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 backward 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 SalI and HindIII, 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 XbaI-Sall, and BamHI- HindIII, 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 XmaI- Xhol, 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 HindIII-Sall, and ligated to the matching sites of pHY300, yielding pHY300-ppc and pHY300-pyc, respectively.

The shuttle vector pEC-XK99E-alsSD-ΔlacIq was constructed for alsS and alsD overexpression without addition of isopropyl β-D-1-thiogalactopyranoside (IPTG).
liner fragment of was amplified from vector pEC-XK99E-alsSD with the primers pEC-lacIq-F/L, then was digested with SpeI and ligated itself to yield pEC-XK99E-alsSD-ΔlacIq.

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-ΔlacIq 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-ΔlacIq 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-ΔlacIq 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-Inppc-FU/BL and D-Inpyc-FU/BL, respectively. Then pEC-XK99E-alsSD-ΔlacIq was
introduced into the transformants to generate CGR9 and CGR10, respectively.