Genome-Shuffling-Improved Acid Tolerance and Lactic Acid Production in *Lactobacillus plantarum* for Commercialization

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We applied genome shuffling to improve the acid tolerance of *Lactobacillus plantarum*, while simultaneously enhancing lactic acid production. The starting populations were mutant libraries generated by gradual low pH adaptation and ultraviolet irradiation and then which were subjected to recursive protoplast fusion. A library of shuffled mutants (fusants) with genetic exchange is achieved by repetition of this process. After three rounds of genome shuffling, we obtained the best performing fusant that grow better at low pH 4.0 and consume glucose faster than does the wild type. In addition, lactic acid production of this fusant was 64% higher than that of the wild type. These results demonstrated that the genome shuffling has been successful in engineering *L. plantarum* with multiple beneficial improved phenotypes. In the future, this technology is a promising candidate to accelerate poorly characterized strains for commercialization.

Key words: genome shuffling, *Lactobacillus plantarum*, lactic acid, acid tolerance

Teknik *genome shuffling* telah diaplikasikan untuk memperbaiki toleransi asam *Lactobacillus plantarum* dan secara simultan meningkatkan produksi asam laktatnya. Populasi awal yang digunakan ialah pustaka mutan yang didapat dengan menurunkan pH medium pertumbuhan secara bertahap dan iradiasi sinar ultra violet. Pustaka mutan kemudian digunakan dalam proses fusi protoplas secara berulang. Hasil *shuffling* dari pustaka mutan disebut fusan. Paska pengulangan yang ke-3 dari *genome shuffling* didapatkan fusan unggul yang mampu tumbuh dengan baik pada pH 4.0 dan mengonsumsi gula lebih cepat dibandingkan dengan galur liar. Produksi asam laktat oleh fusan ini meningkat 64% dibandingkan dengan galur liar. Hasil ini menunjukkan bahwa teknik *genome shuffling* telah berhasil merekayasa *L. plantarum* yang memiliki keunggulan fenotipe. Di masa mendatang teknik ini mampu menjadi kandidat yang menjanjikan dalam komersialisasi galur mikrob yang belum dikarakaterisasi dengan baik.

Kata kunci: *genome shuffling, Lactobacillus plantarum*, asam laktat, toleransi asam

Lactic acid has been widely used in the food, pharmaceutical and cosmetics industries. It has recently emerged as an important source for the production of other chemicals, particularly polylactic acid for biodegradable plastic (Singh *et al.* 2006). Most lactic acid is manufactured by fermentation process as opposed to chemical synthesis since it is environmentally friendly (John *et al*. 2009). Fermentation at low pH contributes to the economics of the lactic acid purification process. Thus, the commercial goal of improving the growth and lactic acid production of microorganisms at acid condition could decrease waste and reduce the cost of production. Moreover, it also reduces potential contamination (Porro *et al*. 1999; Carlson and Peters 2002; Singh *et al*. 2006).

L. plantarum is of commercial interest for lactic acid production, but the effect of acid stress on the bacterium is a complex and poorly understood process. As a result, it is difficult to improve lactic acid production with rational engineering or direct genetic manipulation. On the other hand, classical strain-improvement-methods have succeeded in obtaining many industrial strains, but this is a time-consuming and laborious process because of many repeated rounds of random mutation and selection methods, especially for engineering complex phenotypes (Zhang *et al.* 2002). Thus, an efficient technology named genome shuffling has been presented as a novel whole-genome engineering approach for the rapid improvement of cellular phenotypes. This approach uses recursive protoplast recombination throughout the entire genome without the necessity for genome sequence data or network information (Patnaik *et al.* 2002; Stephanopoulos 2002). At present, genome shuffling has been successfully applied for the improvement acid tolerance in *Lactobacillus* (Patnaik *et al.* 2002), acid and glucose tolerance in *L. rhamnosus* (Wang *et al.* 2007; Yu *et al.* 2008), degradation of pentachlorophenol in *Sphingobium chlorophenolicum* (Dai and Copley 2004) and the production of hydroxycitric acid in *Streptomyces* (Hida *et al.* 2007). However, shuffling of an industrial strain *L. plantarum* has not been reported.

Thus, in the present study, we applied genome shuffling to improve the acid tolerance of *L. plantarum* while simultaneously enhancing lactic acid production.

