Supplementary Material

Evaluation of *Ogataea* (*Hansenula*) *polymorpha* for hyaluronic acid production

João Heitor Colombelli Manfrão-Netto[1], Enzo Bento Queiroz[1], Kelly Assis Rodrigues[1], Cintia M. Coelho[2], Hugo Costa Paes[3], Elíbio Leopoldo Rech Filho[4,] Nádia Skorupa Parachin*[1,5]

[1] Grupo Engenharia de Biocatalisadores, Instituto de Ciências Biológicas, Universidade de Brasília, Brasília-DF, Brazil

[2] Department of Genetics and Morphology, Institute of Biological Science, University of Brasília, Brasília, 70910900, DF, Brazil

[3] Clinical Medicine Division, University of Brasília Medical School, University of Brasília, Brasília, Federal District 70910-900, Brazil

[4] Brazilian Agriculture Research Corporation – Embrapa – Genetic Resources and Biotechnology – CENARGEN, Brasília 70770917 DF, Brazil.

[5] Present address: Ginkgo Bioworks 27th Drydock Ave. 8th floor, Boston, MA, 02210.

*Correspondence: nadiasp@unb.br; nadiasp@gmail.com

Summary of Supporting information

Table S1. List of primers used in this work

Primer name	Sequence to amplify	Primer orientation	Restriction enzyme 5′	[2]Primer sequence 5′→3′
attB_hasB_F	*attL* (*hasB*)	Forward	NA	CCAGTTCCCTGAAATTATTCCCCT
attP_hasB_R	*attL* (*hasB*)	Reverse	NA	CAGCTTCTTGAATTGCACCATCAAT
attB_hasAp_F	*attL* (*hasAp*)	Forward	NA	CTCTTCTTAGGCATCCTTCTATC
attP_hasAp_R	*attL* (*hasAp*)	Reverse	NA	GCAGCACTAGCTTGGAAACC
hasB_F	*hasB*	Forward	HindIII	AAA**AAGCTT**AATGTTTCAGATCAAGAAGATTTGTTGTA
hasB_R	*hasB*	Reverse	SalI	AAA**GTCGAC**TTACACACGTTGCTTC
hasAp_F	*hasAp*	Forward	HindIII	AAGCTT**AAGCTT**ATGAATACCTTATCTC
hasAp_R	*hasAp*	Reverse	XhoI	AAAGTCGACGCCTTTACAATGTGATTGA
AOX-Integration_F[1]	*AOX* promoter	Forward	NA	CAGTTTTTGCCCTACTTGATC
AOX-Integration_R[1]	*AMO*[3] terminator	Reverse	NA	GTAGGAAGGCTGGATGTC

NA: Not applied

[1]These primers were designed to confirm the insert integration when the plasmid pHIPH4 is used for cloning.

[2]In bold, the recognizing sequence for the respective enzyme.

[3] *AMO*: Amino oxidase

Table S2. List of plasmids used in this work

Name	Description	Reference
pBSK_*hasB*	Cloning vector carrying the synthetic *hasB* gene encoding the UDP-glucose dehydrogenase from *Xenopus laevis*	This work
pBSK_*hasAp*	Cloning vector carrying the synthetic *hasAp* gene encoding the hyaluronic acid synthase from *Pasteurella multocida*	This work
pBSK_*hasAs*	Cloning vector carrying the synthetic *hasAs* gene encoding the hyaluronic acid synthase from *Streptococcus zooepidemicus*	This work
pGEM-T Easy	Commercial plasmid used in the cloning steps	Promega
pGEM_*hasB*	pGEM-T Easy plasmid carrying the *hasB* gene	This work
pHIPH4	*O. polymorpha* integrative plasmid containing the native promoter pAOX and the terminator Tamo	[1]
pHIPZ7	*O. polymorpha* integrative plasmid containing the native promoter pTEF1 and the terminator Tamo	[1]
pHIPZ18_eGFP_SKL	*O. polymorpha* integrative plasmid containing the native promoter pAHD1 and the terminator Tamo and carrying the gene encoding eGFP	[1]
pHIPH4_*hasB*	pHIPH4-derived plasmid carrying the *hasB* gene under control of the pAOX promoter	This work
pHIPH4_*hasAs*	pHIPH4-derived plasmid carrying the *hasAs* gene under control of the pAOX promoter	This work
pHIPH4_*ScSInt13*	Synthetic plasmid containing a genetic switch controlling the expression of *hasAp* and *hasB* and carrying a gene of a serine-type phage integrase-13, codon-optimized for *S. cerevisiae*	This work
pHIPZ7_*hasAp*	pHIPZ7-derived plasmid carrying the *hasAp* gene under control of the pTEF1 promoter	This work
pHIPZ18_*hasAp*	pHIPZ18-derived plasmid carrying the *hasAp* gene under control of the pADH1 promoter	This work
pHIPZ18_*hasB*	pHIPZ18-derived plasmid carrying the *hasB* gene under control of the pADH1 promoter	This work

Table S3. List of strains used in this work

Name	Description	Reference
E. coli DH10B	Bacteria strain for plasmid cloning	Invitrogen
O. polymorpha NCYC 495 *yku80*	Methylotrophic yeast with a NHEJ-deficient phenotype; referred to as Wild Type (WT) in this work	[1]
EMB100.1	WT strain transformed with the linearized pHIPH4_*hasB* plasmid. The *hasB* gene was integrated into the genome under control of the pAOX promoter. This strain was used as an intermediate for the construction of the EMB101 and EMB102 strains.	This work
EMB101	EMB100.1 transformed with the linearized pHIPZ18_*hasAp* plasmid. The *hasAp* gene was integrated into the genome under control of the pADH1 promoter. The *hasB* gene is regulated by the pAOX promoter.	This work
EMB102	EMB100.1 transformed with the linearized pHIPZ7_*hasAp* plasmid. The *hasAp* gene was integrated into the genome under control of the pTEF1 promoter. The *hasB* gene is regulated by the pAOX promoter.	This work
EMB103	WT strain transformed with the linearized pHIPH4_ScsInt13 containing a genetic switch controlling the expression of *hasAp* and *hasB* and carrying a gene for a serine-type phage integrase-13, codon-optimized for *S. cerevisiae* and under control of the pAOX promoter. The genes *hasB* and *hasAp* are regulated by the pGDP promoter from *S. cerevisiae* and the pADH1 promoter from *O. polymorpha*, respectively.	This work
EMB100.2	WT strain transformed with the linearized pHIPZ18_*hasB* plasmid. The *hasB* gene was integrated into the genome under control of the pAHD1 promoter. This strain was used as an intermediate for the construction of EMB104.	This work
EMB104	EMB100.2 transformed with the linearized pHIPH4_*hasAs* plasmid. The *hasAs* gene was integrated into the genome under control of the pAOX promoter. The *hasB* gene is regulated by the pADH1 promoter.	This work

Sequence S1. Nucleotide sequence of the *hasB* gene from *Xenopus laevis* (Genbank ID: MH728986). The start and stop codons of the ORF are shown in bold.

ATGTTTCAGATCAAGAAGATTTGTTGTATTGGTGCCGGTTACGTCGGTGGTCCAACCTGT
TCTGTCATTGCACAGATGTGCCCTGACATTAAGGTCACTGTTGTGGATGTGAACCAAGC
CAGGATCAATGCTTGGAATAGTGACACTTTGCCTATCTACGAACCAGGTTTGAAGGAAG
TCGTAGAGTCATGCAGGGGAAAGAATTTGTTCTACTCAACTGACATTGATGGTGCAATT
CAAGAAGCTGATTTGGTGTTCATCTCAGTCAACACTCCAACAAAAACTTACGGTATGGG
TAAGGGAAGGGCAGCCGACTTGAAATACATTGAGGCTTGCGCTAGAAGAATAGTACAG
AATAGTAACGGATACAAGATTGTTACAGAGAAATCTACTGTGCCAGTTAGAGCTGCTG
AATCAATAAGACGTATCTTCGATGCAAATACTAAACCAGATTTGAACTTGCAGGTATTG
AGTAACCCAGAGTTTTTGGCAGAGGGTACAGCCATTAAGGATTTGAAGAACCCTGATA
GAGTTTTGATAGGTGGTGACGAAACCCCTGAAGGTCAGAAAGCTGTTAGAGCTTTGTGT
GACGTATACGAACACTGGGTACCATCTGAGAAAATCATAACCACAAACACCTGGTCTT
CTGAGTTGAGTAAGTTAGCAGCCAACGCATTCTTAGCTCAAAGAATTTCTTCAATCAAC
TCAATTAGTGCCTTATGTGAAGCCACAGGAGCTGACGTTGAAGAGGTTGCCAGAGCTAT
TGGTATGGATCAAAGAATTGGTAACAAGTTTTTGAAGGCTTCAGTGGGATTTGGAGGTT
CATGTTTTCAGAAGGACGTTTTGAACTTGGTTTACTTGTGTGAGGTGTTAAACTTGCACG
AAGTGGCCAAGTACTGGCAACAAGTGATTGATATGAATGATTATCAAAGGAGACGTTT
TACAACTAGGATAATCGATTGTTTGTTTAACACCGTGACCGATAAGAAAATCGCATTGT
TAGGTTTCGCTTTCAAGAAGGATACAGGTGATACTAGAGAGAGTAGTTCAATCTATATC
TCTAAGTATTTGATGGATGAAGGTGCTAAGTTACATATCTACGATCCAAAGGTCCCACG
TGAGCAGATCATCACTGACTTGAGTCAACCTGGTGTTGCAGCTGACGACAGGGTTTCTC
AATTGGTCCACATAAGTACAGATTTGTACGAAGCCTGTGAGAATGCACACGCTATGGTC
ATTTGTACTGAATGGGATATGTTCAAGGAATTAGATTTCAATAGAATCCATAGGATGAT
GTTAAAGCCTGCTTTCATATTCGATGGTAGACGTGTTTTAGATGAATTGCATGGAGAATT
GCAAAACATTGGATTTCAGGTGGAAACCATCGGAAAGAAGGTAGCTTCAAAAAGAAT
ACCATTCACTCCAACTGCTGATATCCCTAAGTTCGGTTTACAGGACTTGCCACACAAGA
AGCAACGTGTG**TAA**

Sequence S2. Nucleotide sequence of the *hasAp* gene from *Pasteurella multocida* (Genbank ID: MH728990). The start and stop codons of the ORF are shown in bold.

