Unfiltered: A rapid method for *Agrobacterium*-mediated transformation of Basidiomycete and Ascomycete yeasts

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Abstract

Agrobacterium-mediated transformation (AMT) is a critical method for genetic manipulation of non-conventional fungi, yet it remains a laborious and inefficient technique. Here we demonstrate that eliminating the time-consuming filtration step frequently incorporated in AMT fungal transformation protocols not only reduces hands-on time from over four hours to 15 minutes, but also increases transformation efficiency by 69% in the lipogenic yeast, *Rhodotorula toruloides*. We further optimized the *Agrobacterium*:yeast cell ratio and culture resuspension volume to achieve an efficiency > 210,000 CFU per transformation representing a 2-3 fold improvement over previously implemented protocols. This simplified “unfiltered” spot-plating method was successfully applied to seven yeast species, including one for which genetic transformation has not previously been reported, *Botryozyma nematodophila.* This approach enables high-throughput transformation workflows that are critical for genome-scale functional studies across diverse yeast systems.

Introduction

Fungi have proven to be essential for basic biological research and applied technical applications in pharmaceuticals, bioproduction, and food fermentation. The investigation and biotechnological use of these important organisms is often hindered by our inability to efficiently transform heterologous genetic material into these species. Unlike *Saccharomyces sp.* and *Pichia (Komagataela) sp.*, many fungal species are recalcitrant to transformation via traditional methods of electroporation or chemical transformation, often requiring laborious protoplasting protocols [1]. This makes *Agrobacterium*-mediated transformation (AMT) a critically advantageous method for introducing heterologous DNA and making genetic knockouts.

AMT leverages the naturally pathogenic relationship between *A. tumefaciens* and plants. In a mechanism analogous to bacterial conjugation, *Agrobacterium* transmits pathogenic DNA into plant cells, resulting in *de novo* plant cell division and tumor formation, the characteristic symptom of crown-gall disease. The *Agrobacterium* genome contains a tumor-inducing (Ti) plasmid that encodes the machinery to horizontally transmit DNA (*vir* genes) as well as the oncogenes themselves. These oncogenes are encoded in a region called the transfer-DNA (T-DNA) and are flanked by defined DNA sequences known as the right border (RB) and left border (LB). With help from the *vir*-genes, the T-DNA between these borders is excised, “injected” into recipient cells, chaperoned to the nucleus, and integrated into the host genome [2].

Capitalizing on *Agrobacterium*’s plant-genome transformation capacity, plant biologists domesticated *Agrobacterium* by removing oncogenes from the Ti plasmids and porting the LB and RB sequences into smaller synthetic plasmids known as binary vectors [3–5]. By cloning transgenes between these conserved border sequences, biologists are able to introduce novel transgenic sequences directly into the plant genome, revolutionizing plant genetics and engineering [6]. Later, AMT was implemented in yeast and fungal species [7]. The original description of the protocol used vacuum filtration to embed yeast and *Agrobacterium* onto a filter before transferring the filter onto an induction medium plate, a protocol presumably modified from standard practice for bacterial conjugation [8]. This step of vacuum filtration adds significant time, labor, batch-variability and risk of contamination to this workflow. Mitigating these challenges is important for the widespread adoption of this very effective transformation method.

Since this original publication, modifications to the protocol have been made, such as changing the choice of filter polymer and the method of embedding cells onto the filter [9]. Despite this, only one described protocol bypasses the filter altogether and simply mixes and spots the mixture of Agrobacterium and yeast onto the induction medium directly [10]. A direct comparison of the "unfiltered" protocol with the standard filtration method has, to our knowledge, not been reported. Here, we present such a comparison and demonstrate the clear advantages of an optimized "unfiltered" approach.

Results and Discussion

Spot plating improves transformation efficiency, reduces protocol time, and does not impact transformation outcomes compared to filtered transformations.

