

Supplementary Material

Non-homologous End-Joining-deficient filamentous fungal strains mitigate the impact of off-target mutations during the application of CRISPR/Cas9

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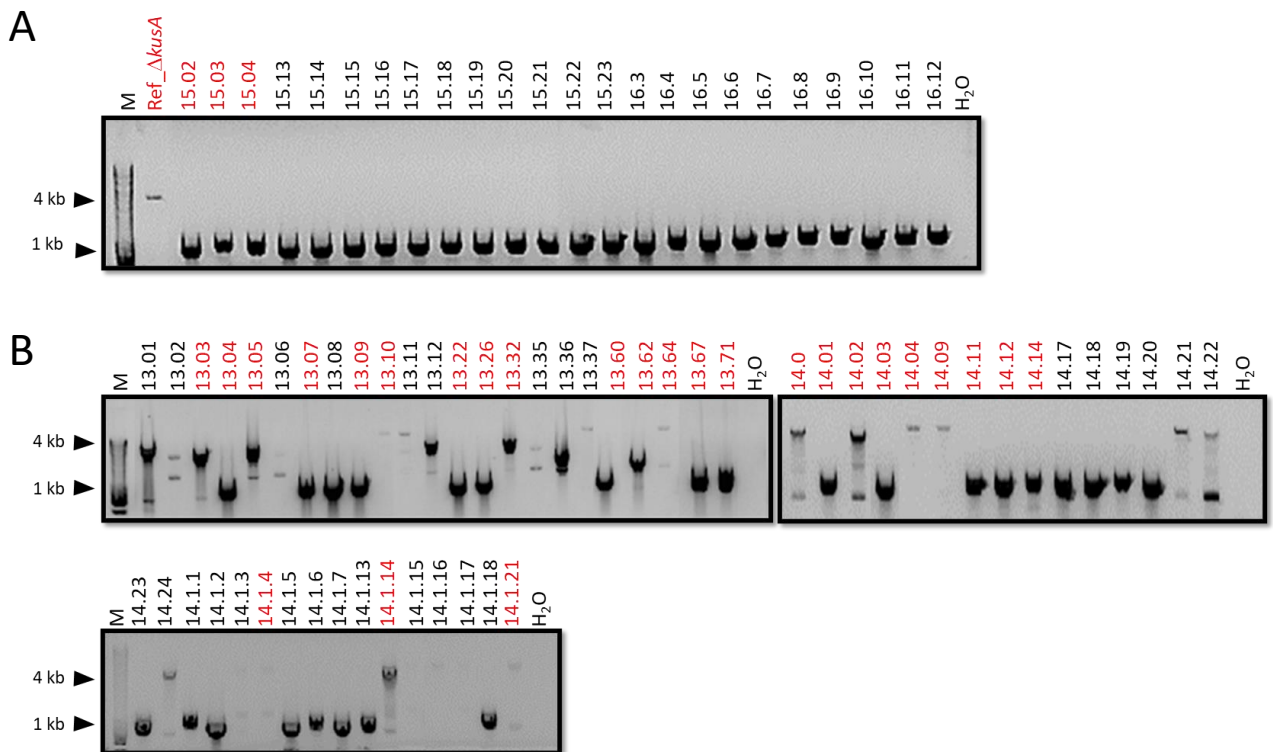
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Running Title: CRISPR/Cas9 risk assessment in fungi