ATGAATACCTTATCTCAAGCCATCAAGGCATACAATTCAAATGACTATCAATTGGCTTT
GAAATTGTTCGAAAAGTCAGCAGAAATCTACGGACGTAAGATAGTAGAGTTTCAGATT
ACTAAGTGCAAGGAGAAATTGTCTGCCCATCCAAGTGTCAATTCAGCTCATCCTTCAGT
CAACTCAGCACATTTGTCAGTAAACAAAGAGGAAAAGGTTAATGTGTGCGATTCTCCAT
TGGATATTGCTACCCAATTGTTGTTGTCAAATGTCAAGAAATTAGTGTTATCTGACAGTG
AGAAGAACACATTGAAGAATAAGTGGAAGTTGTTAACTGAAAAGAAAAGTGAGAATG
CTGAAGTTCGTGCTGTAGCATTGGTACCAAAGGATTTCCCAAAGGATTTGGTGTTGGCA
CCATTACCTGACCATGTGAATGACTTTACTTGGTACAAGAAGAGAAAGAAACGTTTAG
GTATCAAACCAGAACATCAACACGTGGGTTTGTCAATAATTGTCACCACTTTCAATCGT
CCTGCAATCTTAAGTATAACTTTGGCATGCTTAGTTAATCAAAAGACTCACTATCCATTC
GAGGTGATTGTCACAGATGATGGATCACAAGAAGATTTGTCTCCAATCATAAGACAAT
ATGAAAACAAATTGGATATCCGTTATGTCAGACAAAAGGACAATGGTTTCCAAGCTAG
TGCTGCTAGGAATATGGGTTTGAGATTAGCAAAGTATGATTTCATTGGTTTGTTGGATTG

CGATATGGCACCTAACCCATTATGGGTGCATTCATATGTCGCTGAATTGTTAGAAGATG
ATGATTTGACAATCATTGGACCAAGAAAGTACATTGATACACAACATATCGACCCAAA
GGACTTCTTAAACAATGCATCTTTGTTGGAATCATTGCCAGAAGTTAAGACCAATAACT
CAGTGGCCGCAAAAGGTGAAGGTACCGTTTCATTGGATTGGAGGTTGGAGCAATTCGA
AAAGACTGAAAACTTAAGATTGTCAGACTCTCCTTTTAGATTCTTCGCAGCTGGTAATGT
TGCTTTCGCCAAGAAGTGGTTGAACAAATCTGGATTCTTTGATGAAGAGTTCAACCATT
GGGGTGGTGAAGATGTTGAGTTTGGATATAGATTGTTTAGGTATGGTTCATTCTTCAAGA
CTATTGACGGTATCATGGCCTACCATCAAGAGCCACCTGGTAAGGAAAACGAAACAGA
TAGGGAAGCTGGAAAGAACATCACATTGGATATTATGAGGGAGAAGGTACCATATATT
TACAGGAAGTTGTTGCCTATCGAAGATTCACACATCAATAGAGTCCCTTTGGTTTCTATC
TATATCCCAGCTTACAACTGTGCCAATTATATTCAACGTTGTGTTGATTCTGCCTTGAAC
CAGACAGTTGTAGATTTGGAAGTCTGTATTTGCAATGATGGTTCTACAGATAATACTTTG
GAAGTTATCAACAAGTTGTACGGTAACAATCCAAGAGTCAGAATCATGAGTAAACCAA
ATGGTGGTATTGCTAGTGCTTCTAATGCAGCAGTGAGTTTTGCCAAAGGATATTACATA
GGTCAATTAGATTCAGATGACTATTTGGAGCCAGATGCCGTAGAGTTATGTTTGAAAGA
GTTCTTGAAAGACAAAACTTTGGCTTGTGTATATACAACAAACAGAAATGTCAATCCTG
ATGGTTCTTTGATAGCAAATGGTTACAACTGGCCAGAGTTTAGTAGGGAGAAGTTGACT
ACTGCAATGATTGCTCATCACTTCCGTATGTTCACTATCAGGGCATGGCATTTGACCGAT
GGTTTTAATGAGAAGATTGAGAATGCTGTGGACTACGATATGTTCTTGAAGTTGAGTGA
AGTTGGTAAGTTCAAGCACTTAAACAAAATCTGCTATAACAGGGTATTGCATGGTGATA
ATACAAGTATTAAGAAGTTGGGTATCCAAAAGAAGAACCATTTCGTGGTCGTCAACCA
GAGTTTGAACAGGCAAGGAATCACTTACTACAATTACGACGAGTTCGATGACTTAGAT
GAGTCTAGGAAATACATCTTTAACAAAACAGCTGAGTACCAGGAAGAAATTGACATCT
TAAAGGACATTAAGATCATACAAAACAAGGACGCTAAAATAGCAGTATCTATCTTCTA
CCCAAATACTTTGAATGGTTTGGTCAAGAAATTGAATAACATCATCGAGTACAACAAG
AACATATTCGTTATTGTCTTGCATGTGGACAAGAACCATTTGACCCCAGATATCAAGAA
AGAGATATTGGCTTTCTACCACAAGCATCAAGTGAATATTTTGTTGAATAACGATATCT
CATACTACACATCAAACCGTTTAATCAAGACCGAGGCACATTTATCAAACATTAATAA
GTTGTCACAGTTGAACTTGAATTGTGAATATATCATATTCGACAATCATGACTCTTTGTT
CGTGAAGAATGATTCTTATGCCTATATGAAGAAGTACGATGTTGGTATGAATTTCTCAG
CCTTAACTCATGATTGGATTGAAAAGATTAACGCACATCCACCATTCAAGAAGTTGATT
AAGACATACTTTAACGATAATGACTTGAAATCTATGAACGTTAAAGGAGCTAGTCAAG
GAATGTTTATGACATATGCATTGGCTCACGAATTGTTGACTATTATCAAAGAGGTTATCA
CTTCTTGCCAATCTATCGATTCTGTACCAGAATACAACACTGAGGACATATGGTTTCAAT
TTGCATTGTTGATCTTGGAAAAGAAAACTGGTCATGTCTTTAACAAGACAAGTACCTTG
ACATACATGCCTTGGGAGAGGAAGTTGCAATGGACCAATGAACAAATTGAATCAGCTA
AACGTGGAGAAAACATTCCAGTGAACAAGTTCATAATCAATTCAATCACATTG**TAA**

Sequence S3. Nucleotide sequence of the *hasAs* gene from *Streptococcus zooepidemicus* (Genbank ID: AF414053.1). The start and stop codons of the ORF are shown in bold.

