Supplementary Table S1. Primers for making and verification of DNA cassette and DNA deletions

|  |  |  |
| --- | --- | --- |
| Name  | Sequences 5’ – 3’ | Source |
| aceF\_PCR\_for | GCGAGATGGTCAGGATTGTGAACG | This study |
| aceF\_PCR\_rev | CCTAAATCAGCGCAACGGAAGG | This study |
| mdh\_PCR\_for | AGTCACCCGATATGGTGGTTG | This study |
| mdh\_PCR\_rev | ATGGATAAGCTGATCCGCGATG | This study |
| aceF\_Rec\_arm1\_for | GACCGATTAATTAACTCCGGCAGTGGCATCTACCGACTATATG | This study |
| aceF\_Rec\_arm1\_rev | GACCGAGGATCCCCGGCTCTTTTACTTACATCACCAGTGTCCGGTACTTTGATTTCGATAG | This study |
| acef\_Rec\_arm2\_for | GACCGAGAGCTCGGCTATCGAAATCAAAGTACCGGACACTGGTGATGTAAGTAAAAGAGCCGG | This study |
| aceF\_Rec\_arm2\_rev | GACCGAACTAGTCGCAGCGGCGTTAGCTTCAC | This study |
| mdh\_Rec\_arm1\_for | GACCGATTAATTAAATTGAGAAACATGCCTGCGTCACGG | This study |
| mdh\_Rec\_arm1\_rev | GACCGAGGATCCCTTATTAACGAACTCTTCGCCCAGGTTTTAACAGTAGTGCAAGCGCCTGG | This study |
| mdh\_Rec\_arm2\_for | GACCGAGAGCTCCCAGGCGCTTGCACTACTGTTAAAACCTGGGCGAAGAGTTCGTTAATAAG | This study |
| mdh\_Rec\_arm2\_rev | GACCGAACTAGTGGGATCGTGGTTAATGAAGTGTCAC | This study |

