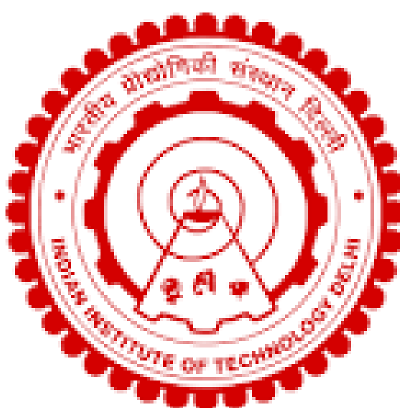
METABOLIC ENGINEERING OF *BACILLUS SUBTILIS* FOR XYLOSE UTILISATION AND PRODUCTION OF 2,3-BUTANEDIOL AND D-LACTIC ACID

NGANGOM PRAVINA DEVI



DEPARTMENT OF BIOCHEMICAL ENGINEERING AND BIOTECHNOLOGY

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by

NGANGOM PRAVINA DEVI

DEPARTMENT OF BIOCHEMICAL ENGINEERING AND BIOTECHNOLOGY

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To my beloved parents for their care, support and unconditional love.

To my brother for being my pillar of support and strength.

To my true friends. May we all find a few.

Certificate

This is to certify that the thesis entitled **"Metabolic engineering of *Bacillus subtilis* for xylose utilisation and production of 2,3-butanediol and D-lactic acid"** being submitted by **Ms. Ngangom Pravina Devi** 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 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:

Place: New Delhi

Prof. Ashish Misra

Assistant professor

Department of Biochemical

Engineering and Biotechnology

IIT Delhi

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Abstract

Bacillus subtilis is a well-studied microbial host that has been used for production of industrially important enzymes and chemicals. *B. subtilis* utilizes glucose efficiently and produces 2,3-butanediol (2,3-BDO) under oxygen-limited conditions and L-lactic acid (L-LA) under severely oxygen-limited conditions; however, it lacks the ability to utilise xylose natively. *B. subtilis* was engineered for efficient utilization of xylose at high concentrations and xylose co-utilisation in the presence of glucose. Additionally, *B. subtilis* was engineered for 2,3-butanediol production under oxygen-limited conditions from glucose-xylose mixtures and food waste; and production of a non-native product, D-lactic acid.

For xylose utilization, highly active xylose isomerase (*xylA)* and xylulokinase (*xylB*) from *Bacillus coagulans* were expressed*,* along with overexpression of the native *B. subtilis* (*araE*) transporter. The 3 genes (*araE*, *xylA* and *xylB)* were genomically integrated downstream of a strong constitutive promoter P_{43}, and their expression was optimised by using different promoter-gene combinations. An engineered strain (Bs-BcABAr) with expression of all 3 genes in an operon format under the P_{43} promoter exhibited growth on xylose which was comparable to its growth on glucose. The Bs-BcABAr strain was able to consume xylose at high concentrations of up to ~80 g/l. Further, this engineered strain simultaneously utilised both glucose and xylose without exhibiting any diauxic growth. The Bs-BcABAr strain exhibited robust performance in bioreactor studies and co-utilised ~20 g/l glucose and ~20 g/l xylose to produce ~13 g/l 2,3-BDO (yield of 0.33 g/g sugar) under oxygen-limited conditions.

B. subtilis was engineered to improve the yields of 2,3-BDO production from glucose-xylose mixtures under oxygen-limited conditions. The Bs-BcABAr strain (engineered for robust xylose utilisation) produced 2,3-BDO as the sole product only oxygen-limited conditions but produced L-LA as a major co-product under severely oxygen-limited

conditions. To enhance 2,3-BDO production, the L-*ldh* gene was deleted and the native *B. subtilis* 2,3-BDO genes were simultaneously overexpressed under the robust constitutive P_{43} promoter in the Bs-BcABAr strain. The resulting Bs-BcABAr+BsBΔL strain produced 2,3-BDO as main product with no L-LA formation on glucose-xylose mixtures under oxygen-limited conditions. For growth in a bioreactor under controlled oxygen-limited conditions, the Bs-BcABAr+BsBΔL strain showed good growth, and co-utilised ~54 g/l of glucose and ~57 g/l of xylose to produce ~40 g/l of 2,3-BDO (yield of 0.36 g/g total sugars) with no L-LA production.