In this study, we primarily focused on the yeast, *Rhodotorula toruloides* IFO0880 (NBRC 0880/NRRL 1588), as we can routinely achieve more than 10,000 colonies per transformation in this species using the standard filter-based AMT method. All transformations in this host were conducted with the binary vector plasmid, pVL_sR2rn_f14rg, containing the *R. toruloides* TUB2 promoter (R2) driving a codon optimized *nrsR* (rn), and the P14 promoter (14) driving a codon optimized sfGFP (rg) [11].

The standard protocol is briefly described here: *Agrobacterium tumefaciens* strain EHA105 carrying a desired binary vector is cultured in LB to an OD of ~1. The culture is then centrifuged, washed, and incubated overnight in an equal volume of induction medium. The target yeast is then cultured to an OD of ~1 in YPD (4.5E7 cells for *Rhodotorula*). One milliliter of yeast is pelletted, the YPD is aspirated and the pellet resuspended with one milliliter of induced *Agrobacterium*. The cell mixture is then pulled onto a 0.45 µM filter under vacuum, and the filter is placed onto an induction media plate for 4-5 days. The co-culture is then removed from the filter, resuspended in YPD, and dilutions are plated onto selective media with the addition of penicillins (cefotaxime, carbenicillin, or timentin) to prevent *Agrobacterium* growth.

The process of vacuum-pulling and drying the mixed culture of yeast and *Agrobacterium* onto a filter is incredibly laborious and time-consuming. With standard bench-top vacuum pumps (0.1-0.4 bar when sealed), this can vary from 10 minutes to an hour for each filter, hindering higher-throughput transformation workflows. Using one filtration apparatus, making a pooled library of 2*10^6 transformants would require dozens of filters and hours of hands-on labor [12]. To demonstrate this labor requirement, three researchers were tasked with conducting and timing the standard protocol and a modified protocol using the same batches of *Agrobacterium* and *Rhodotorula*. Two of the three researchers completed 6 replicates of the standard protocol in approximately three hours. One researcher had a fault in their vacuum apparatus that could not be identified, and they completed 6 filtration replicates in 5 hours. This extended period resulted in extensive contamination, and their data was unviable.

In contrast, the modified “unfiltered” protocol took approximately 15 minutes to complete 6 replicates for all three researchers. This method simply required centrifuging the mixture of *Rhodotorula* and *Agrobacterium*, aspirating the supernatant, resuspending the cell pellet, and placing the entire mixture as a droplet directly onto the induction medium plate (“spot-plating”). These plates were left to dry for an additional 10 minutes in a biosafety cabinet.

Notably, the spot-plating method resulted in significantly more transformants than the traditional filter method. Two researchers each completed 6 replicates of both methods using cells from the same culture. The filter method yielded an average of 67,233 ± 14,269 CFU per transformation (range: 50,400-95,200), while the spot method produced 111,600 ± 19,782 CFU (range: 64,000-143,040) (Figure 1A). This represents a 69% increase in transformation efficiency for the unfiltered method relative to the standard protocol ($p < 0.001$, two-tailed *t*-test). This improvement was consistent across the transformation efficiencies obtained by both researchers, indicating the method’s reproducibility.

Transformation efficiency is not the only metric critical for a transformation protocol, as the genetic outcome of the transformation must also be assessed. To test if the transformation outcome was equivalent between the two protocols, we estimated the transgene copy number in 96 colonies from each method using fluorescence intensity as a proxy for GFP insertions [13]. Transformed colonies were cultured in YPD, and their fluorescence was measured using a plate-reader. The levels of fluorescence in these populations were indistinguishable, suggesting that both methods yielded similar copy numbers of transgenic integration events (Figure 1B).

