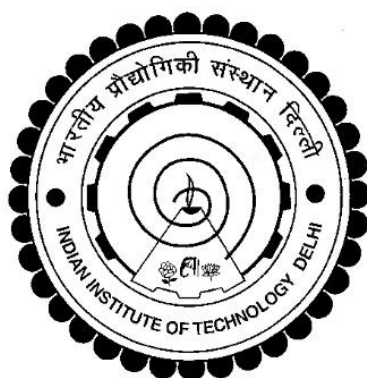


# **Recombinant Protein Production in** *Komagataella phaffii*

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**INDIAN INSTITUTE OF TECHNOLOGY DELHI**

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# **Recombinant Protein Production in** ***Komagataella phaffii***

by

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**Submitted**

**in fulfilment of the requirements of the degree of Doctor Of Philosophy**

**to the**



**INDIAN INSTITUTE OF TECHNOLOGY DELHI**

**OCTOBER 2019**

## **CERTIFICATE**

This is to certify that the thesis entitled “**Recombinant protein production in *Komagataella phaffii***” being submitted by **Ms. Anamika Singh** to the Indian Institute of Technology Delhi, for the award of the degree of **Doctor of Philosophy**, is a record of the bonafide research work carried out by her, which has been prepared under my supervision in conformity with the rules and regulations of the Indian Institute of Technology Delhi. The research reports and the results presented in this thesis have not been submitted for any degree or diploma in any other University or Institute.

Date: 03-10-2019

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## **Abstract**

The methylotrophic yeast *Komagataella phaffii* is considered a highly successful expression host for the large-scale production of recombinant proteins. The presence of the strong, yet tightly regulated, promoter  $P_{AOX1}$  is one of the main reasons for this success. Typically, methanol is used to induce  $P_{AOX1}$  activity. It also serves, at least in the wild type strain, as a source of carbon and energy. However, there are problems with the use of methanol on an industrial scale, which have prompted researchers to explore ways to minimize the amount of methanol used. Two popular strategies are a) the use of Mut (“Methanol utilization”) strains, which are compromised in their production of alcohol oxidase (AOX) and thus their ability to consume methanol and b) addition of secondary carbon sources to support growth and to reduce methanol requirement. The need to systematically study the influence of the different Mut phenotypes and of different secondary carbon sources on recombinant protein expression has been acknowledged in the literature. In this work, we tried to determine the optimal Mut phenotype and suitable secondary carbon sources by studying the production of a model recombinant protein,  $\beta$ -galactosidase. We observed significantly higher specific expression rates in a Mut<sup>+</sup> (high AOX) strain compared to Mut<sup>s</sup> (little AOX) and Mut<sup>-</sup> (no AOX) strains, suggesting that the Mut<sup>+</sup> should be the strain of choice for production of (at least non-secreted) recombinant proteins. Moreover, this observation led us to hypothesize that a downstream metabolite of methanol is involved in induction of  $P_{AOX1}$ . It was found that the metabolites formate and formaldehyde do act as potent inducers of  $P_{AOX1}$ . Since these compounds have several advantages over methanol in industrial protein production processes, this observation has important practical implications for large-scale production of recombinant proteins. The second goal of this work was to identify the best secondary carbon source. Comparable recombinant protein production was observed when either glycerol (a repressing carbon source) or sorbitol (a non-repressing carbon source) was used as the secondary carbon source along with methanol in continuous culture. This demonstrates that it is irrelevant whether a non-repressing or a repressing carbon source is used to support growth. Again, this result has significant practical implications, as it allows the secondary carbon source to be chosen solely on the basis of industrially relevant parameters.

