MODELLING AND EXPERIMENTAL VALIDATION OF GROWTH AND ETHANOL PRODUCTION IN CONTINUOUS CULTURES OF *SCHEFFERSOMYCES (PICHIA) STIPITIS*

BIJU JACOB

DEPARTMENT OF BIOCHEMICAL ENGINEERING AND BIOTECHNOLOGY

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by

BIJU JACOB

DEPARTMENT OF BIOCHEMICAL ENGINEERING AND BIOTECHNOLOGY

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To my beloved father

Late Shri D. Jacob

CERTIFICATE

This is to certify that the thesis titled **Modelling and experimental validation of growth and ethanol production in continuous cultures of *scheffersomyces (pichia) stipitis*** submitted by **Mr. BIJU JACOB**, to the Indian Institute of Technology Delhi, for the award of the degree of Doctor of Philosophy, is a bonafide record of the research work done by him under my supervision and guidance. The contents of this thesis, in full or in parts, have not been submitted to any other institute or university for the award of any degree or diploma, whatsoever.

Dr. Atul Narang
Professor
Dept. of Biochemical Engg. & Biotechnology
Indian Institute of Technology Delhi
Hauz Khas, New Delhi-110016

New Delhi
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ABSTRACT

The ascomycetous yeast, *Scheffersomyces stipitis* (formerly known as *Pichia stipitis*) has received considerable research attention, owing to its propensity for fermenting a wide variety of sugars (both hexoses and pentoses) to produce ethanol. Owing to its abundance in nature, lignocellulosic biomass is considered as a potential feedstock for industrial ethanol fermentation. Nonetheless, its wide adaptation as a raw material is limited by the fact that the most efficient industrial producer of ethanol i.e. *Saccharomyces cerevisiae,* is incapable of fermenting pentose sugars, which constitutes 10-40 % of the carbohydrate fraction in lignocellulosic feedstocks. Thus, pentose fermenting yeasts, like *S. stipitis* hold the key for fermentative production of ethanol from lignocellulose hydrolyzates. Nonetheless, due to its requirement for tight control of aeration, *S. stipitis* has not found much favour as a prospective industrial producer of ethanol. Systematic attempts have been made in the past to quantify the parametric sensitivity of ethanol production in *S. stipitis,* which demonstrated the existence of a dual-nutrient limited regime in which growth is simultaneously limited by both carbon and oxygen. The width of the dual-limited regime has been shown to be a quantitative indicator of the robustness of ethanol production in yeasts-the broader the regime, the more robust the ethanol production by the organism. An understanding of the kinetics and physiology of dual-limited growth is a pre-requisite for undertaking any strain improvement program aimed at enhancing ethanol production in *S. stipitis*. To this end, we developed a simple unstructured model which precisely describes the kinetics of growth in the dual-limited regime, while overcoming the limitations implicit in classical Monod-type models. Our model was subsequently validated through experiments undertaken in a chemostat operated at progressively increasing feed substrate (glucose) concentrations at fixed values of various combinations of the operating parameters, dilution rate (D) and oxygen mass transfer coefficient ($k_l a$). A quantitative comparison of ethanol production in *S. stipitis* relative to *S. cerevisiae* could be achieved when the variables of the model were scaled suitably and the experimental data were analysed on a dimensionless plot. The model was found to have universal applicability in describing dual-nutrient limited growth in a variety of model microbial systems, as demonstrated from a fit of available experimental data in published literature. The insights provided by the current work are expected to stimulate further research into the physiology of dual-limited growth in *S. stipitis*, which could in turn aid in the development of novel genetic tools for improving its ethanol production capacity.

