Application of Marker-Recycling Plasmids in Pichia pastoris (Postprint)

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Abstract

Pichia pastoris is a rapidly developing host strain for high-level expression in recent years. As a methylotrophic yeast, Pichia pastoris offers advantages such as easy manipulation, high-density cultivation, rapid growth, post-translational protein processing, and low production costs. The Pichia pastoris expression system has a history of over 20 years of research, development, and application, with various expression vectors and improved host strains available for intracellular or secretory expression. Integrating genes into the genome using a homologous recombination-based DNA transformation system is currently one of the most important gene editing methods and has been widely applied in Pichia pastoris; however, Pichia pastoris faces the challenge of insufficient selection markers. In the Cre/loxP system, when two loxP sites are placed in the same orientation between a sequence, Cre recombinase can recognize and excise the sequence, leaving a single loxP site. When using mutated loxP sequences (such as lox71 and lox66), after recognition and action by Cre recombinase, a new mutated lox72 site is left behind, which will not interact with subsequently introduced loxP sites, thereby preventing the possibility of residual loxP sites being recognized by Cre recombinase along with newly introduced loxP sites.

In this study, based on the universal Pichia pastoris plasmids pPICZA and pGAPZA, we fused an inducible Cre expression cassette downstream of the zeocin resistance gene expression cassette and introduced lox71 and lox66 sites flanking these two cassettes, constructing the plasmids pZACH and pGACH, respectively; thereby enabling selection marker recycling. The specific process is as follows: 1) Transform the plasmid into Pichia pastoris and select positive transformants using zeocin resistance screening; 2) Inoculate the positive transformants into YPM medium containing the inducer methanol and culture overnight; 3) Streak the overnight culture containing cells onto antibiotic-free YPD plates and incubate until yeast single colonies become visible; 4) Simultaneously spot the yeast single colonies from step 3 onto both antibiotic-free YPD plates and zeocin-containing YPDZ plates. If they can grow on YPD plates but

not on YPDZ plates, this indicates that the zeocin resistance expression cassette has been lost from the colony, which is then verified by PCR identification. The entire marker loss process requires approximately 4-5 days.

Using plasmid pZACH, we expressed phytase from Citrobacter amalonaticus CGMCC 1696 and lipase from Acinetobacter radioresistens CMC-1 in Pichia pastoris, respectively. The marker loss efficiency of these plasmids was greater than 70%, and both phytase and lipase production as well as cell growth were similar to the results obtained using the basic plasmid pPICZA for expression. This plasmid has no adverse effects on heterologous protein expression or cell growth.

Using the selection marker recycling plasmids pZACH and pGACH can overcome the limitation of insufficient selection markers in Pichia pastoris, facilitating better genetic modification in this host, thereby providing a foundation for further industrial applications of Pichia pastoris.

Full Text

Preamble

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Application of Recyclable Selection Marker Plasmids in *Pichia pastoris*

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Pichia pastoris has emerged as a rapidly developing host for high-level protein expression. As a methylotrophic yeast, it offers numerous advantages including straightforward manipulation, suitability for high-density cultivation, rapid growth, capability for proper post-translational protein processing, and low production costs. With over two decades of research and development, the *P. pastoris* expression system provides various expression vectors and improved host strains for both intracellular and secreted protein production. While gene integration via homologous recombination-based DNA transformation represents one of the most important genetic engineering tools and has been widely applied in *P. pastoris*, the system faces a critical limitation: a shortage of available selection markers.

The Cre/loxP recombination system offers an elegant solution to this problem. When two loxP sites are oriented in the same direction flanking a DNA sequence, Cre recombinase recognizes and excises the intervening fragment, leaving behind a single loxP site. By employing mutant loxP sequences such as lox71 and lox66, recombination produces a novel mutant lox72 site that is incompatible with subsequently introduced wild-type loxP sites. This prevents undesirable

recombination between residual loxP sites and newly introduced ones, thereby enabling marker recycling.

In this study, we constructed two recyclable marker plasmids, pZACH and pGACH, based on the commonly used *P. pastoris* vectors pPICZA and pGAPZA. We fused an inducible Cre expression cassette downstream of the bleomycin resistance gene cassette and flanked both cassettes with lox71 and lox66 sites. The marker recycling procedure proceeds as follows: (1) Transform *P. pastoris* with the plasmid and select positive transformants on bleomycin-containing medium; (2) Inoculate positive clones into YPM medium supplemented with methanol as inducer and culture overnight; (3) Streak the overnight culture onto YPD plates without antibiotics and incubate until single yeast colonies appear; (4) Patch colonies from step 3 onto both antibiotic-free YPD plates and bleomycin-containing YPDZ plates. Growth on YPD but not on YPDZ indicates loss of the bleomycin resistance cassette, which is subsequently confirmed by PCR analysis. The entire marker elimination process requires approximately 4–5 days.

We validated this system by expressing phytase from *Citrobacter amalonaticus* CGMCC 1696 and lipase from *Acinetobacter radioresistens* CMC-1 in *P. pastoris* using plasmid pZACH. Both constructs achieved marker loss efficiencies exceeding 70%. Importantly, protein yields and cell growth characteristics were comparable to those obtained with the parental pPICZA vector, demonstrating that the recyclable marker system does not adversely affect heterologous protein expression or host physiology.

The recyclable marker plasmids pZACH and pGACH effectively overcome the limitation of scarce selection markers in *P. pastoris*, facilitating more extensive genetic manipulation and providing a foundation for enhanced industrial applications of this important expression host.

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Note: Figure translations are in progress. See original paper for figures.