The wild-type *B. subtilis* was engineered for better conversion of organic food wastes to 2,3-BDO while eliminating L-LA formation. The native *amyE* gene (coding for α-(1-4)-amylase) was overexpressed under the P_{43} promoter in the wild-type strain to improve its starch-degrading capability while simultaneously deleting the L-*ldh* gene. The recombinant BsΔL-LE strain showed faster utilisation of starch and food waste compared to the wild-type; and produced up to ~10 g/l of 2,3-BDO from rice waste and about ~14 g/l of 2,3-BDO from fruit waste.

Non-native D-LA was produced at high titres in *B. subtilis* by redirecting carbon flux away from L-LA and 2,3-BDO. First, the native L-*ldh* gene was deleted to eliminate L-LA production. Heterologous D-*ldh* genes from native D-LA producers (*Lactobacillus delbruckeii* and *Pediococcus acidilactici*) were expressed; and the acetolactate synthase gene was concurrently deleted to stop 2,3-BDO production. The Bs-PaLΔLΔB strain (expressing *P. acidilactici* D-*ldh*) exhibited ~2.5 times higher D-LA production compared to the Bs-LdLΔLΔB strain (expressing *L. delbruckeii* D-*ldh*). When cultured in a bioreactor under pH-controlled and oxygen-limited conditions, the Bs-PaLΔLΔB strain consumed ~60 g/l of glucose and produced ~35 g/l of D-LA (yield of 0.58 g/g of glucose).

In summary, this work demonstrated metabolic engineering of *B. subtilis* for effective co-utilisation xylose in the presence of glucose; 2,3-BDO production from glucose-xylose mixtures under oxygen-limited conditions; utilisation of food waste for 2,3-BDO production and production of non-native D-LA. All engineered strains showed robust performance in shake flasks and bioreactors without the requirement for antibiotics or inducers because of the use of genomically integrated constitutive expression cassettes. These findings underscore the successful application of metabolic engineering techniques to improve substrate utilisation and product formation in *B. subtilis,* and furthers its use as an industrially important host organism.

सार

बैसिलस सबटिलिस एक अच्छी तरह से अध्ययन किया गया माइक्रोबियल होस्ट है जिसका उपयोग औद्योगिक रूप से महत्वपूर्ण एंजाइमों और रसायनों के उत्पादन के लिए किया गया है। बी. सबटिलिस ग्लूकोज का कुशलतापूर्वक उपयोग करता है और ऑक्सीजन-सीमित परिस्थितियों में 2,3-ब्यूटेनडियोल (2,3-बीडीओ) और गंभीर ऑक्सीजन-सीमित परिस्थितियों में एल-लैक्टिक एसिड (एल-एलए) का उत्पादन करता है; हालाँकि, इसमें ज़ाइलोज़ का मूल रूप से उपयोग करने की क्षमता का अभाव है। बी. सबटिलिस को उच्च सांद्रता में ज़ाइलोज़ के कुशल उपयोग और ग्लूकोज की उपस्थिति में ज़ाइलोज़ सह-उपयोग के लिए इंजीनियर किया गया था। इसके अतिरिक्त, बी. सबटिलिस को ग्लूकोज-ज़ाइलोज़ मिश्रण और खाद्य अपशिष्ट से ऑक्सीजन-सीमित परिस्थितियों में 2,3-ब्यूटेनडियोल उत्पादन के लिए इंजीनियर किया गया था; और एक गैर-देशी उत्पाद, डी-लैक्टिक एसिड का उत्पादन।