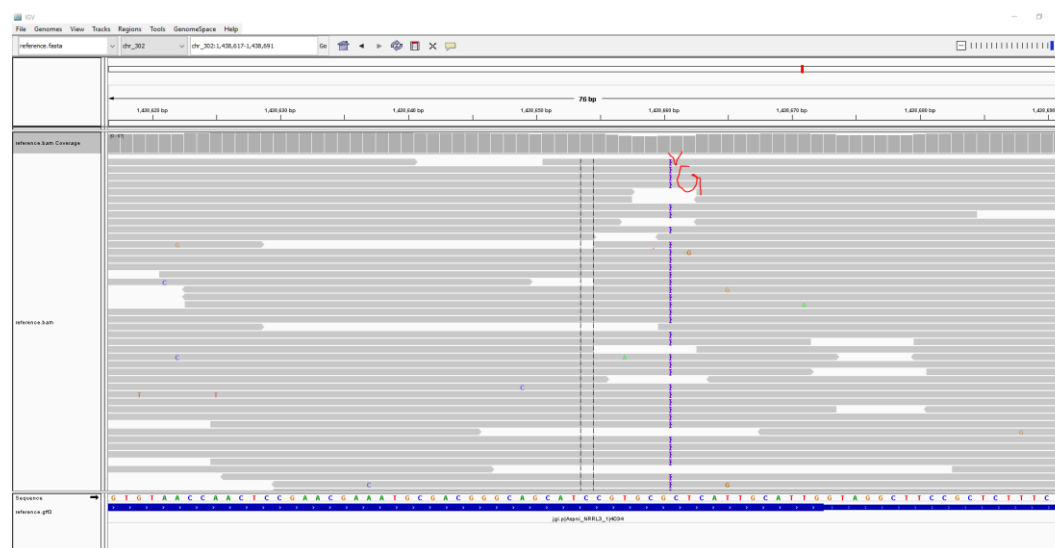


Supp. Fig S1. Molecular characterization of the $\Delta xInR$ mutants in the $\Delta kusA$ (A) and wild-type (B) genetic backgrounds. The 1 kb bands represent clean *xInR* deletions after HR in the presence of a dDNA. The 4 kb bands represent the presence of *xInR* gene compatible with NHEJ DNA repair mechanism. Note that all $\Delta kusA$ -derived strains underwent HR, while wild type-derived strains underwent both HR and NHEJ. PCRs were carried out with oligos An-*xInR*_5F and An-*xInR*_3R (Supp. Table S1). Strains for which WGS was successful are represented in red. M: Molecular marker.

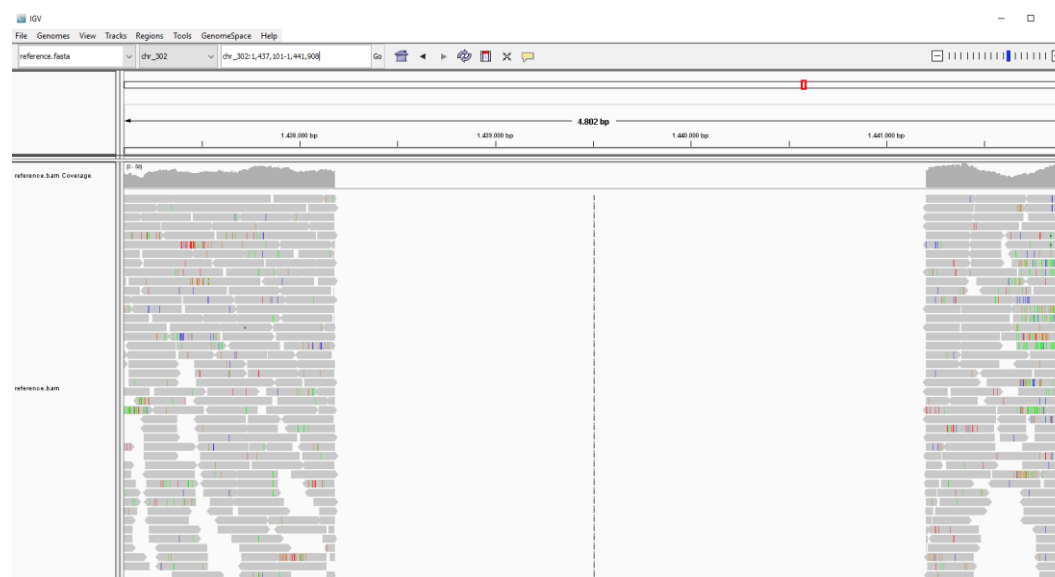
A



B



C



Supp. Fig. S2. WGS analysis confirmed different repairing patterns between HDR and NHEJ after CRISPR/Cas9 in *A. niger* wild type strains. (A) a 9bp deletion was detected in NHEJ repairing sample-13.10; (B) an insertion of extra G was detected in NHEJ repairing sample-13.35, which cause a frameshift mutation; (C) a full deletion of *XlnR* gene was detected on HDR repairing sample 13.26. All three detected variants occurred at CRISPR/Cas9 cutting position (chr_302: 1438653) resulted in dramatic function change of *XlnR* gene, which leads to a similar growth phenotype as showed in Fig. 1.