ATGAGAACTTTGAAAAATTTAATTACAGTTGTTGCATTCTCAATTTTCTGGGTTTTATTA
ATTTATGTTAATGTTTATTTGTTCGGTGCTAAAGGTTCATTGTCAATTTATGGTTTCTTATT
AATTGCATATTTGTTAGTTAAAATGTCTTTGTCATTCTTCTATAAACCATTCAAAGGTAG
AGCAGGTCAATATAAAGTTGCTGCAATTATTCCATCATATAATGAAGATGCTGAATCTT

TATTGGAAACTTTGAAATCAGTTCAACAACAAACATATCCATTGGCAGAAATTTATGTT
GTTGATGATGGTTCAGCCGATGAAACAGGTATTAAAAGAATTGAAGATTATGTTAGAG
ATACTGGTGATTTATCATCTAATGTTATTGTTCATCGTAGCGAAAAAAATCAAGGTAAA
AGACATGCACAAGCATGGGCTTTCGAAAGAAGCGATGCAGATGTTTTCTTGACAGTTGA
TTCAGATACATATATTTATCCAGATGCTTTGGAAGAATTATTGAAAACTTTCAATGATCC
TACAGTTTTCGCAGCTACAGGTCATTTGAATGTTAGAAATAGACAAACTAATTTGTTAA
CAAGATTGACTGATATTAGATATGATAATGCATTCGGTGTTGAAAGAGCAGCACAATCA
GTTACTGGTAATATTTTAGTTTGTTCTGGTCCTTTGTCAGTTTATAGAAGAGAAGTTGTTG
TTCCTAATATTGATAGATATATTAATCAAACTTTCTTAGGTATTCCAGTTTCAATTGGTGA
TGATAGATGTTTAACTAATTATGCTACTGATTTGGGTAAAACAGTTTATCAATCTACAGC
TAAATGTATTACTGATGTTCCAGATAAAATGTCTACATATTTGAAACAACAAAATAGAT
GGAATAAATCATTCTTCAGAGAATCTATTATTTCAGTTAAAAAAATTATGAATAATCCA
TTCGTTGCTTTGTGGACTATTTTGGAAGTTTCTATGTTCATGATGTTAGTTTATTCAGTTGT
TGATTTCTTCGTTGATAATGTTAGAGAGTTCGATTGGTTAAGAGTTTTGGCATTCTTGGTT
ATTATTTTCATTGTTGCTTTGTGTAGAAATATTCATTATATGTTGAAACATCCTTTGTCTTT
CTTGTTATCTCCTTTCTATGGTGTTTTACATTTATTCGTTTTGCAACCATTGAAATTGTATT
CATTGTTCACTATTAGAAATGCTGATTGGGGTACTAGAAAAAAATTGTTG**TAA**

Sequence S4. Nucleotide sequence of the gene of serine integrase-13 (Int13), codon-optimized for *S. cerevisiae*. The start and stop codons of the ORF are shown in bold.

ATGGCGGTTGGGATTTACATCAGAGTCTCAACCCAAGAGCAGGCGAGTGAAGGGCACA
GTATTGAAAGCCAAAAAAAGAAACTGGCGTCTTATTGTGAAATCCAAGGATGGGATGA
CTACAGGTTCTACATCGAAGAGGGCATATCCGGGAAAAACACAAATAGACCGAAGCTT
AAGCTATTAATGGAACATATCGAAAAGGGAAAAATTAACATTTTATTGGTCTACAGGCT
GGATAGGTTGACTAGGTCTGTGATCGATTTACATAAGCTATTAAACTTTTTACAGGAAC
ATGGGTGCGCGTTTAAATCTGCTACAGAAACTTACGACACAACTACTGCAAACGGAAG
GATGAGTATGGGTATAGTGAGTCTTCTAGCCCAATGGGAGACAGAAAATATGAGTGAG
CGTATTAAACTAAACCTGGAACACAAAGTCTTGGTTGAGGGGGAAAGAGTAGGGGCGA
TTCCCTATGGATTCGACTTGTCAGATGATGAAAAGCTTGTGAAGAATGAAAAGTCTGCA
ATTTTATTGGACATGGTCGAAAGGGTGGAGAACGGCTGGTCCGTCAATAGGATCGTCAA
CTATCTTAATTTAACTAACAATGATCGTAACTGGTCACCTAATGGGGTGCTACGTTTGTT
AAGGAACCCTGCACTATATGGCGCTACAAGGTGGAATGATAAAATCGCAGAGAACAC
ACACGAGGGTATAATTAGCAAGGAACGTTTCAACCGTCTGCAGCAAATACTTGCAGAC
CGTAGCATCCATCACAGACGTGATGTGAAAGGAACATACATATTTCAAGGAGTTTTGA
GATGTCCGGTTTGTGATCAGACGCTGTCCGTTAATAGGTTTATTAAGAAGCGTAAGGAT
GGAACAGAGTACTGTGGTGTCCTTTATAGGTGTCAGCCATGTATTAAGCAAAACAAGTA
CAATTTAGCTATCGGCGAAGCTAGGTTCCTGAAGGCCCTTAACGAGTACATGTCTACGG
TGGAATTCCAGACAGTTGAAGACGAGGTGATACCCAAGAAAAGTGAGAGAGAAATGT
TGGAATCTCAGCTGCAACAGATCGCAAGAAAGAGGGAGAAATACCAAAAGGCATGGG
CGAGCGATTTAATGTCCGATGATGAATTTGAGAAACTTATGGTCGAGACCCGTGAAACT
TATGACGAATGCAAGCAAAAACTGGAGAGTTGCGAGGACCCTATTAAGATCGACGAG
ACATATTTGAAGGAAATAGTTTACATGTTTCATCAAACATTCAATGATTTAGAGTCCGA
GAAGCAAAAGGAGTTTATATCAAAATTTATAAGGACTATCCGTTACACCGTCAAAGAG
CAGCAACCTATCAGACCTGATAAGTCTAAGACAGGTAAGGGTAAACAGAAAGTGATA
ATTACGGAAGTGGAGTTTTACCAG**TAA**

Sequence S5. The nucleotide sequence of the pGEM_*hasB* plasmid. The sequence of the *hasB* gene is shown in yellow.

TATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAA
CCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTA
ATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGA
ATGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCG
TGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCT
CGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCG
ATTTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGTGATGGTTCACGTA
GTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTA
ATAGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATTCTTTTG
ATTTATAAGGGATTTTGCCGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAA
AAATTTAACGCGAATTTTAACAAAATATTAACGCTTACAATTTCCTGATGCGGTATTTTC
TCCTTACGCATCTGTGCGGTATTTCACACCGCATCAGGTGGCACTTTTCGGGGAAATGTG
CGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGA
CAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAAC
ATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCA
GAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACA
TCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTT
CCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCC
GGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTC
ACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCT
GCCATAACCATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGAC
CGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGT
TGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCT
GTAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTC
CCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGC
TCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCT
CGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTA
CACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGG
TGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGAT
TGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCT
CATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAA
AGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAA
AAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTT
CCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTTCTTCTAGTGTAGCC
GTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAA

TCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCA
AGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACAC
AGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATG
AGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAG
GGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTAT
AGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGG
GGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTG
CTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATT
ACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGT
CAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTG
GCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAG
CGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTAT
GCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACA
GCTATGACCATGATTACGCCAAGCTATTTAGGTGACACTATAGAATACTCAAGCTATGC
ATCCAACGCGTTGGGAGCTCTCCCATATGGTCGACCTGCAGGCGGCCGCGAATTCACTA
GTGATTAAAAAGCTTAATGTTTCAGATCAAGAAGATTTGTTGTATTGGTGCCGGTTACGT
CGGTGGTCCAACCTGTTCTGTCATTGCACAGATGTGCCCTGACATTAAGGTCACTGTTGT
GGATGTGAACCAAGCCAGGATCAATGCTTGGAATAGTGACACTTTGCCTATCTACGAAC
CAGGTTTGAAGGAAGTCGTAGAGTCATGCAGGGGAAAGAATTTGTTCTACTCAACTGA
CATTGATGGTGCAATTCAAGAAGCTGATTTGGTGTTCATCTCAGTCAACACTCCAACAA
AAACTTACGGTATGGGTAAGGGAAGGGCAGCCGACTTGAAATACATTGAGGCTTGCGC
TAGAAGAATAGTACAGAATAGTAACGGATACAAGATTGTTACAGAGAAATCTACTGTG
CCAGTTAGAGCTGCTGAATCAATAAGACGTATCTTCGATGCAAATACTAAACCAGATTT
GAACTTGCAGGTATTGAGTAACCCAGAGTTTTTGGCAGAGGGTACAGCCATTAAGGATT
TGAAGAACCCTGATAGAGTTTTGATAGGTGGTGACGAAACCCCTGAAGGTCAGAAAGC
TGTTAGAGCTTTGTGTGACGTATACGAACACTGGGTACCATCTGAGAAAATCATAACCA
CAAACACCTGGTCTTCTGAGTTGAGTAAGTTAGCAGCCAACGCATTCTTAGCTCAAAGA
ATTTCTTCAATCAACTCAATTAGTGCCTTATGTGAAGCCACAGGAGCTGACGTTGAAGA
GGTTGCCAGAGCTATTGGTATGGATCAAAGAATTGGTAACAAGTTTTTGAAGGCTTCAG
TGGGATTTGGAGGTTCATGTTTTCAGAAGGACGTTTTGAACTTGGTTTACTTGTGTGAGG
TGTTAAACTTGCACGAAGTGGCCAAGTACTGGCAACAAGTGATTGATATGAATGATTAT
CAAAGGAGACGTTTTACAACTAGGATAATCGATTGTTTGTTTAACACCGTGACCGATAA
GAAAATCGCATTGTTAGGTTTCGCTTTCAAGAAGGATACAGGTGATACTAGAGAGAGT
AGTTCAATCTATATCTCTAAGTATTTGATGGATGAAGGTGCTAAGTTACATATCTACGAT
CCAAAGGTCCCACGTGAGCAGATCATCACTGACTTGAGTCAACCTGGTGTTGCAGCTGA
CGACAGGGTTTCTCAATTGGTCCACATAAGTACAGATTTGTACGAAGCCTGTGAGAATG
CACACGCTATGGTCATTTGTACTGAATGGGATATGTTCAAGGAATTAGATTTCAATAGA

ATCCATAGGATGATGTTAAAGCCTGCTTTCATATTCGATGGTAGACGTGTTTTAGATGAA
TTGCATGGAGAATTGCAAAACATTGGATTTCAGGTGGAAACCATCGGAAAGAAGGTAG
CTTCAAAAAGAATACCATTCACTCCAACTGCTGATATCCCTAAGTTCGGTTTACAGGAC
TTGCCACACAAGAAGCAACGTGTGTAAGTCGACTTTAATCGAATTCCCGCGGCCGCCAT
GGCGGCCGGGAGCATGCGACGTCGGGCCCAATTCGCCC

Sequence S6. The nucleotide sequence of the pHIPZ7_*hasAp* plasmid. The sequence of the *hasAp* gene is shown in green.