ज़ाइलोज़ उपयोग के लिए, बेसिलस कोगुलांस से अत्यधिक सक्रिय ज़ाइलोज़ आइसोमेरेज़ (*xylA*) और ज़ाइलुलोकिनेज़ (*xylB*) को देशी बी सबटिलिस (*araE*) ट्रांसपोर्टर की ओवरएक्प्रेशन के साथ व्यक्त किया गया था। 3 जीन (*araE*, *xylA* और *xylB*) को एक मजबूत संवैधानिक प्रमोटर $पी_{43}$ के डाउनस्ट्रीम में जीनोमिक रूप से एकीकृत किया गया था, और विभिन्न प्रमोटर-जीन संयोजनों का उपयोग करके उनकी अभिव्यक्ति को अनुकूलित किया गया था। $पी_{43}$ प्रमोटर के तहत एक ऑपेरॉन प्रारूप में सभी 3 जीनों की अभिव्यक्ति के साथ एक इंजीनियर्ड स्ट्रेन (Bs-BcABAr) ने ज़ाइलोज़ पर वृद्धि प्रदर्शित की जो ग्लूकोज पर इसकी वृद्धि के बराबर थी। Bs-BcABAr स्ट्रेन ~80 ग्राम/लीटर तक की उच्च सांद्रता पर ज़ाइलोज़ का उपभोग करने में सक्षम था। इसके अलावा, इस इंजीनियर्ड स्ट्रेन ने किसी भी डायऑक्सिक वृद्धि को प्रदर्शित किए बिना ग्लूकोज और जाइलोज़ दोनों का एक साथ उपयोग किया। Bs-BcABAr स्ट्रेन ने बायोरिएक्टर अध्ययनों में मजबूत प्रदर्शन प्रदर्शित किया और

~20 ग्राम/लीटर ग्लूकोज और ~20 ग्राम/लीटर ज़ाइलोज़ का सह-उपयोग करके ~13 ग्राम/लीटर 2,3-बीडीओ (0.33 ग्राम/ग्राम चीनी की उपज) का उत्पादन किया। ऑक्सीजन-सीमित स्थितियाँ।

बी. सबटिलिस को ऑक्सीजन-सीमित परिस्थितियों में ग्लूकोज-ज़ाइलोज़ मिश्रण से 2,3-बीडीओ उत्पादन की पैदावार में सुधार करने के लिए इंजीनियर किया गया था। Bs-BcABAr स्ट्रेन (मजबूत ज़ाइलोज़ उपयोग के लिए इंजीनियर) ने केवल ऑक्सीजन-सीमित स्थितियों में एकमात्र उत्पाद के रूप में 2,3-बीडीओ का उत्पादन किया, लेकिन गंभीर रूप से ऑक्सीजन-सीमित स्थितियों के तहत एक प्रमुख सह-उत्पाद के रूप में L-LA का उत्पादन किया। 2,3-बीडीओ उत्पादन को बढ़ाने के लिए, एल-एलडीएच जीन को हटा दिया गया था और मूल बी सबटिलिस 2,3-बीडीओ जीन को एक साथ बीएस-बीसीएबीएआर स्ट्रेन में मजबूत संवैधानिक पी$_{43}$ प्रमोटर के तहत अतिरंजित किया गया था। परिणामी Bs-BcABAr+BsBΔL स्ट्रेन ने ऑक्सीजन-सीमित परिस्थितियों में ग्लूकोज-ज़ाइलोज़ मिश्रण पर कोई L-LA गठन के साथ मुख्य उत्पाद के रूप में 2,3-बीडीओ का उत्पादन किया। नियंत्रित ऑक्सीजन-सीमित परिस्थितियों में बायोरिएक्टर में वृद्धि के लिए, Bs-BcABAr+BsBΔL स्ट्रेन ने अच्छी वृद्धि दिखाई, और ~54 ग्राम/लीटर ग्लूकोज और ~57 ग्राम/लीटर ज़ाइलोज़ का सह-उपयोग करके ~40 ग्राम/लीटर का उत्पादन किया। 2,3-बीडीओ (0.36 ग्राम/ग्राम कुल शर्करा की उपज) बिना एल-एलए उत्पादन के।