Supplementary Table S2. DNA used in this work

|  |  |  |
| --- | --- | --- |
| DNA  | Description - Sequences 5’ – 3’ | Source |
| *Xyl*S activator, *Pm* promoter | GGAGTGCCCCTTTGGTCGAAAAAAAAAGCCCGCACTGTCAGGTGCGGGCTTTTTTCTGTGTTTCCACGTTCGTAATCAAGCCACTTCCTTTTTGCATTGACGCAGGGTGTCGGAAGGCAACTCGCCGAACGCGCTCCTATAGTTTTCAGCGAAGCGTCCCAAATGTAAGAAGCCGTAGTCTAGGGCTATCTCAGTTATACTACGCACATTGGCACTGGGATCGTTCAAGCAGGCGCGGATGCTTTCGAGCTTGCGGTTGCGGATGTAGTTCTTCGGCGTGGTGCCGGCGTGCTTCTCGAACAAATTGTAGAGCGAGCGTGGACTCATCATCGCCAGCTCCGCTAACCGCTCAAGGCTGATATTCCGTTTGAGATTCTCCTCAATGAATTGAACGACTCGCTCGAAAGACGGGTTACCTTTGCTGAAAATTTCACGGCTGACATTGCTGCCCAGCATTTCGAGCAGCTTGGAAGCGATGATCCCCGCATAGTGCTCTTGGACCCGAGGCATCGACTTTGTATGTTCCGCTTCGTCACAAACTAACCCGAGTAGATTGATAAAGCCATCGAGTTGCTGGAGATTGTGTCGCGCGGCGAAACGGATACCCTCCCTCGGCTTGTGCCAATTGTTGTCACTGCACGCCCGATCAAGGACCACTGAGGGCAATTTAACGATAAATTTCTCGCAATCTTCTGAATAGGTCAGGTCGGCTTGGTCATCCGGATTGAGCAGCAATAGTTCGCCCGGCGCAAAATAGTGCTCCTGGCCATGGCCACGCCACAGGCAATGGCCTTTGAGTATTATTTGCAGATGATAACAGGTTTCTAATCCAGGCGAGATTACCCTCACGCTACCGCCGTAGCTGATTCGACACAGATCGAGGCATCCGAAGATTCTGTGGTGCAGCCTGCCTGCCGGGCGCCCGCCCTTGGGCAGGCGAATAGAGTGCGTACCGACATACTGGTTAACATAATCGGAGACTGCATAGGGCTCGGCGTGGACGAAGATCTGACTTTTCTCGTTCAATAAGCAAAAATCCATAGTTCACGGTTCTCTTATTTTAATGTGGGCTGCTTGGTGTGATGTAGAAAGGCGCCAAGTCGATGAAAATGCATCTCGACGTGATGCGTATACGGGTTACCCCCATTGCCACGTTGCGCCATCCTTTTTGCAATCAGTGACCACTTTTCCAAGCAAAAATAACGCCAAGCAGAACGAACACGTTCTTTTTAAGAAGCGAGAACACCAGAAGTTCGTGCTGTCGGGGCATGGGGCGACGAATTGGCGGATAAAGGGGATCTGCTGGATATTACGGCCTTTTTAAAGACCGTAAAGAAAAATAAGCACAAGTTTTATCCGGCCTTTATTCACATTCTTGCCCGCCTGATGAATGCTCATCCGTAATTACGTATGGCAATGAAAGACGGTGAGCTGGTGATATGGGATAGTGTTCACCCTTGTTACACCGTTTTCCATGAGCAAACTGAAACGTTTTCATCGCTCTGGAGTGAATACCACGACGATTTCCGGCAGTTTCTACACATATATTCGCAAGATGTGGCGTGTTACGGTGAAAACCTGGCCTATTTCCCTAAAGGGTTTATTGAGAATATGTTTTTCGTCTCAGCCAATCCCTGGGTGAGTTTCACCAGTTTTGATTTAAACGTGGCCAATATGGACAACTTCTTCGCCCCCGTTTTCACCATGCATGGGAATTAGCTTGATCTGACCAACGACCGGTAGCGGAGCTATCCAACGGCGGTATACCAGGAAAACACACAGCAGGTACATCAGAACAGTACCATGACTGAAGAACAAATAGTTTTTTCCTGATCCATAAAGCAGAACGGCCTGCTCCATGACAAATCTGGCTCCCCAACTAATGCCCCATGCAGCCAGCATAACCAGCATAAAGGCAAGGAGTGCAGTGTCCGGTTTGATAGGGATAAGTCCAGCCTTGCAAGAAGCGGATACAGGAGTGCAAAAAATGGCTATCTCTAGTAAGGCCTACCCCTTAGGCTTTATGCAA | This study |
| *als*S | Gen extracted for PCR from *E. coli* W-pIZIbPSO | (Felpeto-Santero et al., 2015) |
| *ilv*D | Gen extracted for PCR from *E. coli* W-pIZIbPSO | (Felpeto-Santero et al., 2015) |
| *ilv*C | Gen extracted for PCR from *E. coli* W-pIZIbPSO | (Felpeto-Santero et al., 2015) |
| RBS-std | 1  | (Nogales et al., 2011) |
| T1 Terminator | GCTTCTTGGACTCCTGTTGATAGATCCAGTAATGACCTCAGAACTCCATCTGGATTTGTTCAGAACGCTCGGTTGCCGCCGGGCGTTTTTTATTGGTGAGAATCCAGCGCT | This study |
| BBa B1006 Terminator | AAAAAAAAACCCCGCCCCTGACAGGGCGGGGTTTTTTTT | http://parts.igem.org/Part:BBa\_B1006 |
| Linker Terminator 1 | TCCCAGACCCACCTT | This study |
| Linker Terminator 3 | CACGGGCGGTAGCAG | This study |
| LinkerPromoter 2 | AAACTCAGTTGTAGT | This study |
| LinkerPromoter 4 | GGAGCCCCTGGCGCCCCTT | This study |
| Host plasmids |
| Lv1 Host vector | pSEVA23g19[g1] - Fusion sites 1AI2 | (Blázquez et al., 2022) |
| pSEVA23g19[g2] - Fusion sites 2AI3  |
| pSEVA23g19[g3] - Fusion sites 3AI4 |
| pSEVA23g19[g4] - Fusion sites 4AI5 |