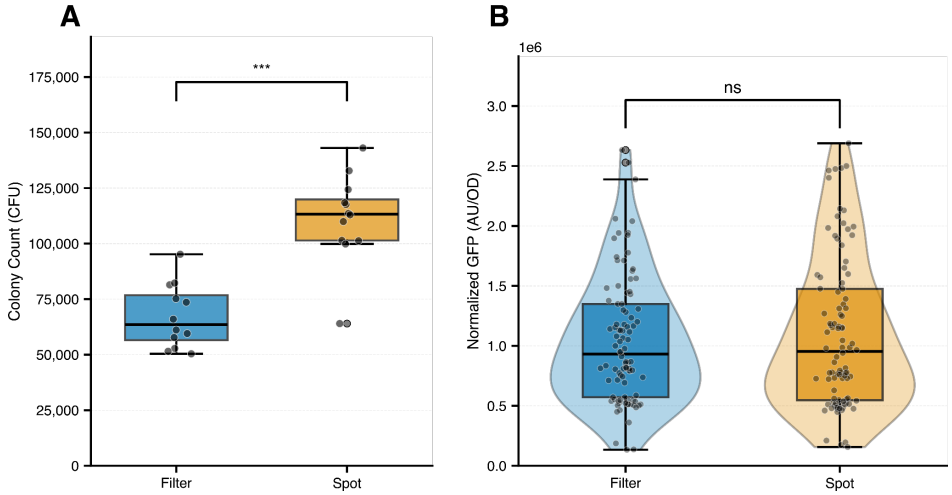


Figure 1: Comparison of filter and spot methods in *Rhodotorula toruloides*. A. Transformation results in terms of number of CFUs from the filter and spot plating methods (n = 12, p = 0.000004 Welch’s t-test). **B.** Comparison of the transformation outcomes in terms of GFP intensity from randomly selected colonies from transformants in 1A (n = 96 per group, p = 0.5364 Welch’s t-test, p = 0.99 Mann-Whitney U). All transformations were conducted with the binary vector pVL_sR2rn_f14rg.