AGGGGATATCCTCGAGACTTGCCTTTGAAGGCTCTTGTTGCGGTAAATAAGTATATAGG
ACACGACAATCTAGTAATCTCCACTATTGACGAGCTCGTCGAACTGCGAAAATAGGTTT
TCCATCTGGTCTGTAGGCATCAGCCCGGCGTCATCCTCCTGCGCAGGAGCAGCGGGCTC
AGGGCCGGCCTGGGCGGGCTGATCCAGAAAGTCGAGGTTCAGATCCCCCACACACCAT
AGCTTCAAAATGTTTCTACTCCTTTTTTACTCTTCCAGATTTTCTCGGACTCCGCGCATCG
CCGTACCACTTCAAAACACCCAAGCACAGCATACTAAATTTTCCCTCTTTCTTCCTCTAG
GGTGTCGTTAATTACCCGTACTAAAGGTTTGGAAAAGAAAAAAGAGACCGCCTCGTTTC
TTTTTCTTCGTCGAAAAAGGCAATAAAAATTTTTATCACGTTTCTTTTTCTTGAAATTTTTT
TTTTTAGTTTTTTTCTCTTTCAGTGACCTCCATTGATATTTAAGTTAATAAACGGTCTTCAA
TTTCTCAAGTTTCAGTTTCATTTTTCTTGTTCTATTACAACTTTTTTTACTTCTTGTTCATTA
GAAAGAAAGCATAGCAATCTAATCTAAGGGGCGGTGTTGACAATTAATCATCGGCATA
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Sequence S7. The nucleotide sequence of the pHIPZ18_*hasAp* plasmid. The sequence of the *hasAp* gene is shown in green.

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Sequence S8. The nucleotide sequence of the pHIPZ18_*hasB* plasmid. The sequence of the *hasB* gene is shown in yellow.

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TCGCATTGTTAGGTTTCGCTTTCAAGAAGGATACAGGTGATACTAGAGAGAGTAGTTCA
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GGTCCCACGTGAGCAGATCATCACTGACTTGAGTCAACCTGGTGTTGCAGCTGACGACA
GGGTTTCTCAATTGGTCCACATAAGTACAGATTTGTACGAAGCCTGTGAGAATGCACAC
GCTATGGTCATTTGTACTGAATGGGATATGTTCAAGGAATTAGATTTCAATAGAATCCA

TAGGATGATGTTAAAGCCTGCTTTCATATTCGATGGTAGACGTGTTTTAGATGAATTGCA
TGGAGAATTGCAAAACATTGGATTTCAGGTGGAAACCATCGGAAAGAAGGTAGCTTCA
AAAAGAATACCATTCACTCCAACTGCTGATATCCCTAAGTTCGGTTTACAGGACTTGCC
ACACAAGAAGCAACGTGTGTAAGTCGACT

Sequence S9. The nucleotide sequence of the pHIPH4_*hasB* plasmid. The sequence of the *hasB* gene is shown in yellow.

AAGCTTAATGTTTCAGATCAAGAAGATTTGTTGTATTGGTGCCGGTTACGTCGGTGGTCC
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Sequence S10. The nucleotide sequence of the pHIPH4_*hasAs* plasmid. The sequence of the *hasAs* gene is shown in blue.

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CTGAATCTTTATTGGAAACTTTGAAATCAGTTCAACAACAAACATATCCATTGGCAGAA
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ATCTATAAATACTGCTGCCAGTGCACGGTGGTGACATCAATCTAAAGTACAAAAAC

Sequence S11. The nucleotide sequence of the pHIPH4_*ScSInt13* plasmid. The sequence of the *hasB* gene is shown in yellow. The reverse sequence of the *hasAp* gene is shown in green. The sequence of the Int13 encoding gene is shown in red.

GGCCGCCCCCTGCATTATTAATCACCACCCCGTCTACGATGACAGGCTCGCGACTGCAG
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CTGGTCTGTAGGCATCAGCCCGGCGTCATCCTCCTGCGCAGGAGCAGCGGGCTCAGGG
CCGGCCTGGGCGGGCTGATCCAGAAAGTCGAGGTTCA

Figure S1. Confirmation of integration of both genes into the EMB101 strain by colony PCR of 12 colonies selected after transformation and plating on selective medium. The upper gel shows amplification of the *hasB* gene (1505 bp) using the primer pair hasB_F and hasB_R. The bottom gel shows amplification of the *hasAp* gene (2966 bp) using the primer pair hasAp_F and hasAp_R. The agarose content was 1% and the ladders used were 1kB Ladder Plus M1191/M1192 (M1; Sinapse Inc) 1 kb Ladder K9 (M2; Kasvi). The *O. polymorpha* NCYC495 *yku80* was used as a negative control (C-) for the PCR reactions. The pHIPH4_*hasB* and pHIPZ18_*hasAp* plasmids were used as positive controls (C+) for the PCR reactions of *hasB* and *hasAp*, respectively.

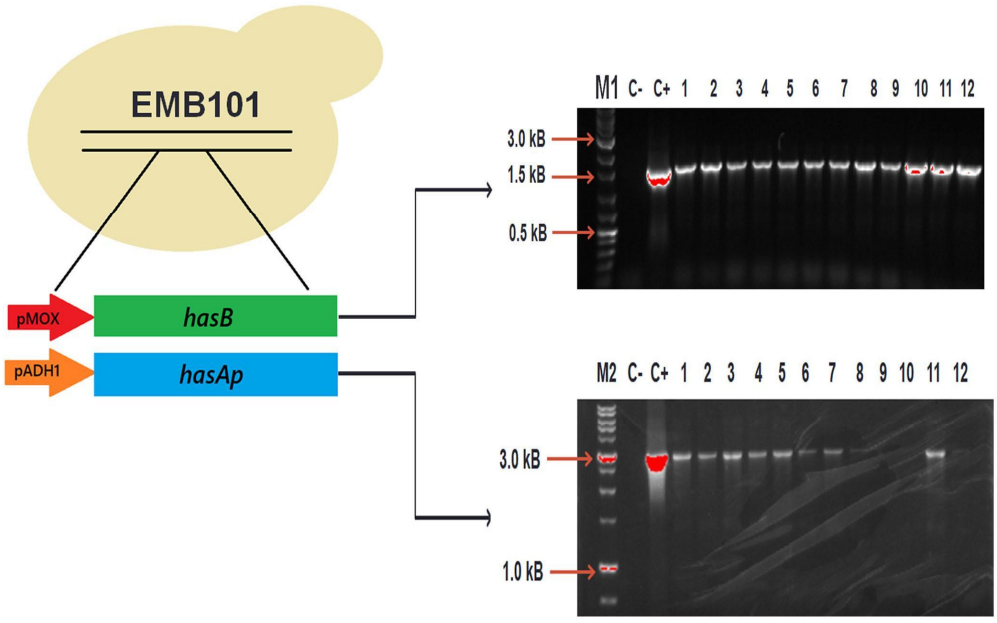


Figure S2. Colony PCRs to verify the *hasAp* (2966 bp) and *hasB* (1505 bp) genes stability in the genome of EMB101 strain after three successive passages on YPD supplemented with zeocin and hygromycin. Five colonies were selected after the transformation and plating on YPD plate containing both antibiotics for the verification of *hasB* and *hasAp* stability on the genome of *O. polymorpha* NCYC495 *yku80*. All PCR reactions for *hasB* gene were performed using the primer pair hasB_F and hasB_R while the PCRs reactions for *hasAp* gene using the primer pair hasAp_F and hasAp_R. In all PCRs reactions performed, the *O. polymorpha* NCYC495 *yku80* was used as a negative control (C-) and the pHIPH4_*hasB* and pHIPZ18_*hasAp* plasmids were used as positive controls (C+) for the of *hasB* and *hasAp*, respectively. The fragments amplified at the height of 1.5 kb in the upper gels correspond to the *hasB* gene and the fragments in the height of 3.0 kb at the bottom gels correspond to the *hasAp* gene. The black arrows indicate a passaging to another YPD plate supplemented with zeocin and hygromycin. The agarose content was 1% and the ladder used was 1 kb Plus DNA Ladder (M1; Invitrogen). **(A)** Colony PCR for the *hasAp* gene of the first passaging on selective medium, **(B)** Colony PCR for the hasB (upper gel) and hasP (bottom gel) genes of the second passaging on YPD plate with both antibiotics. **(C)** Colony PCR for the *hasB* (upper gel) and *hasAp* (bottom gel) genes of the third passaging on a YPD plate with both antibiotics.

