STUDIES ON THE MECHANISM OF GLUCOSE-MEDIATED REPRESSION OF THE *LAC* OPERON OF *ESCHERICHIA COLI*: ROLE OF INDUCER EXCLUSION AND POSITIVE FEEDBACK

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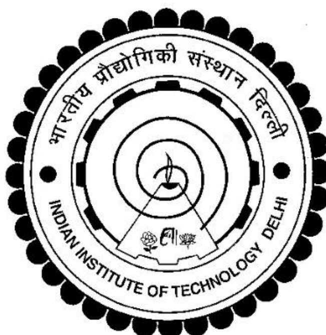

DEPARTMENT OF BIOCHEMICAL ENGINEERING AND BIOTECHNOLOGY

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by

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Submitted

in fulfilment of the requirements for the degree of the Doctor of Philosophy

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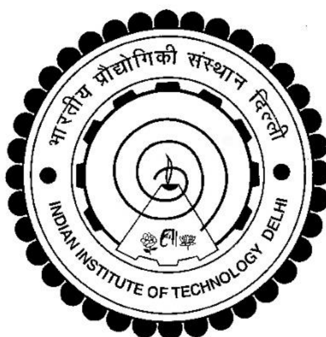

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CERTIFICATE

This is to certify that the thesis entitled **"Studies on the mechanism of glucose-mediated repression of the *lac* operon of *Escherichia coli*: Role of inducer exclusion and positive feedback"** being submitted by **Mr. Ritesh Kumar Aggarwal** is worthy of consideration for the award of the degree of **Doctor of Philosophy**. The thesis has been prepared under my supervision and guidance in conformity with the rules and regulations of Indian Institute of Technology Delhi and is a record of the original bonafide
research work. The results presented in this thesis have not been submitted in part or full to any other universities or institutes for the award of any other degree or diploma.

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ABSTRACT

The *lac* operon of *Escherichia coli* is repressed >600-fold in the presence of glucose. This repression is usually attributed to two molecular mechanisms, namely activation by 3',5'-cyclic adenosine monophosphate (cAMP) and inducer exclusion. cAMP-mediated transcriptional inhibition has been shown to have a modest effect on the regulation of the *lac* operon. This has led some to postulate that inducer exclusion is solely responsible for the catabolite repression. However, the effect of inducer exclusion is only 2-fold in *lac* constitutive strains, while the proposition that inducer exclusion may have a dramatic effect on cells induced to lower levels of *lac* expression, has not been sufficiently tested. Therefore, the first goal of this work was to quantify the magnitude of inducer exclusion in wild-type cells with lower *lac* expression. We found that inducer exclusion exerted a maximum 5-fold effect in the partially induced cells—still a quantitative mismatch on account of the several hundred fold repression. It is concluded that the the current models cannot explain the magnitude of catabolite repression. Since the design of industrial bioprocesses is largely dependent on knowledge of regulatory mechanisms, it becomes absolutely essential to address basic sciences problems such as this. Moreover, the textbooks available globally have already begun to acknowledge the problem of repression deficit.

We hypothesize that *lac* regulation is subject to positive feedback, which amplifies the small effects of inducer exclusion or cAMP activation to account for the almost complete repression. Positive feedback occurs during induction of the *lac* operon because allolactose stimulates the synthesis of *lac* enzymes, namely Lac permease and β-galactosidase, which in turn, promote synthesis of allolactose. While the first process was established more than 50 years ago, there is no experimental evidence for the latter. Indeed, based on mathematical model, it has been argued that during growth on lactose, intracellular allolactose is

independent of enzyme levels, i.e., no positive feedback. Therefore, the second goal was to test the existence of positive feedback during growth on lactose by measuring the intracellular allolactose concentration as a function of Lac permease activity in various culturing conditions. However, rapid efflux results in massive accumulation of allolactose in the medium, thus severely limiting the application of standard metabolomics approaches to measure intracellular allolactose concentrations using direct or differential methods. To resolve this, we developed an indirect method to measure intracellular allolactose from specific allolactose efflux rate during growth in the presence of lactose. With this method, we found that during growth on lactose (induction) the specific β-galactosidase and intracellular allolactose concentrations are coupled, i.e., they increased simultaneously in both batch and continuous cultures. It follows that positive feedback exists. Further, during catabolite repression in the presence of glucose, the positive feedback loop reversed direction leading to a progressive decline in the levels of enzyme and intracellular allolactose, consistent with the positive feedback loop reversing the direction.