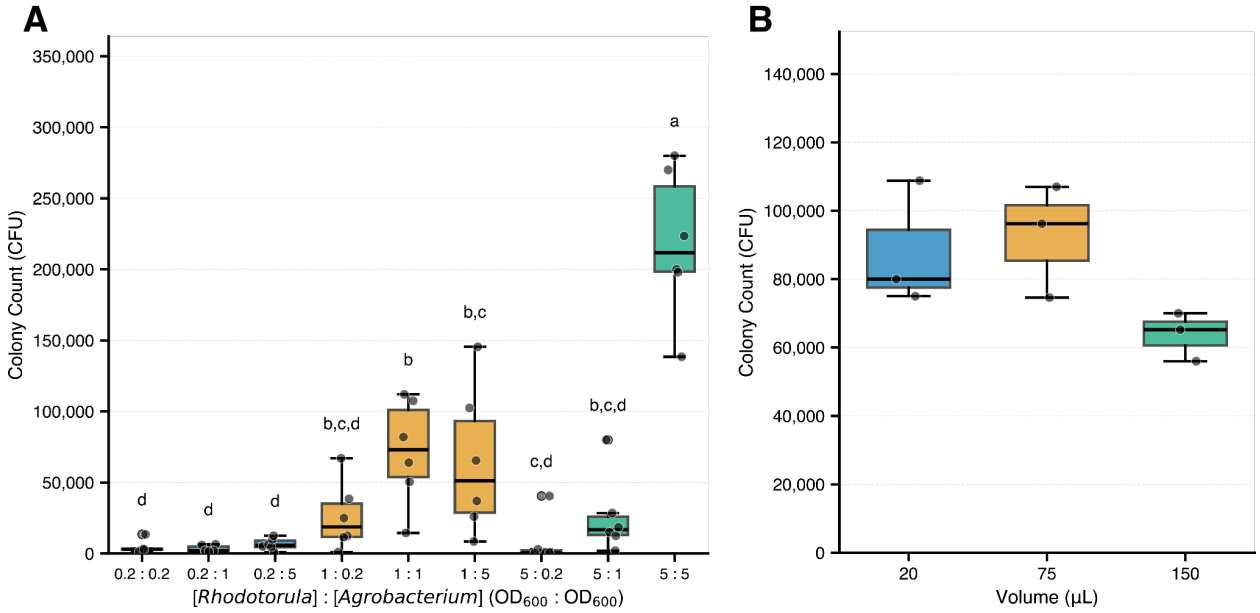
141

142 ***Agrobacterium*:Yeast Ratio contributes to transformation efficiency.**

143 To further optimize the unfiltered method, we systematically tested *Rhodotorula* to
144 *Agrobacterium* ratio combinations ranging from 0.2 to 5.0 OD·mL for both species. All 9
145 ratio combinations tested produced viable transformants, but efficiency varied
146 dramatically across ratios (500 to 280,000 CFU). The highest transformation efficiency
147 was achieved with a *Rhodotorula*:*Agrobacterium* ratio of 5.0:5.0 (mean: 218,333),
148 representing a 3-fold improvement over the standard 1:1 ratio (mean: 71,750 CFU)
149 (Figure 1A). When the concentration of *Rhodotorula* cells is high, efficient transformation
150 requires higher concentrations of *Agrobacterium*. This could reflect increased T-DNA
151 delivery events from improved cell-cell contact during co-cultivation.

152 The size of the co-culture spot could additionally influence cell-cell contact. To evaluate
153 this, we tested three resuspension volumes (20 µL, 75 µL, and 150 µL) using the standard
154 OD·mL ratio (1:1). The 75 µL volume produced the highest colony counts (mean: 92,600
155 CFU), compared to 87,933 for 20 µL and 63,733 for 150 µL; however, these results were
156 not statistically significant (Figure 2B). It is possible that upon drying, the resulting spot
157 has sufficient cell-cell contact to enable effective AMT.

158



159

160 **Figure 2: Cell ratio and spot volume effects on transformation efficiency**. **A.** Transformation efficiency
161 with different proportions of *Agrobacterium* and *Rhodotorula*. Letters above each group denote statistically
162 distinct groups based on Tukey's HSD post-hoc test (FWER = 0.05) following a significant one-way ANOVA
163 ($F = 30.56$, $p < 0.0001$). Groups sharing a letter are not significantly different from one another. **B.**
164 Transformation efficiency with different spot-volumes (n=3). There was no statistically significant difference
165 between any of the volumes tested ($p > 0.05$)

166

167 **Unfiltered method is effective across species in diverse genera**

168 To demonstrate broader applicability of the unfiltered spot-plating method for other yeast
169 species, we first compared the filter and spot plating methods in *Saccharomyces*
170 *cerevisiae* and *Kluyveromyces marxianus* . For *S. cerevisiae*, we constructed a plasmid
171 (pVR_sATk_fS3sg_HR) containing the *Ashbya gossypii* TEF1 promoter (AT) driving a
172 G418 resistance cassette (k), the *S. cerevisiae* TDH3 promoter (S3) driving SG12 (sg)
173 expression, and homology arms targeting the *tau* locus of chromosome XVI.
174 Transformation of *K. marxianus* was conducted with pVR_sATk_fS3sg, an otherwise
175 identical plasmid without homology arms. The unfiltered method yielded fewer colonies
176 for both hosts. Though this decrease was not statistically significant for *S. cerevisiae* ($p >$
177 0.05) but was highly significant for *K. marxianus* ($p < 0.001$) (Figure 3). *S. cerevisiae*
178 yielded 99,400 ± 39,972 and 66,400 ± 19604 transformants in the filter and spot plating
179 methods, respectively. *K. marxianus* yielded 3,860 ± 811 and 880 ± 421 transformants in
180 the filter and spot plating methods respectively. However, the 5 replicates for each host
181 required between 15 to 45 minutes for each filter to be pulled and dried (3 hours total),
182 while the spots were conducted in parallel and finished in 30 minutes (accounting for dry-
183 time in a biosafety cabinet). The cause for reduced efficiency when spot plating in these
184 hosts could be due to any number of morphological differences relative to *Rhodotorula*
185 *toruloides*. Notably, *Rhodotorula* cell walls contain distinct exopolysaccharides rich in
186 mannose that may affect *Agrobacterium* adhesion [14].