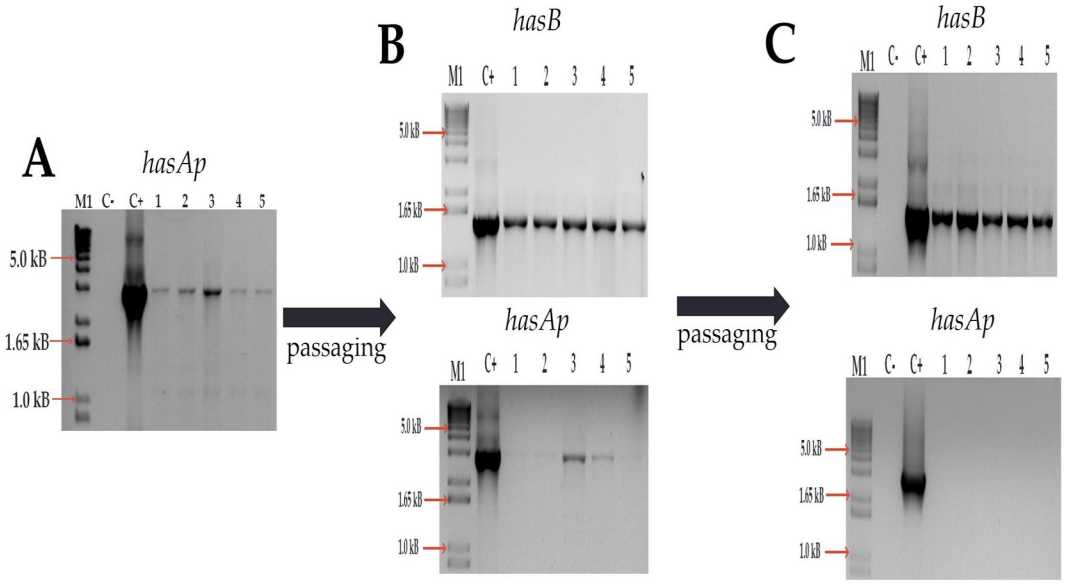


Figure S3. Confirmation of integration of both genes into the EMB102 strain by colony PCR of 12 colonies selected after the transformation and plating on selective medium. The upper gel shows amplification of the *hasB* gene (1.6 kB) using the primer pair hasB_F and hasB_R. The bottom gel shows amplification of the *hasAp* gene (2.9 kB) using the primer pair hasAp_F and hasAp_R. The agarose content was 1% and the ladders used were 1kB Ladder Plus M1191/M1192 (M1; Sinapse Inc) 1 kb Ladder K9 (M2; Kasvi). The *O. polymorpha* NCYC495 *yku80* was used as a negative control (C-) for the PCR reactions. The pHIPH4_*hasB* and pHIPZ7_*hasAp* plasmids were utilized as positive controls (C+) for the PCR reactions of *hasB* and *hasAp*, respectively.

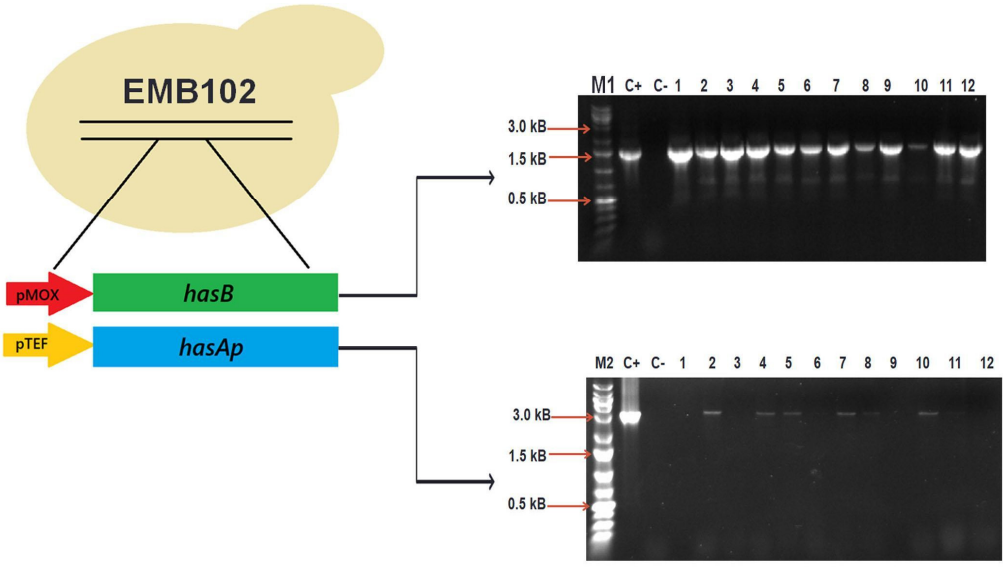


Figure S4. Scheme representing the genetic switch constructed to control the expression of both *hasB* and *hasAp* by a serine integrase. (A) The gene encoding the Int13 codon-optimized for *S. cerevisiae* is regulated by the promoter pAOX which is inducible by methanol (details in Figure S14). Thus, the addition of methanol leads to the production of Int13 that recognizes the sites *attB* and *attP* flanking both *has* genes synthesized in reverse complement orientation. The action of Int13 causes a rotation of 180° in both genes resulting in two different flanking sequences named *attL* and *attR*. In the final, both genes are in ORF with promoter and terminator and can be properly transcripted. The correct gene orientation as well the formation of *attL* sequence could be evaluated by PCR using the pair of primer attB_hasB_F and attP_hasB_R for *hasB* (resulting fragment of 493 bp) and attB_hasAp_F and attP_hasAp_R for *hasAp* (resulting fragment 827 bp). When the genes are in the initial orientation, the primer annealing fails and no amplification occurs. The black arrows represent the primer orientation. (B) Confirmation by PCR of the both genes rotation in the EMB103 strain after methanol induction. For all PCRs reactions, the genomic DNA was utilized as the template and for the extraction the phenol/chloroform method was applied according to [2]. The gel in the left shows the fragment (827 bp) containing the *attL* sequence and the *hasAp* flipped and in the right the fragment for *hasB* (493 bp).The *O. polymorpha* NCYC495 *yku80* was used as a negative control (C-) for the PCR reactions. The pHIPZ18_*hasAp* plasmid which contains the *hasAp* in ORF was utilized as positive controls (C+) for the PCR reactions of *hasAp*. The plasmid PKLAC2-BP constructed previously for our group (REF) was utilized as the control positive for the PCR reaction of *hasB* once all plasmids constructed in this work has the *hasB* gene controlled by endogenous promoters of *O. polymorpha* instead of pGDP promoter from *S. cerevisiae*.

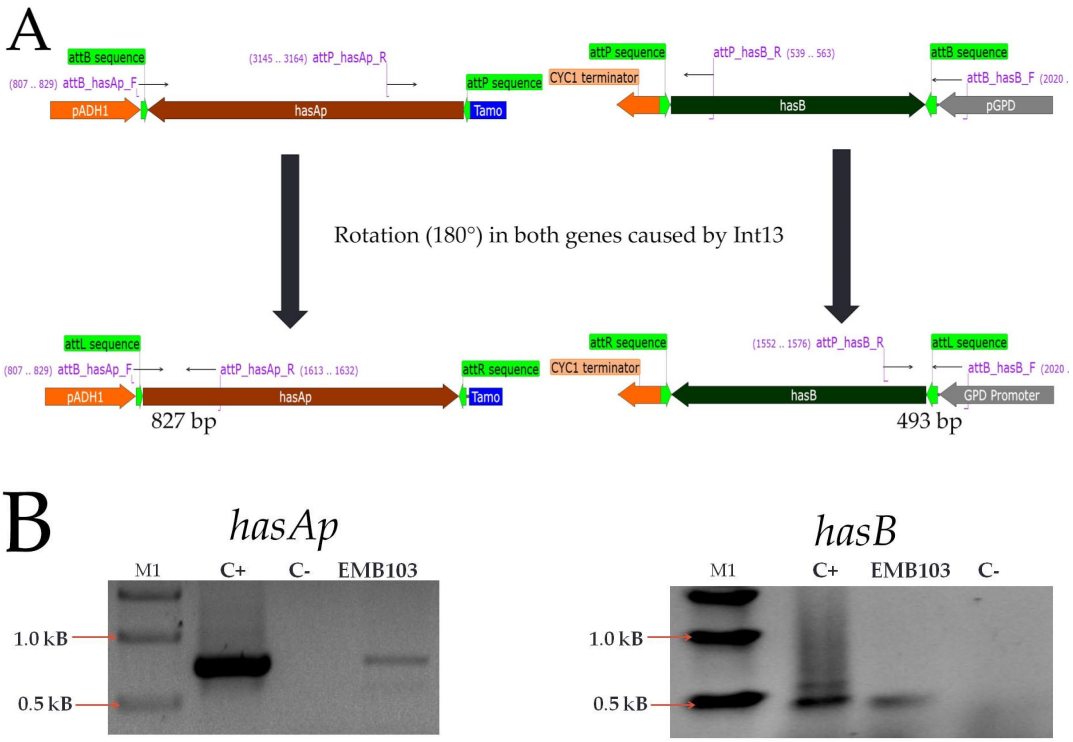


Figure S5. Confirmation of integration of both genes into the EMB104 strain by colony PCR of 12 colonies selected after the transformation and plating on selective medium. The upper gel shows amplification of the *hasB* gene (1.6 kB) using the pair of primers hasB_F and hasB_R. The bottom gel shows amplification of the *hasAs* gene between the pAOX (pMOX) promoter and the *AMO* terminator (1.5 kB) using the pair of primers AOX-Integration_F and AOX-Integration_R. The agarose content was 1% and the ladder used was 1kB Ladder M1181/M1182 (M1; Sinapse Inc). The *O. polymorpha* NCYC495 *yku80* was used as a negative control (C-) for the PCR reactions. The pHIPZ18_*hasB* and pHIPH4_*hasAs* plasmids were used as positive controls (C+) for the PCR reactions of *hasB* and *hasAs*, respectively.