Suppl. Table S1. Average of mutations identified in the selected *A. niger* strains after WGS.

Data show mean \pm Standard Deviation (SD) of the replicate samples. Number of replicates (n) are indicated in brackets

	Transformation control (no Cas9)		Transformation with Cas9, no gRNA		Transformation with Cas9 and gRNA cassette			
	wild type (n= 8)	$\Delta kusA$ (n= 4)	wild type (n= 11)	$\Delta kusA$ (n= 3)	Wild type (NHEJ) (n= 13)	Wild type (HR) (n= 13)	Wild type (NHEJ+HR) (n=26)	$\Delta kusA$ (n= 3)
SNVs	181 \pm 78	2 \pm 0.8	183 \pm 89	7 \pm 9	317 \pm 159	282 \pm 149	300 \pm 152	4 \pm 1
Insertions	10 \pm 3	8 \pm 2	9 \pm 1	8 \pm 2	10 \pm 4	10 \pm 2	10 \pm 3	7 \pm 2
Deletions	6 \pm 2	2 \pm 1	6 \pm 3	2 \pm 1	7 \pm 3	6 \pm 3	7 \pm 3	3 \pm 2

Suppl. Table S2. Statistical analysis. Significance was referred to $p < 0.05$

Mutations	Comparisons	p value	Significance (*)
SNVs	wt no_cas9 VS. $\Delta kusA$ no_cas9	0.001	*
	wt no_cas9 VS. wt_cas9	0.973	
	$\Delta kusA$ _no_cas9 VS. $\Delta kusA$ _cas9	0.329	
	wt_ $\Delta xlnR$ _NHEJ VS. wt_ $\Delta xlnR$ _HR	0.569	
	wt_cas9 VS. $\Delta kusA$ _cas9	0.006	*
	wt_cas9 VS. wt_ $\Delta xlnR$ (NHEJ+HR)	0.023	*
	$\Delta kusA$ _cas9 VS. $\Delta kusA$ _ $\Delta xlnR$	0.616	
	wt_ $\Delta xlnR$ VS. $\Delta kusA$ _ $\Delta xlnR$	0.002	*
Insertions	wt no_cas9 VS. $\Delta kusA$ no_cas9	0.343	
	wt no_cas9 VS. wt_cas9	0.525	
	$\Delta kusA$ _no_cas9 VS. $\Delta kusA$ _cas9	0.867	
	wt_ $\Delta xlnR$ _NHEJ VS. wt_ $\Delta xlnR$ _HR	0.814	
	wt_cas9 VS. $\Delta kusA$ _cas9	0.528	
	wt_cas9 VS. wt_ $\Delta xlnR$ (NHEJ+HR)	0.181	
	$\Delta kusA$ _cas9 VS. $\Delta kusA$ _ $\Delta xlnR$	0.741	
	wt_ $\Delta xlnR$ VS. $\Delta kusA$ _ $\Delta xlnR$	0.139	
Deletions	wt no_cas9 VS. $\Delta kusA$ no_cas9	0.034	*
	wt no_cas9 VS. wt_cas9	0.935	
	$\Delta kusA$ _no_cas9 VS. $\Delta kusA$ _cas9	0.945	
	wt_ $\Delta xlnR$ _NHEJ VS. wt_ $\Delta xlnR$ _HR	0.446	
	wt_cas9 VS. $\Delta kusA$ _cas9	0.084	
	wt_cas9 VS. wt_ $\Delta xlnR$ (NHEJ+HR)	0.444	
	$\Delta kusA$ _cas9 VS. $\Delta kusA$ _ $\Delta xlnR$	0.829	
	wt_ $\Delta xlnR$ VS. $\Delta kusA$ _ $\Delta xlnR$	0.037	*