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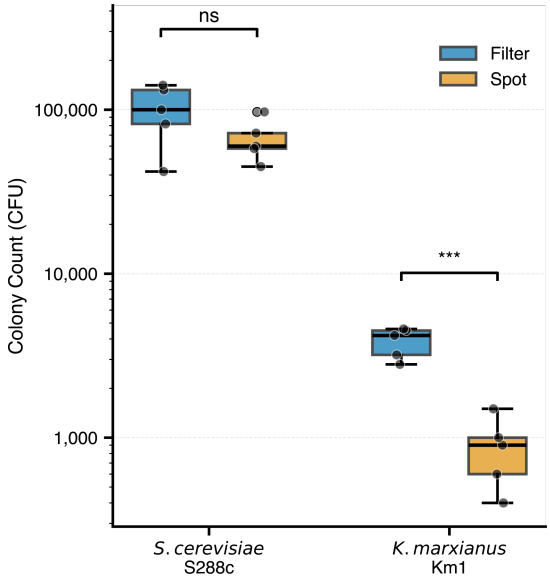

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189 **Figure 3: Comparison of filter and spot methods in *S. cerevisiae* and *K. marxianus*.** Transformation
190 results in terms of number of CFUs from the filter and spot plating methods. *** $p < 0.001$ Welch's t-test.

191 Another five phylogenetically diverse *Ascomycete* yeasts were tested with the unfiltered
192 spot-plating method: *Metschnikowia pulcherrima*, *Pichia pastoris*, *Ogataea*
193 *parapolymorpha*, *Lipomyces tetrasporus*, and *Botryozyma nematodophila*. Notably,

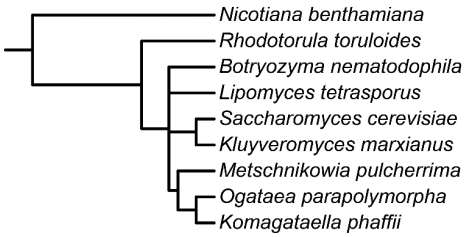
genetic transformation of *Botryozyma sp.* have not been previously reported (Figure 4A and 4B). Nourseothricin was found to be an effective antifungal against all species, so each was transformed with variations of the *nrsR* gene with different promoters: the wildtype *Streptomyces nourseii nrsR* (n) two codon optimized *nrsR* variants (on and ln), the *A. gossypii* TEF1 promoter (AT), and the *L. starkeyii* TRPC (LR) promoter.

All variants of the selection cassette worked to varying degrees in all hosts except *M. pulcherrima* (Table S3). *M. pulcherrima* is a CTG-Ser yeast and could only be successfully transformed with the codon optimized *nrsR* cassettes, both of which lacked CTG codons [15]. Transformation efficiency varied by species (from <100 to >100,000 colonies), possibly reflecting differences in morphology (Figure 4C), cell wall composition, T-DNA integration efficiency, selection marker expression, or preferred DNA repair mechanisms (HR vs NHEJ). Nonetheless, the fact that all seven species could be transformed via this protocol demonstrates that the unfiltered method is broadly applicable across diverse yeast lineages.

A

Strain	Plasmid	Average	Range
Kluyveromyces marxianus	pVR_sATn_fS3sg	3433	1620 - 6410
Metschnikowia pulcherrima	pVR_sLRln_fLTsg	30333	10000 - 59000
Ogataea parapolymorpha	pVR_sATon_fS3sg	120	70 - 220
Pichia pastoris	pVR_sATon_fS3sg	677	400 - 900
Lipomyces tetrasporus	pVR_sLRln_fLTsg	2027	1080 - 2540
Botryozyma nematodophila	pVR_sLRln_fLTsg	110	80 - 130