Supplementary Table S3. designed DNA cassettes

|  |  |  |
| --- | --- | --- |
| Name  | Sequences 5’ – 3’ | Source |
| Rec\_arm1\_aceF | CTCCGGCAGTGGCATCTACCGACTATATGAAACTGTTCGCTGAGCAGGTCCGTACTTACGTACCGGCTGACGACTACCGCGTACTGGGTACTGATGGCTTCGGTCGTTCCGACAGCCGTGAGAACCTGCGTCACCACTTCGAAGTTGATGCTTCCTACGTGGTTGTAGCGGCGCTGGGCGAACTGGCTAAACGTGGCGAAATCGATAAGAAAGTGGTTGCTGACGCAATCGCCAAATTCAACATCGATGCAGATAAAGTTAACCCGCGTCTGGCGTAAGAGGTAAAAGAATAATGGCTATCGAAATCAAAGTACCGGACACTGGTGATGTAAGTAAAAGAGCCGG | This study |
| Rec\_arm2\_aceF | GGCTATCGAAATCAAAGTACCGGACACTGGTGATGTAAGTAAAAGAGCCGGCCCAACGGCCGGCTTTTTTCTGGTAATCTCATGAATGTATTGAGGTTATTAGCGAATAGACAAATCGGTTGCCGTTTGTTGTTTAAAAATTGTTAACAATTTTGTAAAATACCGACGGATAGAACGACCCGGTGGTGGTTAGGGTATTACTTCACATACCCTATGGATTTCTGGGTGCAGCAAGGTAGCAAGCGCCAGAATCCCCAGGAGCTTACATAAGTAAGTGACTGGGGTGAGGGCGTGAAGCTAACGCCGCTGCG | This study |
| Rec\_arm1\_mdh | ATTGAGAAACATGCCTGCGTCACGGCATGCAAATTCTGCTTAAAAGTAAATTAATTGTTATCAAATTGATGTTGTTTTGGCTGAACGGTAGGGTATATTGTCACCACCTGTTGGAATGTTGCGCTAATGCATAAGCGACTGTTAATTACGTAAGTTAGGTTCCTGATTACGGCAATTAAATGCATAAACGCTAAACTTGCGTGACTACACATTCTTGAGATGTGGTCATTGTAAACGGCAATTTTGTGGATTAAGGTCGCGGCAGCGGAGCAACATATCTTAGTTTATCAATATAATAAGGAGTTTAGGATGAAAGTCGCAGTCCTCGGCGCTGCTGGCGGTATTGGCCAGGCGCTTGCACTACTGTTAAAACCTGGGCGAAGAGTTCGTTAATAAG | This study |
| Rec\_arm2\_mdh | CCAGGCGCTTGCACTACTGTTAAAACCTGGGCGAAGAGTTCGTTAATAAGTAATTAATTAGCGAATAATAAAAAACCGGAGCACAGACTCCGGTTTTTTGTTTTGAGCACTCGACTTAATTGGTTGCCGGATATTCCTGAATGGTGACCTGCAGCGTTAACTGCTTATCATCACGCATCACTACAACCGGGATCACCGAACCAGGGCGAATTTCTGCCACCTGATCCATCGTCTCCAGAGCAGAGATGGCCGGTTTGTTATCCACCGAAATAATCAGATCGTTGACCTGAATACCCGCATTCGCCGCCGGGCCGTCAGGTGACACTTCATTAACCACGATCCC | This study |
| Rec\_kanamycin | GGATCCGCCCTGCTTAGAAAAACTCATCGAGCATCAAATGAAACTGCAATTTATTCATATCAGGATTATCAATACCATATTTTTGAAAAAGCCGTTTCTGTAATGAAGGAGAAAACTCACCGAGGCAGTTCCATAGGATGGCAAGATCCTGGTATCGGTCTGCGATTCCGACTCGTCCAACATCAATACAACCTATTAATTTCCCCTCGTCAAAAATAAGGTTATCAAGTGAGAAATCACCATGAGTGACGACTGAATCCGGTGAGAATGGCAAAAGCTTATGCATTTCTTTCCAGACTTGTTCAACAGGCCAGCCATTACGCTCGTCATCAAAATCACTCGCATCAACCAAACCGTTATTCATTCGTGATTGCGCCTGAGCGAGACGAAATACGCGATCGCTGTTAAAAGGACAATTACAAACAGGAATCGAATGCAACCGGCGCAGGAACACTGCCAGCGCATCAACAATATTTTCACCTGAATCAGGATATTCTTCTAATACCTGGAATGCTGTTTTCCCGGGGATCGCAGTGGTGAGTAACCATGCATCATCAGGAGTACGGATAAAATGCTTGATGGTCGGAAGAGGCATAAATTCCGTCAGCCAGTTTAGTCTGACCATCTCATCTGTAACATCATTGGCAACGCTACCTTTGCCATGTTTCAGAAACAACTCTGGCGCATCGGGCTTCCCATACAATCGATAGATTGTCGCACCTGATTGCCCGACATTATCGCGAGCCCATTTATACCCATATAAATCAGCATCCATGTTGGAATTTAATCGCGGCCTCGAGCAAGACGTTTCCCGTTGAATATGGCTCATAACACCCCTTGTATTACTGTTTATGTAAGCAGACAGTTTTATTGTTCATGATGATATATTTTTATCTTGTGCAATGTAACATCAGAGATTTTGAGACACAACGTGGCTTTGTTGAATAAATCGAACTTTTGCTGAGTTGAAGGATCAGATTACCCTGTTATCCCTAGCTGACACTGAGGATCTACTTACATACTGCTCGCGATAACTGACTCTGCCACTCAAGACCAAAAAAAACCCCGCCCTGTCAGGGGCGGGGTTTTTTTTTTGTTAGGTCAGTGACCTCTTAAGAGCGAAAAAACCCCGCCGAAGCGGGGTTTTTTGCGGGTAAGCGTCACTAACGACATTGGGTTCAGTTACTTAACAGAACCGCTGCCACTCTTGAGATAAGGGAGCTC | This study |