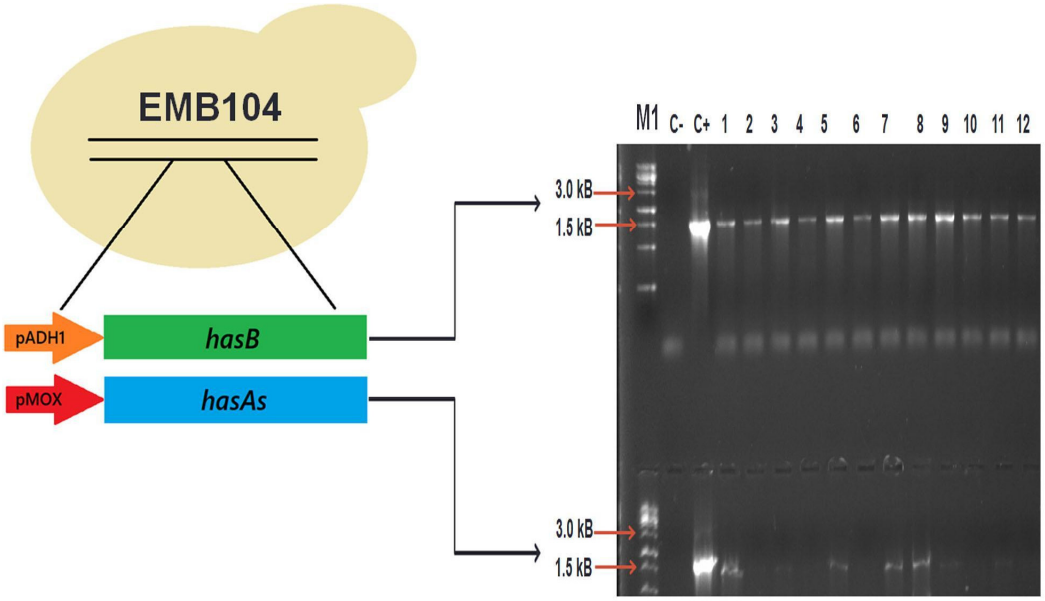


Figure S6. Map of the synthetic plasmid pBSK_*hasB* harbouring the *hasB* gene from *Xenopus laevis*. Oligonucleotide primers for amplification of *hasB* (HasB_F and HasB_R) are shown on their annealing sites.

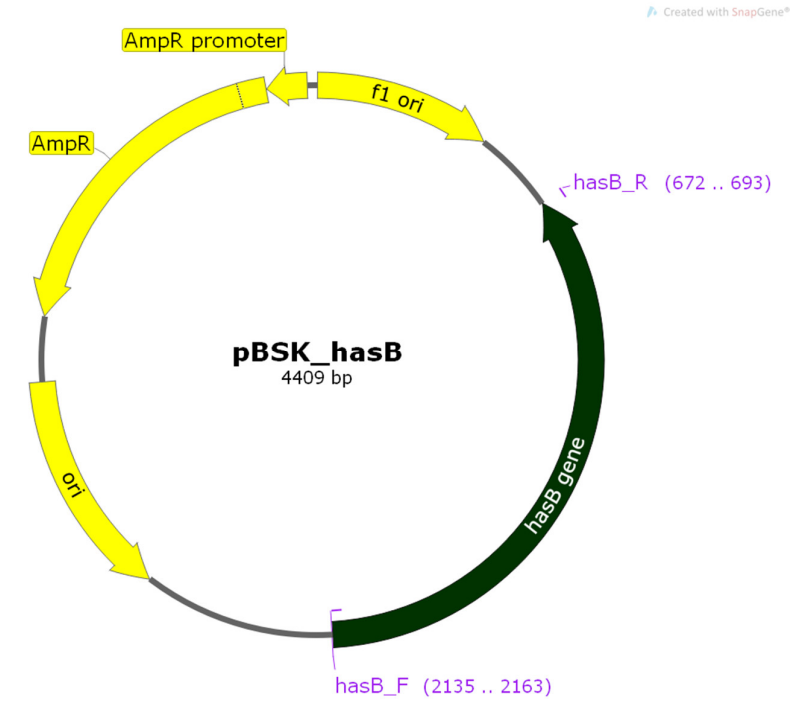


Figure S7. Map of the synthetic plasmid pBSK_*hasAp* harbouring the *hasA* gene from *Pasteurella multocida*. Primers for amplification of *hasAp* (HasAp_F and HasAp_R) are shown on their annealing sites.

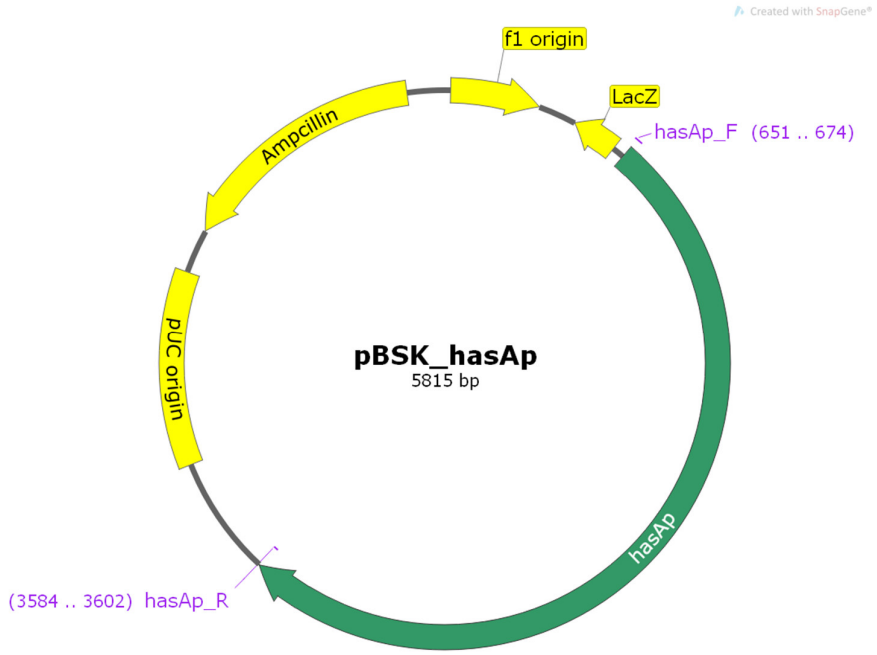


Figure S8. Map of the synthetic plasmid pBSK_*hasAs* harbouring the *hasA* gene from *Streptococcus zooepidemicus*. Restriction sites for HindIII and XbaI are shown.

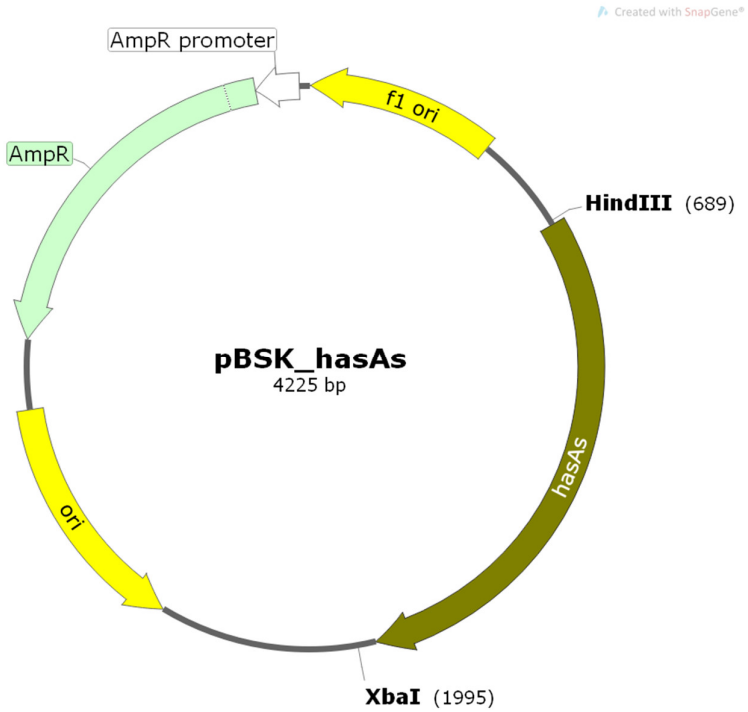


Figure S9. Map of the pGEM_*hasB* plasmid harbouring the *hasB* gene from *X. laevis*. Restriction sites for HindIII and SalI are shown.