Suppl. Table S3. Primers used in this study

Primer ID	Use	TM (°C)	Sequence (5' → 3') ^a	Purpose ^b	Reference
P1-gRNA	F	57.3	CAACCTCCAATCCAATTTGACTCCGCCGAA CGTACTG	gRNA	(38)
P2-gRNA	R	54.1	ACTACTCTACCACTATTTGAAAAGCAAAAA AGGAAGGTACAAAAAAGC	gRNA	(38)
Fw-screen	F	47.9	TTTTCTCTCCATTTACGC	cse	(27)
Rev-screen	R	53.1	GGGGATCATAATAGTACTAGCCA	cse	(27)
P3- <i>xlnR</i>	R	53.2	CGGTCTCCTGGCGAGTATGCGACGAGCTT ACTCGTTTCG	gRNA	(23)
P4- <i>xlnR</i>	F	49.3	GCATACTCGCCAGGAGACCGGTTTTAGAG CTAGAAATAGCAAG	gRNA	(23)
An- <i>xlnR</i> _5F	F	55.4	GTGTGTGTGTGAGAGAGAAAGG	RT (outside), csa	(23)
An- <i>xlnR</i> _5R	R	55.1	GCATCTCATCATCAGCCGTGTGAAAGTG AGGTATTCAGACCG	RT	(23)
An- <i>xlnR</i> _3F	F	57.6	CGGTCTGAATACCTCACTTTCCACACGGCT GATGATGAGATGC	RT	(23)
An- <i>xlnR</i> _3R	R	57.2	GACGAGAGGAGTTGGTAGCG	RT (outside), csa	(23)
An- <i>xlnR</i> -NEST_5F	F	55.7	CTTTCTCGTGGGTTCTTCACC	RT	(23)
An- <i>xlnR</i> -NEST_3R	R	56.6	GGATGTAGTCGTCCAGGAGG	RT	(23)
P3- <i>gaaR</i>	R	53.2	CGTCGCGTCCATGCCGAACCGACGAGCTT ACTCGTTTCG	gRNA	(56)
P4- <i>gaaR</i>	F	49.3	GGTTCGGCATGGACGCGAGGTTTTAGA GCTAGAAATAGCAAG	gRNA	(56)
An- <i>gaaR</i> _5F	F	56.5	AAAGCAATCACGGCAACTGG	RT (outside), csa	(56)
An- <i>gaaR</i> _5R	R	55.2	GGGTATCATCGCTCTAGTATTCAGTATGT GGGAAGCGAATAGAGG	RT	(56)
An- <i>gaaR</i> _3F	F	55.6	CCTCTATTCGTTCCACATCAGTGAATAC TAGAGCGATGATACCC	RT	(56)
An- <i>gaaR</i> _3R	R	56.1	TGTAGAATACTTGCCCATAGGTCG	RT (outside), csa	(56)
An- <i>gaaR</i> - NEST_5F	F	55.9	AGAATAGCTTCTTGCTAGACTGG	RT	(56)
An- <i>gaaR</i> - NEST_3R	R	56	CGTGGACGCATTCTGTATG	RT	(56)
P3- <i>araR</i>	R	53.2	CCCAGAAAGTCAGGGCACACGACGAGCT TACTCGTTTCG	gRNA	(56)
P4- <i>araR</i>	F	49.3	GTGTGCCCTGACTTTCTGGGTTTTAGAG CTAGAAATAGCAAG	gRNA	(56)
An- <i>araR</i> _5F	F	59.2	GTCCGCAAGTTGTGTGGTGG	RT (outside), csa	(56)
An- <i>araR</i> _5R	R	56.2	GCATCGGTGCTGTGAGAAACGGAATCGC AGTCTGATGAAACG	RT	(56)
An- <i>araR</i> _3F	F	56.7	CGTTTCATCAGACTGCGATTCCGTTTCTCA CAGCACCAGTGC	RT	(56)
An- <i>araR</i> _3R	R	57.7	AACCGAGAAGCCAGTTTCG	RT (outside), csa	(56)
An- <i>araR</i> - NEST_5F	F	57.8	GAAGCGACCTCATAGCGACC	RT	(56)
An- <i>araR</i> -NEST-3R	R	56.7	ATGCCAGAAACATGCGATGC	RT	(56)
P3- <i>rhaR</i>	R	53.2	CGCTGTGATCCCGTTCAGAGACGAGCTT ACTCGTTTCG	gRNA	(56)
P4- <i>rhaR</i>	F	49.3	TCTGGAACGGGATCACAGCGGTTTTAGA GCTAGAAATAGCAAG	gRNA	(56)
An- <i>rhaR</i> _5F	F	56.5	GTGCGGATCTCTTGATGCG	RT (outside), csa	(56)