Supplementary Table S4. Reactions evaluated for Monte Carlo Sampling in *E. coli* W.

|  |  |  |  |
| --- | --- | --- | --- |
| **Gen** | **Enzyme** | **Reaction abbreviation** | **Reaction** |
| *pyk*AF | Pyruvate kinase | PYK | adp + H + pep -> pyr + atp |
| *ilv*BN, *ilv*GM*, ilv*IH | Acetolactate synthase | ACLS | H + 2pyr -> alac + CO2 |
| *ilv*D | Dihydroxy-acid dehydratase | DHAD1 | 23dhmb -> 2-KIV + H2O |
| *pho*E*, omp*FNC | Transport proteins | EX-2KIV | EX\_2-KIV[Extra\_organism] -> |
| *pho*E*, omp*FNC | Transport proteins | EX-valine | val\_L[Extra\_organism] -> |
| *mae*B | Malate dehydrogenase ubiquinone-8 | ME2 | mal\_L + nadp -> nadph + CO2 + pyr |
| *ace*F | Pyruvate dehydrogenase | PDH | coa + nad + pyr -> CO2 + nadh + accoa |
| *ppc* | Phosphoenolpyruvate carboxylase | PPC | H2O + pep + CO2 -> pi + h + oaa |
| *mdh* | Malate dehydrogenase | MDH | mal\_L + nad <=> h + nadh + oaa |
| *pho*E*, omp*FNC | Transport proteins | EX-acetate | ac [Extra\_organism] -> |
| *tdc*E*, pfl*ABDC*, yfi*D | Pyruvate formate lyase | PFL | coa + pyr <=> accoa + for |
| *ace*A | Isocitrate lyase | ICL | icit -> glx + succ |
| *icd* | Isocitrate dehydrogenase - NADP | ICDHyr | icit + nadp <=> nadph + akg + CO2 |
| *suc*AB, *lpd* | Oxogluterato deshidrogenasa | AKGDH | akg + coa + nad -> CO2 + nadh + succoa |
| *acn*AB | Aconitase | ACONTb | acon + H2O <=> icit |
| *glt*A | Citrate synthase | CS | accoa + H2O + oaa -> cit + coa + H |

Supplementary Table S5. Maximum specific growth rates of *E. coli* W on different substrates pure and combined with glucose at a final concentration of 2 g/L.

|  |  |
| --- | --- |
| **Substrate (2 g/L)** | **µmax (h-1)** |
| **Glucose 50% : Lactose 50%** | **0.824±0.01a**b |
| Lactose | 0.82±0.11ab |
| Glucose 50% : Fructose 50% | 0.81±0.02abc |
| Glucose 50% : Maltose 50% | 0.73±0.01abcd |
| Sucrose | 0.71±0.02abcd |
| Glucose 50% : Sucrose 50% | 0.70±0.01abcd |
| Maltose | 0.69±0.01abcd |
| Galactose | 0.66±0.18bcd |
| **Glucose** | **0.63±0.03bcd** |
| Fructose | 0.63±0.01bcd |
| Glucose 50% : Galactose50% | 0.62±0.02cd |

± represents the standard deviation. Similar lowercase letters per column indicate no statistical difference between treatments (p<0.05).

Supplementary Table S6. Studies on redirection the carbon flux towards pyruvate for the overproduction of 2-KIV, L-valine and some alcohols.