सार

एस्कोमाइसेटस यीस्ट, *शेफ़रसोमाइसेस स्टिपिटिस* (जिसे पहले *पिचिया स्टिपिटिस* के नाम से जाना जाता था) पर काफी शोध ध्यान दिया गया है, क्योंकि इसमें इथेनॉल का उत्पादन करने के लिए विभिन्न प्रकार की शर्करा (हेक्सोज और पेंटोस दोनों) को किण्वित करने की प्रवृत्ति है। प्रकृति में इसकी प्रचुरता के कारण, लिग्नोसेल्यूलोसिक बायोमास को व्यावसायिक इथेनॉल किण्वन के लिए संभावित फीडस्टॉक माना जाता है। बहरहाल, कच्चे माल के रूप में इसका व्यापक अनुकूलन इस तथ्य से सीमित है कि इथेनॉल का सबसे कुशल व्यावसायिक उत्पादक यानी *सैक्रोमाइसेस सेरेविसिया* पेंटोस शर्करा को किण्वित करने में असमर्थ है, जो लिग्नोसेल्यूलोसिक फीडस्टॉक्स में कार्बोहाइड्रेट अंश का 10-40% होता है। इस प्रकार, *एस. स्टिपिटिस* जैसे पेंटोस किण्वन यीस्ट, लिग्नोसेल्यूलोज हाइड्रोलाइजेट्स से इथेनॉल के किण्वक उत्पादन की कुंजी रखते हैं। फिर भी, वातन के कड़े नियंत्रण की आवश्यकता के कारण, *एस. स्टिपिटिस* को इथेनॉल के संभावित व्यावसायिक उत्पादक के रूप में अधिक समर्थन नहीं मिला है। *एस. स्टिपिटिस* में इथेनॉल उत्पादन की पैरामीट्रिक संवेदनशीलता को मापने के लिए अतीत में व्यवस्थित प्रयास किए गए हैं, जिसने दोहरे पोषक तत्व सीमित क्षेत्र के मौजूदगी को प्रदर्शित किया है जिसमें वृद्धि एक साथ कार्बन और ऑक्सीजन दोनों द्वारा सीमित है। दोहरे-सीमित क्षेत्र की चौड़ाई को यीस्ट में इथेनॉल उत्पादन की मजबूती का एक मात्रात्मक संकेतक दिखाया गया है - क्षेत्र जितना व्यापक होगा, जीव द्वारा इथेनॉल उत्पादन उतना ही अधिक मजबूत होगा। *एस. स्टाइपिटिस* में इथेनॉल उत्पादन को बढ़ाने के उद्देश्य से किसी भी स्ट्रेन सुधार कार्यक्रम को शुरू करने के लिए दोहरे-सीमित वृद्धि की गतिकी और फिजियोलॉजी की समझ एक पूर्व-आवश्यकता है। इस प्रयोजन के लिए, हमने एक सरल असंरचित मॉडल विकसित किया है जो शास्त्रीय मोनोड-प्रकार के मॉडल में निहित सीमाओं को पार करते हुए दोहरे-सीमित क्षेत्र में वृद्धि की गतिशीलता का सटीक वर्णन करता है। हमारे मॉडल को बाद में ऑपरेटिंग मापदंडों, डिल्यूशन दर (D) और ऑक्सीजन स्थानांतरण गुणांक ($k_l a$) के विभिन्न संयोजनों के निश्चित मूल्यों पर उत्तरोत्तर बढ़ते फ़ीड सब्सट्रेट (ग्लूकोज) सांद्रता पर संचालित केमोस्टेट में किए गए प्रयोगों के माध्यम से मान्य किया गया था। *एस. सेरेविसिया* के सापेक्ष *एस. स्टाइपिटिस* में इथेनॉल उत्पादन की एक मात्रात्मक तुलना तब प्राप्त की जा सकती थी जब मॉडल के चर को उपयुक्त रूप से बढ़ाया गया था और प्रयोगात्मक डेटा का एक आयामहीन प्लॉट पर विश्लेषण किया गया था। मॉडल को विभिन्न प्रकार के मॉडल माइक्रोबियल सिस्टम में दोहरे पोषक तत्वों की सीमित वृद्धि का वर्णन करने में सार्वभौमिक प्रयोज्यता पाई गई, जैसा कि प्रकाशित साहित्य में उपलब्ध प्रयोगात्मक डेटा से पता चलता है। वर्तमान कार्य द्वारा प्रदान की गई अंतर्दृष्टि से *एस. स्टाइपाइटिस* में दोहरी-सीमित वृद्धि के फिजियोलॉजी में आगे के शोध को प्रोत्साहित करने की उम्मीद है, जो बदले में इसकी इथेनॉल उत्पादन क्षमता में सुधार के लिए नए आनुवंशिक उपकरणों के विकास में सहायता कर सकता है।