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MATERIALS AND METHODS

Bacterial Strains and Media. *Lactobacillus plantarum* was obtained from Biotechnology Type Culture Collection (BTCC) Research Center for Biotechnology-LIPI, Indonesia. The strain was stored in a deMan Rogosa and Sharpe (MRS) broth with 10% (w v^{-1}) glycerol at -70 °C. The strain was grown in MRS broth from a 1% inoculum with 18 h incubation at 37 °C. At least two generations of precultures were required just before the experiments.

Yeast extract fermentation medium (YE) contained 100 g L^{-1} glucose and 15 g L^{-1} yeast extract. Regeneration medium (RM) was MRS agar without Tween 80 and supplemented with 0.5% BSA, 25 mM MgCl$_2$, 25 mM NaCl$_2$, and 0.5 M sucrose (Patnaik *et al.* 2002).

Adaptation of *L. plantarum* to Low pH. Culture adaptation was achieved by modification of the procedure from Patnaik *et al.* (2002), through a continuous and controlled decrease in broth medium pH 6.2 to pH 5.0 and pH 4.0. For scenarios in which the imposed pH was not tolerated, and the culture steady-state could not be maintained, the culture was replaced with fresh MRS broth of pH lower than 4.0. Adapted wild-type strain in pH 4.0 medium was then used as the parental, or starter, for genome shuffling.

Strain Mutagenesis and Mutant Screening. The cells of *L. plantarum* wild-type strain were grown in a 10 ml MRS broth at 37 °C for 18 h. Cultures were centrifuged at 4500 x *g* for 10 min. UV irradiation was performed by exposing samples of the wild-type strain in uncovered petri dish directly to UV light at a distance 20 cm with 120 mJ cm^{-2} of intensity for 30 s using UV Crosslinker (Hoefer UVC500, USA). The mutants were then spread on MRS agar plates containing 2% (w v^{-1}) CaCO$_3$ and incubated at 37 °C for 48 h. The fast growing colonies, which had bigger transparent haloes compared to colony diameter, were picked off and selected as the starter for the genome shuffling.

Protoplast Preparation and Genome Shuffling. Protoplast formation was carried out as described by Cocconcelli *et al.* (1986) with modification. Strains were cultured at 37 °C for 18 h in MRS broth. The cells were harvested by centrifugation at 4500 *x g* and 4 °C for 10 min, and washed twice with distilled water. The cells from a 5 mL culture were suspended in *Lactobacillus* protoplast buffer (LPB) (50 µL Tris HCl 1 M pH 6.3, 2.5 mL sucrose 1 M, 2 mL CaCl$_2$ 50 mM, and 450 µL distilled water) containing lysozyme (2.0 X 10^5 U mL^{-1}) and incubated at 37 °C for 2 h for protoplast formation.

The starting populations for genome shuffling were mutants-library-generated by gradually low pH adaptation and ultraviolet (UV) irradiation. Fusions between protoplast preparations were generated by mixing an approximately equal number of protoplasts from two populations as described by Patnaik with slightly modification (Patnaik *et al.* 2002). The shuffled mutants, named fusants were then cultivated in regeneration medium. Each culture was incubated at 37 °C for 6 h and processing continued by spreading in selection medium of MRS agar plates containing 2% (wv^{-1}) CaCO$_3$, pH 4.0, and then incubated at 37 °C for 3-4 d. Subsequent colonies were selected randomly to carry out further selection with small-scale fermentation analysis in 2 mL YE broth culture at pH 4.0. The cultures were incubated at 37 °C for 48 h. Profiles of both growth and lactic acid production for each culture were then followed. The value of both growth and lactic acid production then were analyzed using SPSS program to identify the fusants that have a significant change in their growth and lactic acid production compared to that of control.

The formation of protoplasts, their fusion, and subsequent regeneration was repeated three times with the pooled regenerated cells from one fusion being the inoculum for the subsequent protoplast culture. Samples from each of the pooled fusion libraries F1-F3 (first/F1, second/F2, third/F3) were saved for later analysis. Non-shuffled (unshuffled) controls were prepared by the recursive formation and regeneration of protoplast without exposure to PEG.