|  |  |  |  |
| --- | --- | --- | --- |
| **Microorganism** | **Genes Deletion** | **Product** | **Author** |
| *C. glutamicum* | *ilv*A, *pan*BC | L-valine | (Radmacher et al., 2002) |
| *E. coli* W3110 | *ilv*A, *leu*A, *pan*B, *ace*F, *md*h y *pfk*A | L-valine | (Park et al., 2007) |
| *C. glutamicum* | *ace*E | L-valine | (Blombach et al., 2007) |
| *E. coli* BW25113 | *adh*E, *ldh*A, *frd*BC – *fnr*, *pta* | Alcohols | (Atsumi et al., 2008) |
| *C. glutamicum* | *ace*E, *ace*E-*pqo, ace*E-*pqo-pgi* | L-valine | (Bartek et al., 2011) |
| *C. glutamicum* | *pan*B | L-valine | Holatko, 2009 |
| *C. glutamicum* | *ace*E, *pq*o, *ilv*E | 2-ketoisovalerate | (Krause et al., 2010) |
| *E. coli* W3110 | *ilv*A, *leu*A, *pan*B, *ace*F, *mdh* y *pfk*A | L-valine | (Park et al., 2011b) |
| *E. coli* W | *ilv*A, *lac*I | L-valine | (Park et al., 2011a) |
| *C. glutamicum* | *ace*E, *pqo*, *ilvE*, *ldh*A | Isobutanol | (Blombach et al., 2011) |
| *Bacillus subtilis* | *ldh*A | 2-ketoisovalerate e isobutanol | (Li et al., 2011) |
| *Saccharomyces cerevisiae* | *yqh*D | 2-ketoisovalerate decarboxylase | (Lee et al., 2012) |
| *Brevibacterium flavum* | *avt*A | L-valine | (Hou et al., 2012) |
| *C. glutamicum* | L-valina: *ace*E, *pqo*; *pqo*, *ppc*. 2-KIV: *ace*E, *pqo*, *ilv*E; *pqo*, *ppc*, *ilv*E | L-valine y 2-ketoisovalerate | (Buchholz et al., 2013) |
| *C. glutamicum* | *pep*C, *ldh*, *avt*A, *ctf*, *ack*A, *pta* | L-valine | (Hasegawa et al., 2013) |
| *C. glutamicum* | *ace*E, *ala*T, *ilv*A | L-valine | (Chen et al., 2015) |
| *Klebsiella pneumoniae* | *bud*A, *ldh*A, *bud*A-*ldh*A, *bud*A-*ldh*A-*Bud*B | 2-ketoisovalerate e isobutanol | (Gu et al., 2017) |
| *C. glutamicum* | *ppc*, *pyc* | L-valine | (Schwentner et al., 2018) |
| *E. coli* MG1655 | *pfl*B, *ldh*A, *ack*A-*pt*a | Isobutanol | (Liang et al., 2018) |
| *Bacillus subtilis* | *Bcd, ilvB, leuA, ilvA, pdhA, sigF* | L-valine | (Westbrook et al., 2018) |
| *E. coli* MG1655 | *pgi*, *gnt*R, *gn*d, *pfl*B, *ldh*A | Isobutanol | (Noda et al., 2019) |
| *E. coli* MG1655 | *frd*A, *pta*, *ldh*A, *adh*E | 2,3-butanediol and isobutanol | (Jung et al., 2020) |
| *E. coli* W3110 | *pfl*B, *adh*E y *ldh*A | L-valine | (Hao et al., 2020) |

Supplementary method descriptions MD1. Construction of knockout mutants

The deletion of *mdh* and *ace*F genes in *Escherichia coli* W was performed using the method developed by Kim *et al.* (Kim et al., 2014) with minor modifications: i) The T2SK from the plasmid pT2SK was adapted by PCR introducing *Sac*I and *Bam*HI restriction sites at both sides (T2SK cassette). Ii) 200 pb upstream and downstream of *mdh* and *ace*F (Rec\_arms 1 and 2) were amplified by PCR using *E. coli* W chromosome as template and ligated at both sides of T2SK in the *Sac*I and *Bam*HI sites and cloned in pSEVA182 generating the mdh\_Arm1y2 and aceF\_Arm1y2 constructs (Figure S1 and Figure S2). iii) These constructs where used as PCR template for the construction of the final mutational cassettes for the generation of the knockout mutants.

Supplementary method descriptions MD2. Construction of synthetic expression systems

For the overexpression of desired genes synthetic operons were constructed according to the Golden Standard method developed in our lab (Blázquez et al., 2022). The CDS level 0 parts were constructed by chemical synthesis (Supplementary Table S2). Were used linkers for a continue transcription of genes. In addition, through of previous test of others ribosomes, was selected standard ribosome (std) (Nogales et al., 2011), everything this within a cloning vector of high copy number (pUC) and using 3MB at 0.5 mM to activate the expression system.



Supplementary Figure S1. Designed plasmid to deletion of *mdh* gen in *E. coli* W



Supplementary Figure S2. Designed plasmid to deletion of *ace*F gen in *E. coli* W



Supplementary Figure S3. Gel electrophoresis for verification of gene knockout in *E. coli* W



Supplementary Figure S4. Cluster of genes found to increase production of pyruvate, 2-KIV and L-valine

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