B



C

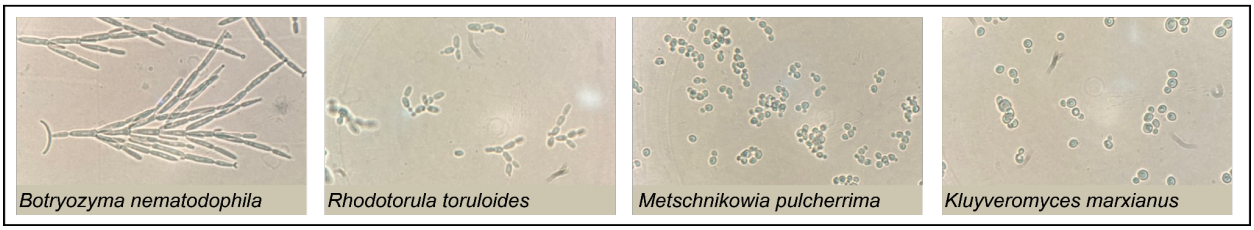


Figure 4: Transformation of diverse Ascomycete yeasts. A. Table of select transformation results for diverse Ascomycete yeasts. Complete table available in Table S3. All transformations were conducted using the spot plating method with *Agrobacterium*:yeast OD ratios of 1:1. **B.** Family tree of tested microbes with *Nicotiana benthamiana* as an outgroup. **C.** Microscopy illustrating the diverse morphologies of select tested microbes.

Conclusions

The unfiltered spot-plating method reported and optimized here streamlines AMT of non-model fungal species, a historically labor-intensive, time-consuming, and inconsistent

221 process. By eliminating the filtration step entirely, this protocol reduces hands-on time
222 from several hours to approximately fifteen minutes, notably increasing the speed and
223 feasibility of AMT for large-scale functional genomic experiments. We furthermore found
224 that this method improved transformation efficiency relative to previously filter plating
225 protocols by up to 69% for *R. toruloides*. By systematic optimization of cell ratios and
226 resuspension volumes, we were able to obtain regular transformation yields higher than
227 200,000 colonies per transformation. This enables transformation of large libraries at
228 appropriate representation and distribution. Our successful transformation of seven yeast
229 species spanning multiple genera using spot-plating confirms that the unfiltered approach
230 is not limited to well-characterized model organisms but functions as a generalizable
231 platform for host onboarding. Successful transformations in *Botryozyma nematodophila*
232 and *Metschnikowia pulcherrima* are of particular interest for their unique morphology and
233 under-explored biotechnological potential respectively [16–18]. Despite its lower
234 transformation efficiency relative to filter-plating in some hosts, spot plating is the more
235 practical method, requiring far less hands-on time (15 minutes vs. 2 or more hours for 6
236 replicates) and enabling parallel processing by eliminating the need for vacuum pump
237 apparatuses. While not iterated upon here, optimization of the unfiltered method can be
238 easily conducted by researchers investigating new hosts, following our described
239 framework. This protocol brings large-scale functional genomics experiments in
240 biocapable yeast species within practical reach for researchers that previously lacked the
241 capacity to attempt them. The straightforward method of drying *Agrobacterium* and
242 yeast on induction medium enables automated workflows, accelerating genetic
243 investigation and engineering in difficult yeasts.

244

245 **Methods**

246 **Media, chemicals, and culture conditions**

247 Bacteria were routinely propagated in Luria-Bertani (LB) medium (BD Biosciences, USA).
248 Yeast were routinely propagated in yeast extract peptone dextrose (YPD) medium (Sigma
249 Aldrich, USA). *Escherichia coli* and *Agrobacterium tumefaciens* were grown at 37 °C and
250 30 °C respectively. Liquid cultures were shaken at 200RPM.

251 Antibiotics and concentrations used are as follows: kanamycin (50 mg/L, Teknova, USA),
252 nourseothricin (50 or 100 mg/L GoldBio, USA), G418 (300 mg/L Sigma Aldrich),
253 carbenicillin (300 mg/L Teknova, USA), cefotaxime (300 mg/L Sigma Aldrich). All other
254 chemicals were purchased through Sigma Aldrich.