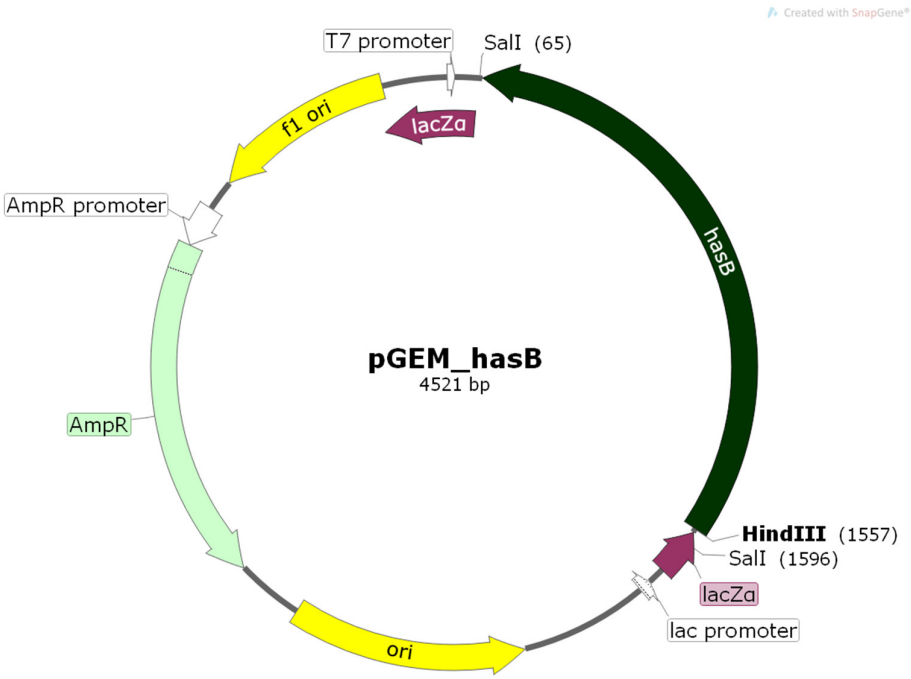


Figure S10. Map of the pHIPZ7_*hasAp* plasmid bearing the *hasAp* gene from *P. multocida*. Primers for amplification of *hasAp* (HasAp_F and HasAp_R) are shown on their annealing sites. Restriction sites for HindIII and XhoI are shown.

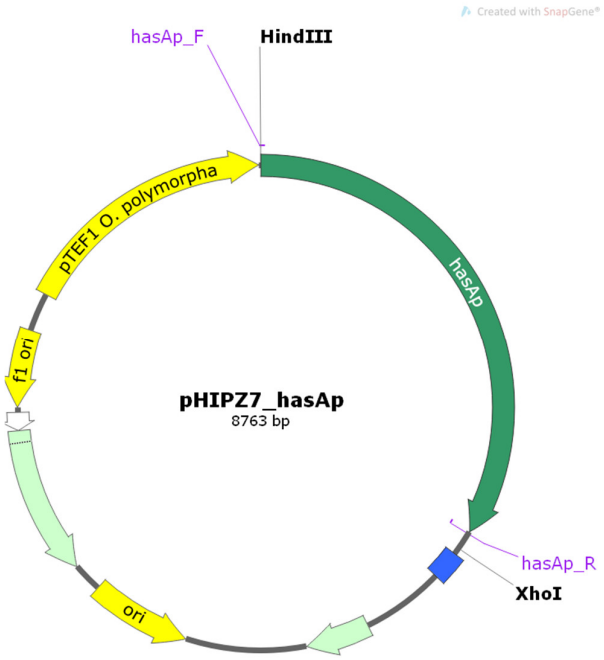


Figure S11. Map of the pHIPZ18_*hasAp* plasmid harbouring the *hasA* gene from *P. multocida*. Primers for amplification of *hasAp* (HasAp_F and HasAp_R) are shown on their annealing sites. Restriction sites for HindIII and XhoI are shown.

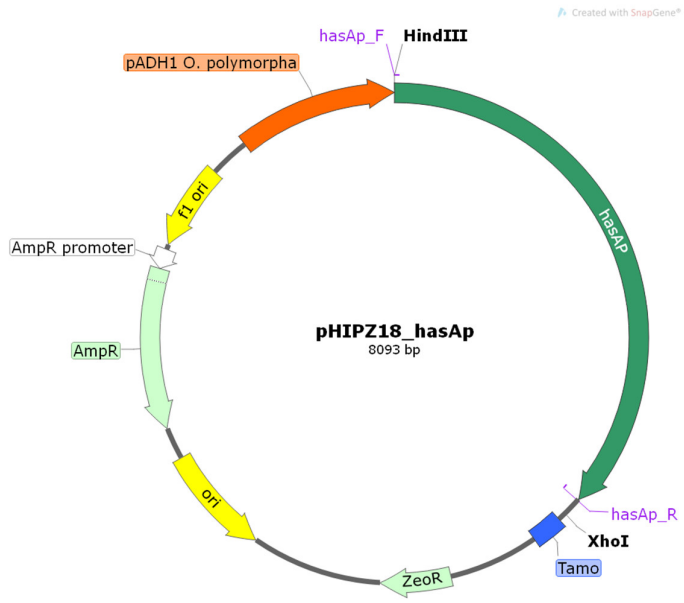


Figure S12. Map of the pHIPZ18_*hasB* plasmid bearing the *hasB* gene from *X. laevis*. Primers for amplification of *hasB* are shown on their annealing sites. Restriction sites for HindIII and SalI are shown.

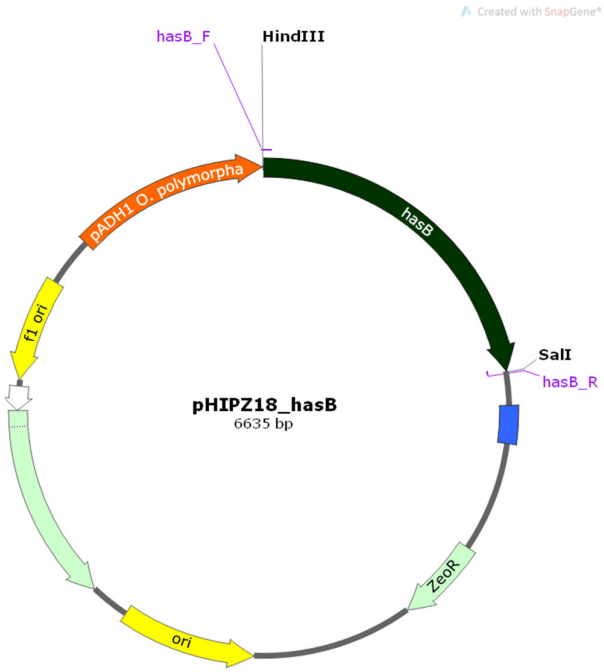


Figure S13. Map of the pHIPH4_*hasB* plasmid harbouring the *hasB* gene from *X. laevis*. Primers for amplification of *hasB* (HasB_F and HasB_R) are shown on their annealing sites. Restriction sites for HindIII and SalI are shown.

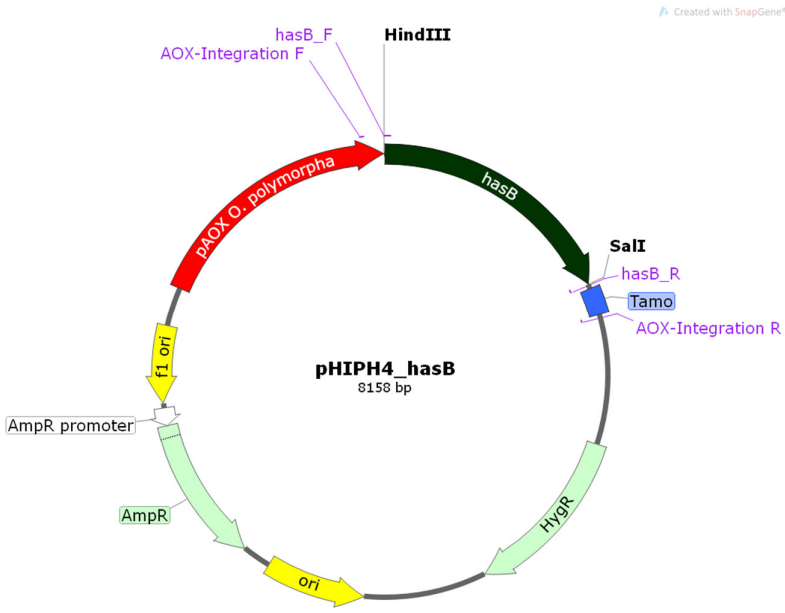


Figure S14. Map of the pHIPH4_*hasAs* plasmid harbouring the *hasA* gene from *S. zooepidemicus*. Primers for amplification of *hasA* (AOX-Integration F and AOX-Integration R) are shown on their annealing sites. Restriction sites for HindIII and XbaI are shown.