An-rhaR_5R	R	56.1	GCGATAAACTGCCTCAAAGATGTTTTATC GGGAGATAGACAGGTGC	RT	(56)
An-rhaR_3F	F	55.7	GCACTGTCTATCTCCGATAAAACATCTT TGAGGCAGTTTATCGC	RT	(56)
An-rhaR_3R	R	55.4	CATCTTGACTTAGTGAACAGGAGC	RT (outside), csa	(56)
An-rhaR- NEST_5F	F	55	CAGTAGTTTAGCGGAGTGGG	RT	(56)
An-rhaR- NEST_3R	R	55.7	ACTCACCTCTTCTCATTCTCG	RT	(56)
P3-galX	R	53.2	ACGCGTTTGGTGGACACTAT GACGAGCTT ACTCGTTTCG	gRNA	(56)
P4-galX	F	49.3	ATAGTGCCACCAAACGCGT GTTTTAGAG CTAGAAATAGCAAG	gRNA	(56)
An-galX_5F	F	56.4	CGTAGTTGAATGCGACCTGC	RT	(56)
An-galX_5R	R	56.7	CCTTTCAGAGCTTTGAGATGGAAGAGGGA AGAATGAGAAGTCAACCG	RT	(56)
An-galX_3F	F	55.7	CGGTTGACTTCTATTCTCCCTCTCCATC TCAAAGCTCTGAAAGG	RT	(56)
An-galX_3R	R	56.9	AACAATTCAATTCGAGAAGCAGC	RT (outside), csa	(56)
An-galX-NEST_5F	F	57.5	GCTCAAAGTGGGACAATGC	RT (outside), csa	(56)
An-galX-NEST_3R	R	56.4	CCAGTGCATCAGAAACACG	RT	(56)
P3-clrB	R	53.2	TCCTTTCTTCAGACCTGAGC GACGAGCTTA CTCGTTTCG	gRNA	(24)
P4-clrB	F	49.3	GCTCAGGTCTGAAGAAAGGAG TTTTAGA GCTAGAAATAGCAAG	gRNA	(24)
An- clrB_5F/NEST_5F	F	56.7	ATCACACAACCCTTCTCGTACC	RT	(24)
An-clrB_5R	R	56.4	CGATAGCGAATCCTAGCAGTTGCTGGAGT TTTTGTTTGACGG	RT	(24)
An-clrB_3F	F	55.5	ACTGCTAGGATTCGCTATCGCTGTACTTAC AGGGTGCAGC	RT	(24)
An- clrB_3R/NEST_3R	R	55	GGATGGATCGTCTTAGGATGC	RT	(24)
An-clrB_F	F	57.5	GTGTGACTGTCCCCTTCTCC	csa	(24)
An-clrB_R	R	56.4	TTCCTTATGTAGCCCCAGAGC	csa	(24)
P3-amyR	R	53.2	TGCGATAAGTGTGACGCCT GACGAGCTT ACTCGTTTCG	gRNA	(Kun <i>et al</i> , unpublished)
P4-amyR	F	49.3	AGGCGTCGACACTTATCGCAG TTTTAGAG CTAGAAATAGCAAG	gRNA	(Kun <i>et al</i> , unpublished)
An-amyR_5F	F	57.1	TGGGATGTTACCAGTGTACG	RT (outside), csa	(Kun <i>et al</i> , unpublished)
An-amyR_5R	R	57.9	CGATAGCGAATCCTAGCAGTGGGAGACA AGTGTGACTCC	RT	(Kun <i>et al</i> , unpublished)
An-amyR_3F	F	55.8	ACTGCTAGGATTCGCTATCGCAACTACGA CGATGACGATGC	RT	(Kun <i>et al</i> , unpublished)
An-amyR_3R	R	57.8	CACCGTGACCCAGAGAAAGG	RT (outside), csa	(Kun <i>et al</i> , unpublished)
An-amyR- NEST_5F	F	57.3	GAGCCTCAGACTCTGTGAGC	RT	(Kun <i>et al</i> , unpublished)
An-amyR- NEST_3R	R	55.4	TCCACCACCATCAAATCACC	RT	(Kun <i>et al</i> , unpublished)
P3-inuR	R	53.2	ACGTCTTCTTCGAGCTAAG ACGAGCTT ACTCGTTTCG	gRNA	(Kun <i>et al</i> , unpublished)
P4-inuR	F	49.3	TTAGCTCGAGAAGAAGACGT GTTTTAGA GCTAGAAATAGCAAG	gRNA	(Kun <i>et al</i> , unpublished)
An-inuR_5F	F	57	TCGGAGGAGATACGCAAAGC	RT (outside), csa	(Kun <i>et al</i> , unpublished)