255 Induction medium (IM) was composed as follows: 1 g/L NH_4Cl, 300 mg/L $MgSO_4 \cdot 7H_2O$,
256 150 mg/L KCl, 10 mg/L $CaCl_2$, 0.75 mg/L $FeSO_4 \cdot 7H_2O$, 144 mg/L K_2HPO_4, 48 mg/L
257 NaH_2PO_4, 3.9 g/L 2-(N-morpholino)ethane sulfonic acid (MES) (RPI, USA), 2 g/L D-
258 glucose, 400 µM acetosyringone, 10 mg/L thiamine hydrochloride, pH adjusted to 5.5
259 with KOH. 2% Difco Bacto Agar (BD Biosciences) was used for induction medium plates.

260 Media was autoclaved before the addition of filter sterilized stock solutions of thiamine
261 (100 g/L in H_2O) and acetosyringone (400 mM in DMSO (Sigma Aldrich)).

262

263 **Strains and plasmids**

264 All bacterial and yeast strains used in this work are listed in Table 1, and all plasmids are
265 listed in Table 2. All plasmids generated in this paper were assembled using PCR and
266 Gibson Assembly® and transformed into *E. coli* NEB-turbo using standard protocols [19].
267 Primers were purchased from Integrated DNA Technologies (IDT, USA). Plasmids were
268 isolated using the Qiaprep Spin Miniprep kit (Qiagen, USA). Plasmid sequences were
269 verified using whole plasmid sequencing (Plasmidsaurus, USA). *A. tumefaciens* was
270 routinely transformed via electroporation as described previously [20].

271

Species	**Source/Citation**	**Provided by**
Agrobacterium tumefaciens EHA105	[21]	Prof. Patrick Shih
Escherichia coli NEBturbo	NEB	NEB
Rhodotorula toruloides NBRC 0880	NBRC	NBRC
Saccharomyces cerevisae S288c	[22]	Prof. Graham Hudson
Kluyveromyces marxianus km1	[23] ABF_015686	Prof. Rachel Brem
Metschnikowia pulcherrima 40-214 (ATCC 18406)	Phaff Collection, Davis, CA	Prof. Rachel Brem
Botryozyma nematodophila CBS 7442	CBS, [16]	Prof. Rachel Brem
Pichia pastoris X-33	Invitrogen	Invitrogen
Ogataea parapolymorpha DL-1 (ATCC 26012)	ATCC	Prof. John Dueber
Lipomyces tetrasporus NRRL Y-11562 (CBS 5910)	CBS, [24]	Dr. Ziyu Dai

272

273 **Table 1:** Strains employed in this study

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Plasmid	**Part ID**	**Source**
pVS1_R106H	JPUB_024140	[25]

pVL_sR2rn_f14sg	ABF_015680	This Work
pVR_sATn_fS3sg	ABF_015682	This Work
pVR_sATon_fS3sg	ABF_015681	This Work
pVR_sLRln_fLTsg	ABF_015683	This Work
pVR_sATk_fS3sg	ABF_015685	This Work
pVR_sATk_fS3sg_hom	ABF_015684	This Work

Table 2: Plasmids used in this study. All available through public-registry.jbei.org and public-registry.agilebiofoundry.org

Vacuum filtration *Agrobacterium* mediated transformation

This describes a protocol yielding up to 10 total transformations. This can be scaled up using larger culture and centrifugation formats.

Day 1: A single colony of *Agrobacterium* carrying a binary vector was inoculated into a culture tube containing 10 mL LB medium with kanamycin and cultured overnight (~16 hours, 30 °C, 200 RPM). The target yeast strain was inoculated from a single colony into a culture tube containing 10 mL YPD and grown in parallel.

Day 2: The overnight *Agrobacterium* culture was diluted to OD_{600} = 0.5 in 10 mL LB without antibiotics and grown at 30 °C with shaking until OD_{600} = 1.0 (~2.5 hours). Cells were harvested by centrifugation at 5,000 × g for 15 minutes in 15-mL conical tubes (Falcon, Corning, USA), washed once with 10 mL IM, and resuspended in fresh IM to a final OD_{600} = 1.0. The culture was then incubated at 30 °C with shaking overnight for *vir*-gene induction. Serial dilutions of the overnight yeast culture were prepared in fresh YPD so that one dilution would reach OD_{600} = 1.0 the following day.