Shaking Flask Analysis. The best performing selected shuffled strain, F3-33, after three rounds of genome shuffling (F3) was cultured in 10 mL MRS broth before being transferred into 100 mL YE medium at pH 4.0 in shaking flasks (250 mL) at 100 rpm, 37 °C. The growth, pH, residual glucose, and lactic acid production were periodically monitored up to 96 h.

Analytical Methods. Cell growth was monitored by measuring cell number via the optical density at 600 nm (A$_{600nm}$). Supernatants of the samples for assay were collected by centrifugation. Total lactic acid for fusant selection was estimated according to the titration method, and the total sugar concentration was determined using the phenol-sulphuric acid method, as described by Dubois *et al.* (1956). Furthermore, total lactic acid of the selected F3 fusant was then determined by reversed-phase high-performance liquid chromatography (HPLC) (Shimadzu LC-6AD, Japan) using a C18 silica column (HPLC Packed Column UG120 Shiseido, Japan) with UV detector set at 220 nm. The column was eluted with 20 mM NaH$_2$PO$_4$ (pH 2.55) as a mobile phase at a flow

rate of 1.0 mL min^{-1}. All of the measurements were performed in duplicate from two independent experiments.

RESULTS

Isolation of Initial Strains. The first population of the adapted acid-mutant-library was achieved by slowly decreasing the pH from 6.2 to 4.0 over a period of 840 h. A stable population of *L. plantarum* was able to grow at pH 4.0, a pH that severely inhibits growth of the wild-type strain. The second population was enriched for acid tolerance of the wild-type strain using UV mutant library selected on MRS-agar-plates at pH 4.0. Ten colonies which had bigger transparent haloes compared to colony diameter were obtained (unpublished data).

Genome Shuffling. The resulting new population (shuffled strains/fusants) represented a larger combinatorial library of the original genetic diversity. After the first fusion, 56 colonies were picked off randomly for further selection in small-scale fermentation. Selection was done by SPSS program based on their growth and lactic acid production values. Fusants with higher and lower lactic acid production respectively were shown (Fig 1, 2, 3). First-round shuffling also resulted 29 fusants with growth-faster compared to that of wild-type (unpublished data). Among those fusants, 4 fusants, i.e. F1-2, F1-16, F1-48, and F1-50, showed the value of relative lactic acid production per A_{600nm} higher than 1.0 (control) and 2 fusants, i.e., F1-6 and F1-30 showed relative growth-fastest (120%). Relative lactic acid production is the value of total lactic acid produced by sample divided by total lactic acid produced by control, whereas relative growth is the value of cell density of sample divided by that of control at A_{600nm}. Two fusants from the selected F1 fusants library, with higher relative lactic acid production/ A_{600nm} ($p < 0.05$) to that of wild type, F1-30 and F1-16 (shown as red bar) were used as resources for the next fusion (Fig 1a). Figure 1b showed 4 fusants (F2-3, F2-22, F2-26, and F2-65) and 10 fusants (F2-64, F2-77, F2-78, F2-79, F2-88, F2-89, F2-90, F2-91, F2-109 and F2-116) with lower and higher lactic acid production, respectively. Second round of shuffling also resulted 87 of fusants with growth faster compared to that of F1-30 as control (unpublished data). Among second shuffled library F2, 4 fusants, namely F2-78, F2-88, F2-89, and F2-91 were selected and used for the next fusion (Fig 1c). These fusants were selected due to their higher relative lactic acid production to that of F1-30 as control ($p < 0.05$). The result showed 4 fusants (F3-33, F3-118, F3-119, and F3-176) and 34 fusants with higher and lower
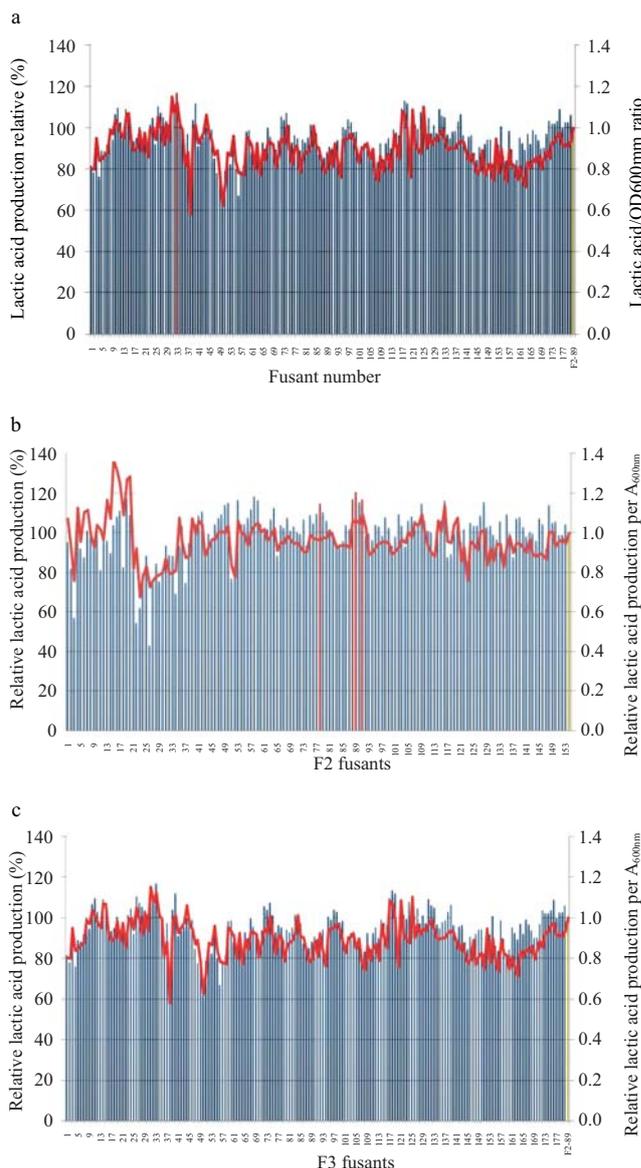