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$\mathit{\Omega}$	Aerobicity parameter
X	Biomass
x	Biomass concentration (g.l^{-1})
$C\!:N$	Carbon-Nitrogen ratio
D	Dilution rate (h^{-1})
χ	Dimensionless biomass concentration ($\triangleq Dx/k_l a.c_o^*$
ω	Dimensionless oxygen concentration ($\triangleq c_o/c_o^*$)
ρ	Dimensionless parameter ($\equiv Ds_f/k_l a.c_o^*$)
π	Dimensionless product concentration ($\triangleq Dp/k_l a.c_o^*$
τ	Dimensionless ratio ($\triangleq Ds_f/Dn_f$)
δ	Dimensionless ratio ($\equiv Ds_f/k_l a.c_o^*$)
σ	Dimensionless substrate concentration ($\triangleq s/s_f$)
θ_x	Dimensionless volumetric biomass formation rate ($\triangleq q_x/k_l a\,.\,c_o^*$)
θ_o	Dimensionless volumetric oxygen consumption rate ($\triangleq q_o/k_l a.c_o^*$)
θ_p	Dimensionless volumetric product formation rate ($\triangleq q_p/k_l a\,.\,c_o^*$)
θ_s	Dimensionless volumetric substrate uptake rate ($\triangleq q_s/k_l a\,.\,c_o^*$)
c_o	Dissolved oxygen concentration (g.l^{-1})
n_f	Feed concentration of nitrogen (g.l^{-1})
s_f	Feed substrate concentration (g.l^{-1})
$\underline{s_f}$	Feed substrate concentration at C-limited boundary (g.l^{-1})
c_s^{μ}	Flux control coefficient for specific growth rate μ by a nutrient, S
m	Maintenance coefficient
μ_{max}	Maximum specific growth rate (h^{-1})
c_o^*	Oxygen solubility (g.l^{-1})
$k_l a$	Oxygen transfer coefficient (h^{-1})
P	Product
p	Product concentration (g.l^{-1})

s	Residual substrate concentration (g.l^{-1})
K	Saturation constant (g.l^{-1})
r_x	Specific biomass formation rate (g.g dw^{-1}.h^{-1})
μ	Specific growth rate (h^{-1})
r_o	Specific oxygen consumption rate (g.g dw^{-1}.h^{-1})
r_p	Specific product formation rate (g.g dw^{-1}.h^{-1})
r_s	Specific substrate consumption rate (g.g dw^{-1}.h^{-1})
S	Substrate
$q_{s,total}$	Total volumetric substrate consumption rate (g.l^{-1}.h^{-1})
q_x	Volumetric biomass formation rate (g.l^{-1}.h^{-1})
q_o	Volumetric oxygen consumption rate (g.l^{-1}.h^{-1})
q_p	Volumetric product formation rate (g.l^{-1}.h^{-1})
q_s	Volumetric substrate consumption rate (g.l^{-1}.h^{-1})
Y_{xo}	Yield of oxygen over biomass
Y_{so}	Yield of oxygen over substrate
Y_{xp}	Yield of product over biomass
Y_{xs}	Yield of substrate over biomass
G	Volumetric flow rate of gas (l.min^{-1})
T	Temperature of gas (^{0}C)
P	Pressure of influent gas (atm)
y_{O_2}	Mole fraction of O_2 in gas phase
y_{CO_2}	Mole fraction of CO_2 in gas phase
y_I	Mole fraction of inerts (N_2 or Ar) in gas phase
N	Molar flow rates of gas (mol.min^{-1})
OTR	Oxygen uptake rate by the cells (mol O_2.min^{-1})
CPR	Carbon dioxide production rate by the cells (mol CO_2.min^{-1})
$NMFR_{O_2}$	Net molar flow rate of oxygen (mol.min^{-1})

LIST OF ACRONYMS/ABBREVIATIONS

C-limited	Carbon-limited
C-source	Carbon source
DO	Dissolved oxygen
DOT	Dissolved oxygen tension
GHG	Greenhouse gas
N-limited	Nitrogen-limited
N-source	Nitrogen source
O-limited	Oxygen-limited
PHA	Polyhydroxy alkanoates
S. cerevisiae	*Saccharomyces cerevisiae*
S. stipitis	*Scheffersomyces stipitis*