Day 3: The OD_{600} of both the induced *Agrobacterium* and yeast cultures was measured. 1 mL of yeast culture (OD_{600} = 1.0) was transferred to a 1.5-mL microcentrifuge tube (Eppendorf, Germany) and pelleted at 5,000 × g for 5 minutes. The supernatant was aspirated, and the yeast pellet was resuspended in a volume of induced *Agrobacterium* culture yielding an equivalent OD_{600} ratio (e.g., 666 µL of OD 1.5 Agrobacterium culture). The combined cell suspension (1,000 µL) was applied in 250-µL increments onto a 0.45-µm mixed cellulose ester membrane filter (HAWP; MilliporeSigma, USA) under vacuum. The filter was transferred with sterile forceps, cell-side up, to an IM agar plate and incubated at 26 °C for 4 days.

Day 7: The filter was carefully removed from the IM plate with sterile forceps and placed into a 50-mL conical tube containing 5 mL of YPD. The tube was vortexed vigorously for 2 minutes to resuspend the cells. Serial dilutions were prepared, and aliquots were spread onto selective YPD agar plates supplemented with cefotaxime and carbenicillin to counter-select *Agrobacterium*. Plates were incubated at 30 °C for 2 days or until single colonies were visible.

"Unfiltered" spot plating *Agrobacterium* mediated transformation

Days 1 and 2 were performed identically to the vacuum filtration protocol described above.

Day 3: The OD_{600} of both the induced *Agrobacterium* and yeast cultures was measured. 1 mL of yeast culture (OD_{600} = 1.0) was transferred to a 1.5-mL microcentrifuge tube (Eppendorf, Hamburg, Germany) and pelleted at 5,000 × g for 5 minutes. The supernatant was aspirated, and the yeast pellet was resuspended in the induced *Agrobacterium* culture at an equivalent OD_{600} ratio (i.e., 1:1 by cell density). The combined suspension was then centrifuged at 10,000 × *g* for 5 minutes, the supernatant was aspirated, and the cells were resuspended in 20 µL of sterile deionized water. The concentrated cell mixture was spotted directly onto the surface of an IM agar plate and incubated at 26 °C for 4 days.

Day 7: The cell mixture was scraped from the surface of the IM agar using a 10 µL sterile loop (Thermofisher Scientific, USA) and resuspended in 1 mL YPD by swirling. Cell suspensions were vortexed until cell clumps were no longer visible, serial dilutions were prepared, and aliquots were spread onto selective YPD agar plates supplemented with cefotaxime and carbenicillin to counter-select *Agrobacterium*. Plates were incubated at 30 °C for 2 days or until single colonies were visible.

Microscopy and Spectrometry

For all cultures, OD_{600} was measured using a Spectramax M2 (Molecular Devices, USA). *Metschnikowia pulcherrima* and *Pichia pastoris* were prone to forming flocs in liquid culture, confounding optical density measurements. For accurate measurements, these cultures were diluted into an EDTA solution (50 mM, pH = 8) and vortexed to disrupt flocs before reading.

Colonies of yeast species were inoculated and cultured overnight in YPD (30 °C, 200 RPM). Cultures were spotted onto microscope slides (VWR) and examined under a VistaVision compound microscope (VWR, USA) with 10 x magnification eyepiece and 100 x magnification objective (1000 x total magnification). Images were taken with an iPhone 14 Plus (Apple, USA) main camera (12 MP, 26 mm, *ƒ*/1.5 aperture) held to the eyepiece lens without software magnification.

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Contributions

Conceptualization, P.A.A., M.R.I., M.G.T..; Methodology, P.A.A, M.R.I, M.G.T., M.J.S., A.L., G.M.G; Investigation, M.R.I, M.G.T., M.J.S., G.M.G., A.L.,; Writing – Original Draft, M.R.I., M.G.T, A.L.; Writing – Review and Editing, All authors; Resources and supervision, M.G.T., D.L., J.M.G., P.M.S.

Competing Interests

M.G.T., M.J.S., and P.M.S. have financial interests in BasidioBio.

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