It seemed desirable to supplement the indirect allolactose concentration measurements with more direct measurements. However, the difference method mentioned above is error prone due to massive accumulation of allolactose in the medium. We reasoned that the error in this method could be reduced significantly if extracellular accumulation of allolactose is somehow minimized. Therefore, the third goal of this work was to develop a suitable perfusion bioreactor to facilitate removal of extracellular allolactose, while cells are retained in the reactor. To this end, we employed a membrane bioreactor that allowed for fast medium dilution rate, and estimated the errors sustained in the measurements. We established the proof of concept that at high dilution rates, rapidly expelled intracellular metabolites can be quantified reproducibly. However, for allolactose quantification the desirable dilution rates must be increased another two-fold before acceptable data is obtained.

सार

अश्केरिकिआ कोलाई का लैक ओपेरों ग्लूकोज की उपस्थिति में 600 गुना रिप्रेस (दमन) होता है। यह दमन आमतौर पर दो आण्विक तंत्र, अर्थात् 3 ', 5'-चक्रीय एडेनोसाइन मोनोफॉस्फेट (सीएएमपी) और इंड्यूसर एक्सक्लूशन (प्रेरक बहिष्करण) द्वारा सक्रियण के लिए जिम्मेदार है। सीएएमपी-मध्यस्थ ट्रांसक्रिप्शन (संश्लेषण) अवरोध को लैक ऑपरॉन के विनियमन पर मामूली प्रभाव देखा गया है। इसने कुछ लोगों को यह निर्धारित करने के लिए प्रेरित किया है कि प्रेरक बहिष्करण पूरी तरह से संश्लेषण दमन के लिए जिम्मेदार है। हालांकि, प्रेरक बहिष्करण का प्रभाव लैक संवैधानिक उपभेदों में केवल 2 गुना है, जबकि प्रस्ताव है कि प्रेरक बहिष्कार का परिणाम लाखों अभिव्यक्ति के निम्न स्तर से प्रेरित कोशिकाओं पर नाटकीय प्रभाव हो सकता है, पर्याप्त रूप से परीक्षण नहीं किया गया है। इसलिए, इस काम का पहला लक्ष्य कम लैक अभिव्यक्ति वाले जंगली प्रकार के कोशिकाओं में इंड्यूसर बहिष्करण की परिमाण को मापना था। हमने पाया कि आंशिक बहिष्कार ने आंशिक रूप से प्रेरित कोशिकाओं में अधिकतम 5 गुना प्रभाव डाला है-अभी भी कई सौ गुना दमन के कारण मात्रात्मक विसंगति है। यह निष्कर्ष निकाला गया है कि वर्तमान मॉडल संश्लेषण दमन की परिमाण को समझा नहीं सकते हैं। चूंकि औद्योगिक बायोप्रोसेस के डिजाइन बड़े पैमाने पर नियामक तंत्र के ज्ञान पर निर्भर हैं, इसलिए बुनियादी विज्ञान की समस्याओं को हल करने के लिए यह बिल्कुल आवश्यक हो जाता है। इसके अलावा, विश्व स्तर पर उपलब्ध पाठ्यपुस्तक पहले से ही दमन घाटे की समस्या को स्वीकार करना शुरू कर चुके हैं। हम अनुमान लगाते हैं कि लैक विनियमन पॉज़िटिव फीडबैक (सकारात्मक प्रतिक्रिया) के अधीन है, जो लगभग पूरी तरह से दमन के लिए प्रेरक बहिष्कार या सीएएमपी सक्रियण के छोटे प्रभाव को बढ़ाता है। सकारात्मक प्रतिक्रिया लैक ओपेन के प्रेरण के दौरान होती है क्योंकि एलोलैक्टोज़ लैक एंजाइमों, अर्थात् लैक पर्मिऐज और β-गेलैक्टोसिडेज़ के संश्लेषण को उत्तेजित करता है, जो बदले में, एलोलैक्टोज के संश्लेषण को बढ़ावा देता है। जबकि पहली प्रक्रिया 50 साल पहले स्थापित की गई थी, बाद के लिए कोई प्रयोगात्मक सबूत नहीं है। वास्तव में, गणितीय मॉडल के आधार पर, यह तर्क दिया गया है कि लैक्टोज पर वृद्धि के दौरान, इंट्रासेल्यूलर एलोलैक्टोज एंजाइम के स्तर से स्वतंत्र है, यानी कोई सकारात्मक प्रतिक्रिया नहीं है। इसलिए, दूसरा लक्ष्य