Fig 1 Relative lactic acid production to the control and relative lactic acid production per A_{600nm}. a, F1 fusants library; b, F2 fusants library; c, F3 fusants library *Lactobacillus plantarum*. Wild-type strain (WT), F1-30 fusant, and F2-89 fusant is used as the control for F1, F2, and F3-fusants library respectively (For more detail information see result session of genome shuffling). X-ordinate represents colonies numbers from each fusant library. Bar represents relative lactic acid production of fusants, line represents relative lactic acid production per A_{600nm}. Red bar indicated the fusants were choose for next rounds. ▬, laktat; ▬ , yield.
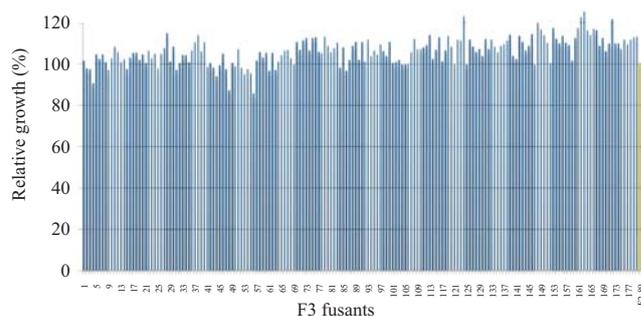

Fig 2 Relative growth of F3 *Lactobacillus plantarum* fusants library to F2-89 fusant as a control. X-ordinate represents colonies numbers from F3 fusants library.
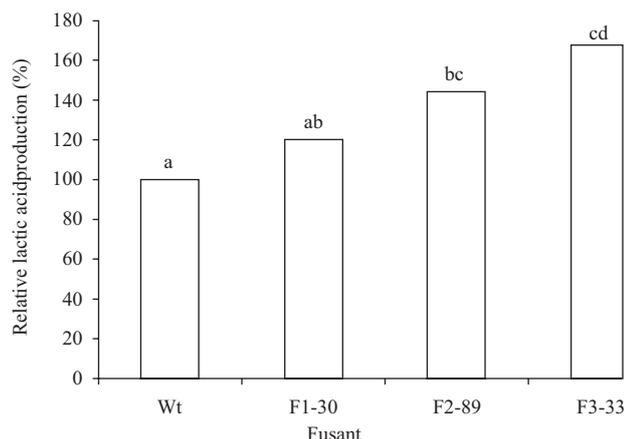
Fig 3 Relative lactic acid production of the best performing fusants from each of three rounds of genome shuffling (F1-F3) to the wild type; wt is wild type strain of *Lactobacillus plantarum*. F1-30 is fusant from colony number 30 of first fusion round, F2-89 is fusant from colony number 89 of second fusion round, while F3-33 is fusant from colony number 33 of third fusion round. Bar with different letters are significantly different by Dunnet test at p<0.05.

relative lactic acid production, respectively (Fig 1c). Among third shuffled library, F3-33 showed highest relative lactic acid production (136%) compared to that of F2-89 as control (p < 0.05).