## सार

मिथाइलोट्रोफिक खमीर *कोमागाटाला फाफि* को पुनः संयोजक प्रोटीन के बड़े पैमाने पर उत्पादन के लिए एक अत्यधिक सफल अभिव्यक्ति मेजबान माना जाता है। प्रबल एवम् सख्ती से विनियमित होने वाले  $P_{AOX1}$  प्रमोटर की उपस्थिति, इस सफलता के मुख्य कारणों में से एक है। आमतौर पर,  $P_{AOX1}$  गतिविधि को प्रेरित करने के लिए मेथनॉल का उपयोग किया जाता है। यह, कम से कम जंगली प्रकार के उपभेद में, कार्बन और ऊर्जा के स्रोत के रूप में भी कार्य करता है। हालांकि, औद्योगिक पैमाने पर मेथनॉल के उपयोग के साथ समस्याएं हैं, जिसने शोधकर्ताओं को इस्तेमाल किए गए मेथनॉल की मात्रा को कम करने के तरीके तलाशने के लिए प्रेरित किया है। दो लोकप्रिय रणनीतियाँ इस प्रकार हैं १) Mut (“मेथनॉल उपयोग”) उपभेदों का उपयोग, जो अल्कोहल ऑक्सीडेज (AOX) के उत्पादन में प्रतिबंधित है कारणवश उनकी मेथनॉल उपभोग करने की क्षमता में भी सिमित हैं तथा २) विकास का समर्थन करने के लिए एवं मेथनॉल का उपभोग घटाने के लिए अप्रधान कार्बन स्रोतों का उपयोग। पुनः संयोजक प्रोटीन अभिव्यक्ति पर विभिन्न Mut फेनोटाइप एवं अप्रधान कार्बन स्रोतों के प्रभाव को व्यवस्थित रूप से अध्ययन करने की आवश्यकता है जिसे साहित्य में स्वीकार किया गया है। इस शोध कार्य में, हमने एक मॉडल पुनः संयोजक प्रोटीन,  $\beta$ -galactosidase के उत्पादन का अध्ययन करके इष्टतम Mut फेनोटाइप और उपयुक्त अप्रधान कार्बन स्रोतों को निर्धारित करने का प्रयास किया। हमने Mut<sup>s</sup> (कम मात्रा में AOX) और Mut<sup>-</sup> (शून्य मात्रा में AOX) उपभेदों की तुलना में Mut<sup>+</sup> (अधिक मात्रा में AOX) उपभेद में महत्वपूर्ण रूप से विशिष्ट अभिव्यक्ति दरों का अवलोकन किया, जो यह संकेत करता है के Mut<sup>+</sup> पुनः संयोजक प्रोटीन (कम से कम गैर-सावित) उत्पादन के लिए अधिमानित उपभेद होना चाहिए। इसके अतिरिक्त, इस अवलोकन ने हमें यह अनुमान लगाने के लिए प्रेरित किया कि मेथनॉल का अनुप्रवाह metabolites  $P_{AOX1}$  के उत्प्रेरण में शामिल है। यह पाया गया कि फॉरमेट एवं फॉर्मलाडेहाइड

metabolites  $P_{AOX1}$  के प्रबल उत्प्रेरक के रूप में कार्य करते हैं। चूंकि इन यौगिकों के औद्योगिक प्रोटीन उत्पादन प्रक्रियाओं में मेथनॉल की तुलना में कई फायदे हैं, इसलिए इस अवलोकन का, पुनः संयोजक प्रोटीन के बड़े पैमाने पर उत्पादन के लिए महत्वपूर्ण व्यावहारिक निहितार्थ हैं। इस शोध कार्य का दूसरा लक्ष्य उच्चतम अप्रधान कार्बन स्रोत की पहचान करना था। जब continuous cultures में मेथनॉल के साथ ग्लिसरॉल (एक दमनकारी कार्बन स्रोत) या सोर्बिटोल (एक गैर-दमनकारी कार्बन स्रोत) का उपयोग किया गया तब हमें समान पुनः संयोजक प्रोटीन का उत्पादन प्राप्त हुआ था। यह दर्शाता है कि यह अप्रासंगिक है कि विकास का समर्थन करने के लिए एक गैर-दमनकारी या दमनकारी कार्बन स्रोत का उपयोग किया जाए या नहीं। फिर से, इस परिणाम के महत्वपूर्ण व्यावहारिक निहितार्थ हैं, क्योंकि यह औद्योगिक रूप से प्रासंगिक मापदंडों के आधार पर अप्रधान कार्बन स्रोत को पूरी तरह से चुनने की अनुमति देता है।

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## Nomenclature

AOX	Alcohol oxidase
$P_{AOX}$	Alcohol oxidase promoter
FLD	Formaldehyde dehydrogenase
FDH	Formate dehydrogenase
MU	Miller units
Mut	Methanol utilization type
PCR	Polymerase chain reaction
D	Dilution rate ( $\text{h}^{-1}$ )
$p_e$	Concentration of extra-cellular protein (units $\text{l}^{-1}$ )
$p_i$	Specific activity of protein (units $\text{gdw}^{-1}$ )
$r_{p_e}^-$	Degradation rate of extracellular protein (units $\text{l}^{-1} \text{h}^{-1}$ )
$r_{p_i}^-$	Specific rates of protein degradation (units $\text{gdw}^{-1} \text{h}^{-1}$ )
$r_{p_i}^+$	Specific rates of protein production (units $\text{gdw}^{-1} \text{h}^{-1}$ )
$r_{p_i}^{\rightarrow}$	Specific rates of protein secretion (units $\text{gdw}^{-1} \text{h}^{-1}$ )
$r_S$	Substrate uptake rate ( $\text{g gdw}^{-1} \text{h}^{-1}$ )
$\mu$	Specific growth rate ( $\text{h}^{-1}$ )
$Y$	Biomass yield ( $\text{gdw g}^{-1}$ )
$x$	Cell density ( $\text{gdw l}^{-1}$ )