विभिन्न संस्कृति स्थितियों में लैक पारगम्य गतिविधि के एक समारोह के रूप में इंट्रासेल्यूलर एलोलाक्टोज एकाग्रता को मापकर लैक्टोज पर वृद्धि के दौरान सकारात्मक प्रतिक्रिया के अस्तित्व का परीक्षण करना था। हालांकि, माध्यम में एलोलेक्टोज के बड़े पैमाने पर संचय में तेजी से एफ्लक्स परिणाम, इस प्रकार प्रत्यक्ष या विभेदक तरीकों का उपयोग कर इंट्रासेल्यूलर एलोलैक्टोज सांद्रता को मापने के लिए मानक चयापचय दृष्टिकोण के आवेदन को गंभीर रूप से सीमित कर रहा है।

इसे हल करने के लिए, हमने लैक्टोज की उपस्थिति में वृद्धि के दौरान विशिष्ट एलोलैक्टोज एफ्लक्स दर से इंट्रासेल्यूलर एलोलाक्टोज को मापने के लिए एक अप्रत्यक्ष विधि विकसित की। इस विधि के साथ, हमने पाया कि लैक्टोज (प्रेरण) पर वृद्धि के दौरान विशिष्ट β-गेलैक्टोसिडेज़ और इंट्रासेल्यूलर एलोलैक्टोज सांद्रता युग्मित होते हैं, यानी, वे दोनों बैच और निरंतर संस्कृतियों में एक साथ बढ़ते हैं। यह इस प्रकार है कि सकारात्मक प्रतिक्रिया मौजूद है। इसके अलावा, ग्लूकोज की उपस्थिति में संश्लेषण दमन के दौरान, सकारात्मक फीडबैक लूप ने दिशा को उलट दिया जिससे एंजाइम और इंट्रासेल्यूलर ऑलोलैक्टोज के स्तर में प्रगतिशील गिरावट आई, सकारात्मक प्रतिक्रिया लूप दिशा को उलट देता है। यह अधिक प्रत्यक्ष माप के साथ अप्रत्यक्ष एलोलैक्टोज एकाग्रता माप पूरक पूरक वांछनीय लग रहा था। हालांकि, उपरोक्त वर्णित अंतर विधि माध्यम में एलोलेक्टोज के बड़े पैमाने पर संचय के कारण त्रुटि प्रवण है। हमने तर्क दिया कि इस पद्धति में त्रुटि को काफी कम किया जा सकता है यदि एलोलेक्टोज का बाह्य कोशिका संचय किसी भी तरह कम हो जाता है। इसलिए, इस काम का तीसरा लक्ष्य बाह्य कोशिकीय एलोलाक्टोज को हटाने की सुविधा के लिए एक उपयुक्त परफ्यूजन बायोरेक्टर विकसित करना था, जबकि कोशिकाओं को रिएक्टर में बनाए रखा गया था। इस अंत में, हमने एक पर्फ्यूशन बायोरेक्टर को नियोजित किया जो तेजी से मध्यम कमजोर पड़ने की दर के लिए अनुमति देता है, और माप में बनाए गए त्रुटियों का अनुमान लगाता है। हमने अवधारणा का सबूत स्थापित किया है कि उच्च कमजोर पड़ने वाली दरों पर, तेजी से निष्कासित इंट्रासेल्यूलर मेटाबोलाइट्स को पुनरुत्पादित किया जा सकता है। हालांकि, एलोलैक्टोज मात्रा के लिए स्वीकार्य डेटा प्राप्त होने से पहले वांछनीय कमजोर दरों को एक और दो गुना बढ़ाया जाना चाहिए।