The relative growth performance to the control of 180 colonies from third shuffled library (F3) which were picked off randomly. Figure 2 showed 109 and 6 of fusants with growth faster and slower, respectively. The relative growth of F3 fusants library was mostly higher compared with that of the F2-89 fusant as control (Fig 2), while relative lactic acid production of F3 fusants library was mostly lower than that of control (Fig 1c). The best performing lactic acid production of fusants in each round (F1-30, F2-89, F3-33) were compared for their relative lactic acid production to that of the wild-type (Fig 3). It is shown that F3-33 fusant in the third round genome shuffling performed the highest in lactic acid production.

Shaking Flask Analysis. The performance of F3-33 was further compared with the wild-type strain in shaking flasks (Fig 4a-c). Consistent with small-scale fermentation results, the genome shuffled strain F3-33 produced about a 64.5 % improvement of lactic acid production (12.0 g L^{-1}) over the wild-type for 48 h (Fig 4a). This lactic acid production increased up to 48 h, after which no significant improvement was found. Moreover, the growth characterization and glucose consumption were consistent with the lactic acid production. The fusant F-33 grew 53% faster compared with the wild-type (Fig 4b), and also consumed glucose faster (Fig 4c).

DISCUSSION

As lactic acid fermentation at low pH (at or below the *pKa* of lactic acid, pH 3.8) can reduce the cost
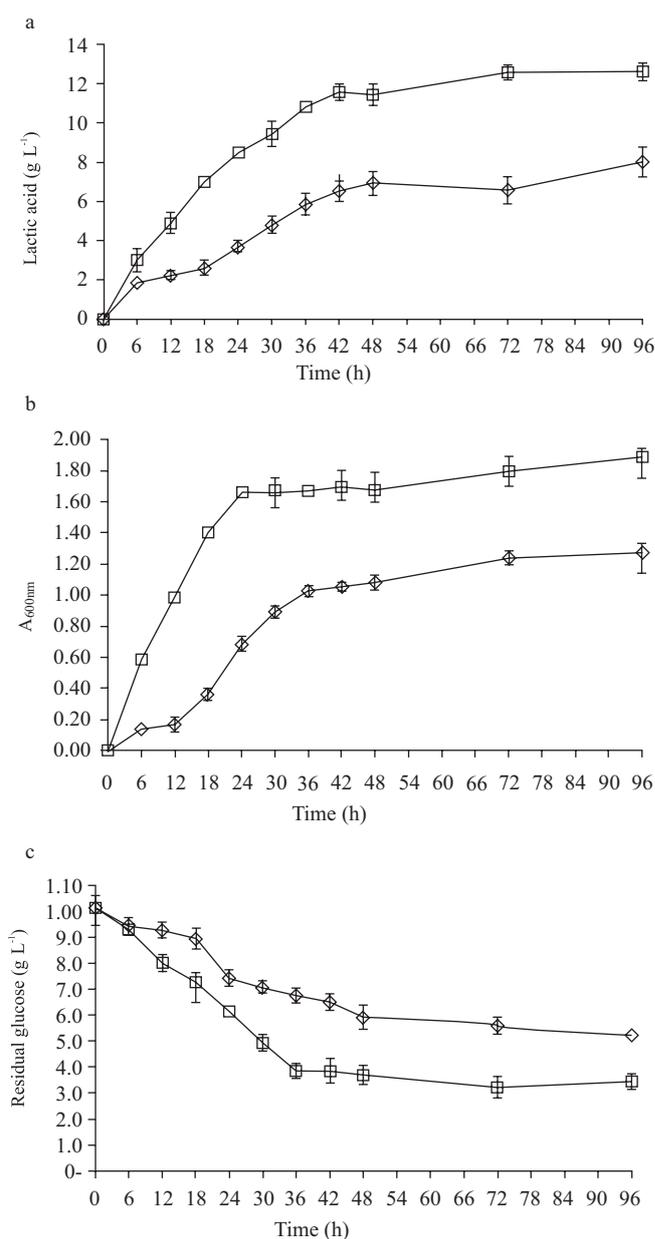