An- <i>inuR</i> _5R	R	55.7	CGATAGCGAATCCTAGCAGTCTCACGAGT CATAAGCATTGTCCG	RT	(Kun <i>et al</i> , unpublished)
An- <i>inuR</i> _3F	F	55.7	ACTGCTAGGATTCGCTATCGTGGCGTTCT GGTTTTATCACG	RT	(Kun <i>et al</i> , unpublished)
An- <i>inuR</i> _3R	R	57.7	ATTGACGGGCATGAGTGTCC	RT (outside), <i>csa</i>	(Kun <i>et al</i> , unpublished)
An- <i>inuR</i> -NEST_5F	F	57.4	TAAGAACAACGACCCCATCGC	RT	(Kun <i>et al</i> , unpublished)
An- <i>inuR</i> -NEST_3R	R	58.5	AAGGGAAACCGCCAATCAGC	RT	(Kun <i>et al</i> , unpublished)
P3- <i>clrA</i>	R	53.2	TGGAAGAATTCGATGACAGG GACGAGCT TACTCGTTTCG	gRNA	(24)
P4- <i>clrA</i>	F	49.3	CCTGTATCGAATCTTCCA GTTTTAGAGC TAGAAATAGCAAG	gRNA	(24)
An- <i>clrA</i> _5F/NEST_5F	F	55.1	TCAGCGAAACAGTAAGAACG	RT	(24)
An- <i>clrA</i> _5R	R	54.9	CGATAGCGAATCCTAGCAGTGTGTTGTG TTCAAGGGTGC	RT	(24)
An- <i>clrA</i> _3F	F	55.6	ACTGCTAGGATTCGCTATCGCATCATACGC ATCTTGTCAGTCC	RT	(24)
An- <i>clrA</i> _3R/NEST_3R	R	56.4	GGCAGTATTAACCAGGCTTGC	RT	(24)
An- <i>clrA</i> _F	F	56.9	TGTCAGTCTCGCCAACATACC	<i>csa</i>	(24)
An- <i>clrA</i> _R	R	55.8	AGTGCAAGATGCTGTCTTTTTGG	<i>csa</i>	(24)
P3- <i>creA</i>	R	53.2	CGGTGAGTGAGCGGGAGTC GGACGAGCT TACTCGTTTCG	gRNA	(Kun <i>et al</i> , unpublished)
P4- <i>creA</i>	F	49.3	CGACTCCCGTCACTCACC GGTTTTAGAGC TAGAAATAGCAAG	gRNA	(Kun <i>et al</i> , unpublished)
An- <i>creA</i> _5F	F	57	ACTTCTCCAGACCACACAGC	RT (outside), <i>csa</i>	(Kun <i>et al</i> , unpublished)
An- <i>creA</i> _5R	R	55	CGATAGCGAATCCTAGCAGTCGACGAGG GTAAAAAGGTAGG	RT	(Kun <i>et al</i> , unpublished)
An- <i>creA</i> _3F	F	56.6	ACTGCTAGGATTCGCTATCGCGAACATTCT TCAGCCACACG	RT	(Kun <i>et al</i> , unpublished)
An- <i>creA</i> _3R	R	55	TCATTCTGACTGACGCAATCC	RT (outside), <i>csa</i>	(Kun <i>et al</i> , unpublished)
An- <i>creA</i> -NEST_5F	F	56.6	TAGGCAACTGATGGAGACACC	RT	(Kun <i>et al</i> , unpublished)
An- <i>creA</i> -NEST_3R	R	56.9	GACGTTTGGGGTAAACAAAGCC	RT	(Kun <i>et al</i> , unpublished)

F: forward, R: reverse, TM: temperature of melting

^aThe gRNA sequence is highlighted in bold

^bgRNA single guide, RNA RT repair template, BC with barcodes, TR traceability, cse colony screening for *E. coli*, *csa* colony screening for *A. niger*