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ABBREVIATIONS AND NOTATIONS

ABBREVIATIONS

A_{420}, A_{410}	-	Absorbance at 420 nm, Absorbance at 410 nm
cAMP	-	3',5'-cyclic adenosine monophosphate
CAP	-	Catabolite activator protein
CGSC	-	Coli Genetic Stock Centre, Yale University
CRP	-	cAMP receptor protein
CSTR	-	Continuously stirred tank reactor
DO	-	Dissolved oxygen concentration, mg/l or %
$EIIA^{Glc}$	-	Enzyme III of glucose transport system, PTS (Previously $EIII^{Glc}$)
gdw	-	Grams of cell dry weight, g
HPAEC	-	High-performance anion exchange chromatography (also see PAD)
IPTG	-	Isopropyl β-D-thiogalactopyranoside or MeSH
kDa	-	Kilodaltons
LB	-	Luria-Bertani broth
LC-MS	-	Liquid chromatography – Mass spectroscopy
$MnCl_2$	-	Manganese chloride
MWCO	-	Molecular weight cut-off
N_2	-	Nitrogen gas
NaCl	-	Sodium chloride
NAD^+	-	Nicotinamide adenine dinucleotide
NaOH	-	Sodium hydroxide
OD_{600}	-	Optical density at 600 nm
ONP	-	*ortho*-nitrophenol
ONPG	-	*ortho*-nitrophenol-β-D-galactopyranoside
PAD	-	Pulsed amperometric detections (also see HPAEC)
PAN	-	Polyacrylonitrile membrane
PAN40	-	Polyacrylonitrile membrane with MWCO 40 kDa
PCA	-	Perchloric acid
PES	-	Polyethersulfone membrane
PES100	-	Polyethersulfone membrane with MWCO 100 kDa
PNP	-	*para*-nitrophenol
α-PNPG	-	*para*-nitrophenol- α -D-galactopyranoside
PTS	-	Phophotransferase system

RNA	-	Ribonucleic acid
rpm	-	Number of revolutions per minute (for reactor impeller)
SDS	-	Sodium dodecyl sulphate
TMG	-	Methyl- β-D-1-thiogalactopyranoside
[^{14}C]TMG	-	Carbon-14 labelled isotope of TMG
UV	-	Ultraviolet (radiation)
vvm	-	Volume of air sparged per unit of reactor volume per minute
VWD	-	Variable wavelength detector
X-gal	-	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

NOTATIONS: Latin

a	Extracellular allolactose concentration, μM
a_i	Intracellular allolactose concentration, μM
a_{tot}	Allolactose concentration in total sample, μM
A	Available filtration surface area, m^2
c	Extracellular cAMP concentration, nM
c_i	Intracellular cAMP concentration, nM
c_{tot}	cAMP concentration in total sample, nM
D	Dilution rate, h^{-1}
$D_{\max}$	Maximum operable dilution rate in membrane bioreactor, h^{-1}
D_{washout}	Washout dilution rate, h^{-1}
$\underline{D_P}$	Minimum dilution rate required for difference method, h^{-1}
$\mathcal{D}$	Diffusion coefficient for back transfer from polarization cake, m^2/s
e	Specific enzyme activity, units/gdw
e_m	Maximum specific enzyme activity, units/gdw
$E_{IIA,t}^{glc}$	Specific activity of $EIIA^{Glc}$
E_p	Specific activity of free permease
E_g	Specific activity of β-galactosidase
$E_{p,\mathrm{t}}$	Specific activity of β-galactosidase (proportional to total permease activity)
$E_{p,\mathrm{t}}^{glu}$	Steady state specific activity of permease in glucose+TMG
$E_{p,\mathrm{t}}^{gly}$	Steady state specific activity of permease in glycerol+TMG
F_P	Volumetric flow rate of permeate stream, m^3/s
F_R	Volumetric flow rate of recirculation stream, m^3/s
J	Flux through the filtration membrane, m/s or $m^3\ s^{-1}\ m^{-2}$
k_a	Diffusivity of allolactose through cell membrane, h^{-1}
k_a^-	Turnover number for permease mediated allolactose uptake