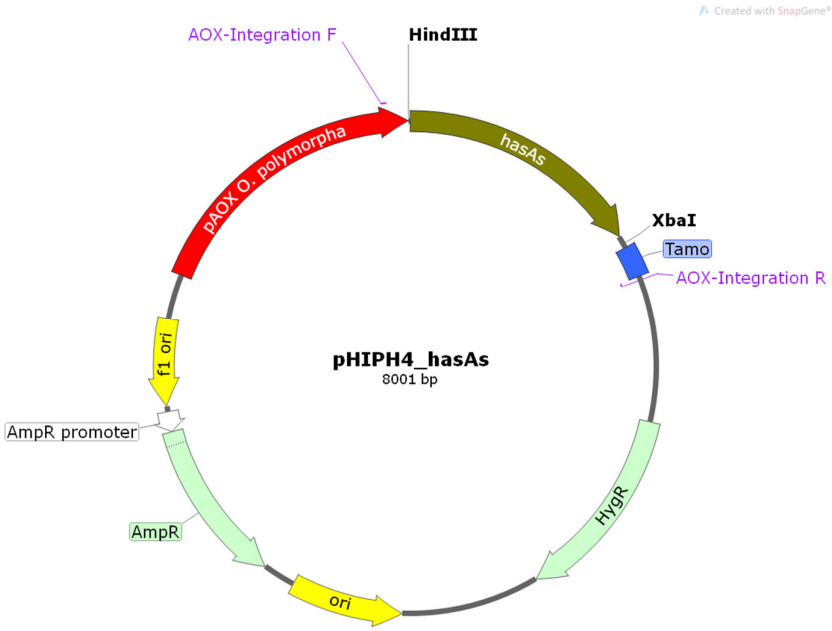


Figure S15. Map of the pHIPH4_*ScSInt13* plasmid harbouring the *hasB* gene from *X. laevis* and the *hasA* gene from *P. multocida*. Primers for amplification of *hasB* (HasB Forward and HasB Reverse) and *hasAp* (HasAp Forward and HasAp Reverse) are shown on their annealing sites.

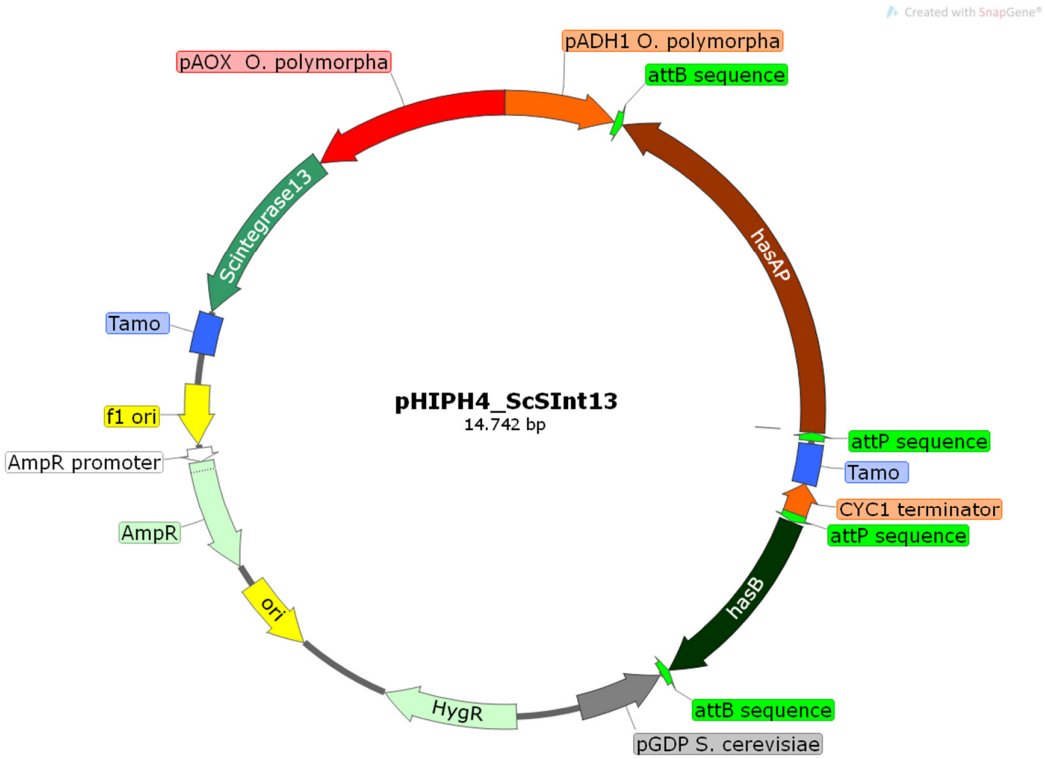


Figure S16. Standard curve obtained by the carbazole method for quantifying HA.

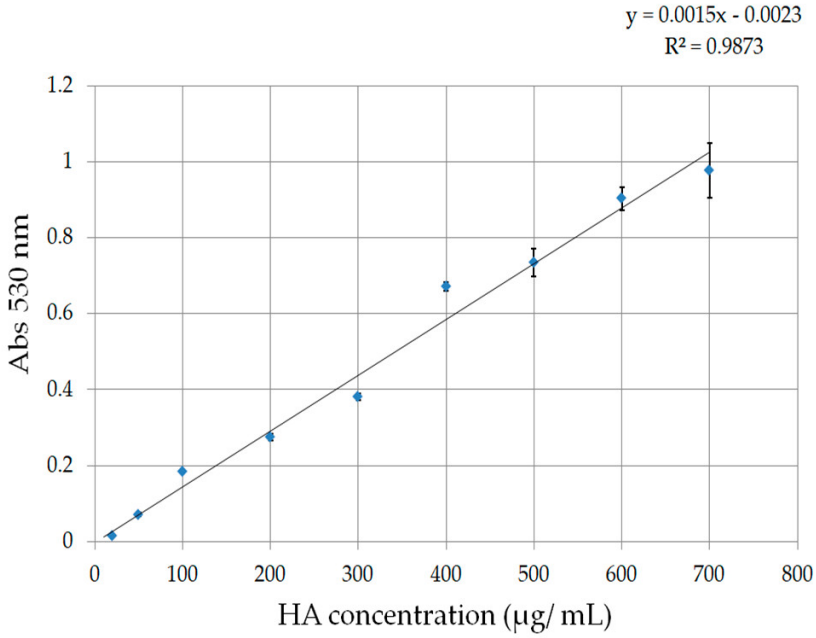
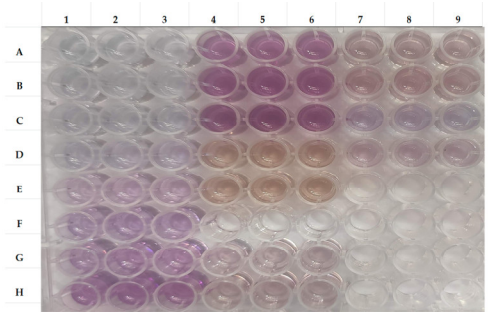


Figure S17. Carbazole assay plate picture. (A) The 96-well plate of the carbazole assay performed to quantify the HA in the supernatants after the 48 h of cultivation. (B) The plate design indicating the position of each sample in (A). I and II indicated the biological replicate. All quantifications were performed in technical replicate. The empty wells are represented with a line."

A



B

	1	2	3	4	5	6	7	8	9
A	Blank	Blank	Blank	500 µg/mL	500 µg/mL	500 µg/mL	EMB103 I	EMB103 I	EMB103 I
B	10 µg/mL	10 µg/mL	10 µg/mL	600 µg/mL	600 µg/mL	600 µg/mL	EMB103 I	EMB103 I	EMB103 I
C	20 µg/mL	20 µg/mL	20 µg/mL	700 µg/mL	700 µg/mL	700 µg/mL	EMB104 I	EMB104 I	EMB104 I
D	50 µg/mL	50 µg/mL	50 µg/mL	WT I	WT I	WT I	EMB104 II	EMB104 II	EMB104 II
E	100 µg/mL	100 µg/mL	100 µg/mL	WT II	WT II	WT II	-	-	-
F	200 µg/mL	200 µg/mL	200 µg/mL	-	-	-	-	-	-
G	300 µg/mL	300 µg/mL	300 µg/mL	EMB102 I	EMB102 I	EMB102 I	-	-	-
H	400 µg/mL	400 µg/mL	400 µg/mL	EMB102 II	EMB102 II	EMB102 II	-	-	-

References

1. van der Klei, I.J. The Hansenula polymorpha expression system Available online: https://www.rug.nl/research/molecular-cell-biology/research/the-hansenula-polymorpha-expression-system (accessed on May 19, 2020).

2. Sambrook, J.; Russell, D.W. *Molecular Cloning - A Laboratory Manual*; 3rd ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor: New York, 2001;

3. V. Gomes, A.M.; C. M. Netto, J.H.; Carvalho, L.S.; Parachin, N.S. Heterologous Hyaluronic Acid Production in Kluyveromyces lactis. Microorganisms 2019, 7, 294, doi:10.3390/microorganisms7090294.