pD-sacB-*ldh*. With a similar procedure, pD-sacB-*pta-ack*, and pD-sacB-*nagD* were also constructed. The fused fragments *pta-ack*-FB and *nagD*-FB were amplified with corresponding primers from the *C. glutamicum* 13032 chromosomal DNA. Digested with *Xba*I-*Sal*I*, and BamH*I-*Hind*III, these three fragments were ligated into the corresponding sites of digested pD-sacB to yield plasmid pD-sacB-*pta-ack* and pD-sacB-*nagD*, respectively.

For *alsS* and alsD gene overexpression in the *C. glutamicum* genome, the vector pD-*sacB-pta-ack-alsSD* was constructed. The strong promoter *tuf* was amplified from *C. glutamicum* 13032 by primers O-tuf-U/L, and the *alsS* and *alsD* genes were amplified together from the plasmid pECXK99E-alsSD by primers O-*alsSD*-U/L. These two fragments were fused by primers O-tuf-U/ O-*alsSD*-L, digested with *XmaJ*I-*Xho*I, then placed between the *pta* and *ack* fragments digested the corresponding sites in pD-sacB-*pta-ack*, generating pD-sacB-*pta-ack-alsSD*.

For *ppc* and *pyc* deletion by single crossing event, the pHY300-*ppc* and pHY300-*pyc* plasmids were constructed. The fragment of the *ppc* and *pyc* coding gene was amplified by primers *ppc*-F/L and *pyc*-F/L, respectively. The two fragments were both digested with *Hind*III-*Sal*I, and ligated to the matching sites of pHY300, yielding pHY300-*ppc* and pHY300-*pyc*, respectively.

The shuttle vector pEC-XK99E-*alsSD*-Δ*lacIq* was constructed for *alsS* and *alsD* overexpression without addition of isopropyl β-D-1-thiogalactopyranoside (IPTG). The

liner fragment of was amplified from vector pEC-XK99E-*alsSD* with the primers pEC-lacIq-F/L, then was digested with *Spe*I and ligated itself to yield pEC-XK99E-*alsSD*-∆*lacIq*.

2. Strains Construction

All constructed strains are listed in Table 1.

The vector pD-sacB-*butA* was integrated into *C. glutamicum* ATCC 13032 chromosome by the first single-crossover recombination and kanamycin-resistant transformants were selected and verified by PCR. Next, the resulting transformant was cultured in LB liquid medium for 12h, and then the cells were spread on a LB-sucrose agar plate. The *butA*-deleted strain, denoted as CGF0, was selected from the grown colonies by PCR verification using primers D-*butA*-FU/BL. With a similar procedure above, the *pta* and *ack* genes could be knocked out together using pD-sacB-*pta-ack* verified by primers D-*pta-ack*-FU/BL, and the *ldh* gene could be deleted using pD-sacB-*ldh* verified by primers D-*pta-ack*-FU/BL. The *pta-ack* and *ldh* were deleted in *C. glutamicum* ATCC 13032 step by step, generating CGF1. The *butA* gene was then deleted in CGF1, yielding CGF2.

The shuttle vector pEC-XK99E-*alsSD* was introduced into *C. glutamicum* ATCC13032, CGF0, CGF1 and CGF2, yielding CGF3, CGF4 CGF5 and CGF6, respectively.

The transformant CGR3 was obtained by introducing the plasmid pEC-XK99E-*alsSD*-∆*lacIq* into CGF2. The *alsSD* operon was integrated into the chromosome of CGF2 using the same two step single crossing event procedure at the *pta-ack* locus and verified by primers D-*pta-ack*-FU/BL, yielding CGR4. CGR5 was generated by introducing pEC-XK99E-*alsSD*-∆*lacIq* into CGR4, and CGR6 was obtained by deleting *nagD* in CGR4 using pD-sacB-*nagD*, and verified by primers D-*nagD*-FU/BL. With introducing pEC-XK99E-*alsSD*-∆*lacIq* into CGR6, CGR7 was generated.

The vector pHY300-*ppc* and pHY300-*pyc* was integrated into the chromosome of CGR6 by single crossing event, and the *ppc* and *pyc* deleted transformants were obtained on tetracycline-resistance agar plate, and verified by primer sets D-In*ppc*-FU/BL and D-In*pyc*-FU/BL, respectively. Then pEC-XK99E-*alsSD*-∆*lacIq* was

introduced into the transformants to genernate CGR9 and CGR10, respectively.