Fig 4 Characterization of lactic acid production: a, cell growth; b, and residual sugar (glucose) of fusant F3-33 compared to that of wild type (WT) *Lactobacillus plantarum*. ◇, WT; □, F3-33.

production and decrease waste, acid tolerance phenotypes meet the performance criteria for industrial strain commercialization. Thus, the strains tolerant to acid conditions are desirable industrial phenotypes (Patnaik *et al.* 2002). Environmental tolerance of pH is a complex and yet poorly understood phenomenon, therefore, therefore we applied genome shuffling approach to improve lactic acid production of local *L. plantarum*. This approach has successfully been used to improve acid tolerance in *Lactobacillus* through fusion between a low-pH-adapted mutant library population and nitrosoguanidine (NTG) mutant-library-population (Patnaik *et al.* 2002), and in *L. rhamnosus* through UV irradiation and NTG mutagenesis (Wang *et al.* 2007). Here, we used an

adapted low-pH-mutant library population and an UV-irradiation- mutant library population, instead of a nitrosoguanidine (NTG) mutant library population, as the starter of genome shuffling. Since genome shuffling practically mimics the features of natural evolution through recursive genetic recombination, it requires a diverse population of mutants with an improvement of the desired phenotype compared with the wild-type as a starting point (Yu *et al.* 2008).

The present study showed more than 50% of fusants with change in their relative lactic acid production and growth. The percentage of fusants with different performing compared to that of their control was increased to 60% (9% and 51% with their lactic acid production and growth, respectively), 66% (9% and 57% with their lactic acid production and growth, respectively) and 85% (21% and 64% with their lactic acid production and growth, respectively) in first, second, and third rounds, respectively. The results of the present study also proved that genome shuffling is an effective strategy for generating strains which become more tolerant to the acid and result in the improvement of lactic acid production. It was shown that the relative lactic acid production of the best performing fusants (F1-30, F2-89, and F3-33) from each of three rounds of genome shuffling were higher than that of the wild-type (120%, 144%, and 167.8% respectively) (Fig 3), corresponding with the higher growth of those fusants compared with the wild-type (unpublished data). After three rounds of genome shuffling, we obtained F3-33 fusant which had the highest lactic acid production and capable of growing at pH 4.0. In this regard, F3-33 fusant was further characterized by comparison with the wild-type strain.

In agreement with small-scale fermentation, the lactic acid production of F3-33 fusant was 64.5% higher compared with the wild-type strain in shaking flasks (Fig 4a). After 48 h of growth at pH 4.0, the wild-type strain had produced 7.74 g L^{-1} of lactic acid, while the F3-33 fusant produced 12 g L^{-1}.

Characterization of F3-33 fusant and the wild-type in the shaking flask showed the lactic acid production was closely correlated with the growth and glucose consumption (Fig 4b and 4c). The best performing fusant grew faster, consumed glucose faster, and produced more lactic acid, and also brought the medium to a lower pH (unpublished data), as reported by Patnaik *et al*. (2002) and Wang *et al*. (2007). However, these previously published results showed that the fusants produced three-fold more lactic acid than the wild-type at pH 4.0. The improvement of lactic acid of our fusant was lower than that of these previous reports. This result may due to the fact that the starting population for each round of fusion had low diversity. The fusant library F1 and F2 were generated from two colonies/ fusants of F0 and F1 fusants library, respectively, while the fusant library F3 was generated from 4 fusants of F3 fusants library. From F1, F2, and F3 fusants library, the percentage of fusants which had changed in their lactic acid production were 9%, 9% and 21%, respectively. These achievements were agreed with the number of fusants used as resources to generate fusants library. Thus, we concluded that the low genetic diversity within a selected population resulted in low recombination and combinatorial library.

These results, however, demonstrated that we had been succeeded in applying the genome shuffling to engineer *L. plantarum* with multiple beneficial improved phenotypes including acid tolerance, lactic acid enhancement, and glucose/substrate consumption at one time. In the future, it is promising that this simple technology is a promising candidate to accelerate poorly characterized strains for commercialization.

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