k_c	Diffusivity of cAMP through cell membrane, h^{-1}
k_d	Diffusivity of TMG through cell membrane, h^{-1}
k_p^+, k_p^-	Turnover numbers for TMG transport in and out, respectively
K_a^-	Apparent saturation constant for permease-mediated allolactose uptake, μM
$K_{a,\text{true}}^-$	True saturation constant for permease-mediated allolactose uptake, μM
$K_{L,\text{true}}^-$	True saturation constant for permease-mediated lactose uptake, μM
K_p^+, K_p^-	Saturation constant for permease-mediated influx, efflux, g/l
K_s	Saturation constant for enzyme-mediated sugar consumption, g/l
L	Extracellular lactose concentration, μM
OD_{600}	Optical density at 600 nm
p	Extracellular product concentration, μM
p_i	Intracellular product concentration, μM
p_{tot}	Product concentration in total sample, μM
P_1	Pressure at the inlet of the filtration module, bar
P_2	Pressure at the outlet of the filtration module, bar
ΔP_a	(Axial) Pressure drop across the length of filtration module, bar
ΔP_t	Average transmembrane pressure, bar
r_a	Specific accumulation rate of allolactose in the medium, g gdw^{-1} h^{-1}
r_a^+	Specific allolactose expulsion rate from cells, g gdw^{-1} h^{-1}
r_a^-	Specific allolactose uptake rate, g gdw^{-1} h^{-1}
r_c^+	Specific cAMP expulsion rate from cells, g gdw^{-1} h^{-1}
r_c^-	Specific cAMP uptake rate, g gdw^{-1} h^{-1}
r_e^+	Specific enzyme synthesis rate, Miller units/h
$r_{e,\max}^+$	Maximum specific enzyme synthesis rate, Miller units/h
$\tilde{r}_e^+$	Steady-state specific enzyme synthesis rate, Miller units/h
$r_{E_g}^+$	Specific synthesis rate of β-galactosidase
r_p^+	Specific rate of permease-mediated influx of TMG
r_p^-	Specific rate of permease-mediated efflux of TMG
r_d	Specific rate of diffusive efflux of TMG
r_s	Specific substrate consumption rate, g gdw^{-1} h^{-1}
R	Recirculation rate, h^{-1}
R_M	Membrane resistance
R_C	Resistance offered by the cake formation
s	Extracellular substrate concentration, μM
s_{out}, s_0	Extracellular substrate concentration, μM
s_i	Intracellular substrate concentration, μM

s_f	Substrate concentration in the feed stream, μM
t	Time, s or min or h
T	Intracellular TMG concentration, μM
$\tilde{T}$	Steady-state intracellular TMG concentration, μM
T_e	Extracellular TMG concentration, μM
T_{gly}, T_{glu}	Intracellular TMG concentration in the presence of glycerol, glucose
v	Volume of sample + buffer in reaction, ml
V	Extracellular volume (without cells), ml
V_a^-	Maximum specific allolactose uptake rate, g gdw^{-1} h^{-1}
V_i	Intracellular volume, μl
V_{tot}	Total volume (medium + cell water), ml
x	Biomass or cell density, gdw/l
x_w	Biomass concentration at the polarization cake surface, gdw/l

NOTATIONS: Greek

α	Total intracellular volume per unit sample volume, l/gdw
δ	Polarization cake thickness, μm
ϵ	Magnitude of inducer exclusion, calculated as $1 - T_{glu}/T_{gly}$
ΔP_a	(Axial) Pressure drop across the length of filtration module, Bar
ΔP_t	Average transmembrane pressure, Bar
κ	Mass transfer coefficient for membrane filtration cake, m/s
μ	Specific growth rate of *E. coli* cells, h^{-1}
μ_{max}	Maximum specific growth rate, h^{-1}
φ_a	Amount of allolactose in medium **:** Amount inside cells (Ratio)
$\bar{\varphi}_a$	Maximum desirable value of φ_a
$\emptyset$	Constant relating the concentration polarization to transverse pressure
ψ_a	Extracellular concentration **:** Intracellular concentration (Ratio)

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