जंगली प्रकार के बी. सबटिलिस को एल-एलए गठन को समाप्त करते हुए जैविक खाद्य अपशिष्टों को 2,3-बीडीओ में बेहतर रूपांतरण के लिए इंजीनियर किया गया था। देशी एमीई जीन (α-(1-4)-एमाइलेज़ के लिए कोडिंग) को एल-एलडीएच जीन को हटाने के साथ-साथ इसकी स्टार्च-डिग्रेडिंग क्षमता में सुधार करने के लिए जंगली-प्रकार के तनाव में पी$_{43}$ प्रमोटर के तहत अतिरंजित किया गया था। पुनः संयोजक BsΔL-LE स्ट्रेन ने जंगली प्रकार की तुलना में स्टार्च और खाद्य अपशिष्ट का तेजी से

उपयोग दिखाया; और चावल के कचरे से ~10 ग्राम/लीटर 2,3-बीडीओ और फलों के कचरे से लगभग ~14 ग्राम/लीटर 2,3-बीडीओ का उत्पादन होता है।

गैर-देशी डी-एलए का उत्पादन एल-एलए और 2,3-बीडीओ से दूर कार्बन प्रवाह को पुनर्निर्देशित करके बी सबटिलिस में उच्च टाइट्रेस पर किया गया था। सबसे पहले, एल-एलए उत्पादन को खत्म करने के लिए मूल एल-एलडीएच जीन को हटा दिया गया था। देशी डी-एलए उत्पादकों (लैक्टोबैसिलस डेलब्रुकेई और पेडियोकोकस एसिडिलैक्टिसी) से विषम डी-एलडीएच जीन व्यक्त किए गए थे; और 2,3-बीडीओ उत्पादन को रोकने के लिए एसिटोलैक्टेट सिंथेज़ जीन को समवर्ती रूप से हटा दिया गया था। Bs-PaLΔLΔB स्ट्रेन (P. एसिडिलैक्टिसी *D-ldh* को व्यक्त करता है) ने Bs-LdLΔLΔB स्ट्रेन (L. delbruckeii D-ldh को व्यक्त करता है) की तुलना में ~2.5 गुना अधिक D-LA उत्पादन प्रदर्शित किया। जब पीएच-नियंत्रित और ऑक्सीजन-सीमित परिस्थितियों में बायोरिएक्टर में संवर्धित किया गया, तो Bs-PaLΔLΔB स्ट्रेन ने ~60 ग्राम/लीटर ग्लूकोज की खपत की और ~35 ग्राम/लीटर डी-एलए (0.58 ग्राम/ग्राम ग्लूकोज की उपज) का उत्पादन किया।

संक्षेप में, इस कार्य ने ग्लूकोज की उपस्थिति में जाइलोज़ के प्रभावी सह-उपयोग के लिए बी. सबटिलिस की चयापचय इंजीनियरिंग का प्रदर्शन किया; ऑक्सीजन-सीमित परिस्थितियों में ग्लूकोज-ज़ाइलोज़ मिश्रण से 2,3-बीडीओ उत्पादन; 2,3-बीडीओ उत्पादन और गैर-देशी डी-एलए के उत्पादन के लिए खाद्य अपशिष्ट का उपयोग। जीनोमिक रूप से एकीकृत संवैधानिक अभिव्यक्ति कैसेट के उपयोग के कारण सभी इंजीनियर उपभेदों ने एंटीबायोटिक्स या इंड्यूसर की आवश्यकता के बिना शेक फ्लास्क और बायोरिएक्टर में मजबूत प्रदर्शन दिखाया। ये निष्कर्ष बी. सबटिलिस में सब्सट्रेट उपयोग और उत्पाद निर्माण में सुधार के लिए चयापचय इंजीनियरिंग तकनीकों के सफल अनुप्रयोग को रेखांकित करते हैं, और औद्योगिक रूप से महत्वपूर्ण मेजबान जीव के रूप में इसके उपयोग को आगे बढ़ाते हैं।

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Abbreviations

% MTY	Percentage of maximum theoretical yield
AA	Acetic acid
ALDC	Acetolactate decarboxylase
ALS	Acetolactate synthase
ATP	Adenosine triphosphate
BDH	Butanediol dehydrogenase
BDO	Butanediol
BGSC	Bacillus genetic stock centre
CCR	Carbon catabolite repression
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
EMP	Embden-Meyerhof-Parnas
GRAS	Generally Regarded As Safe
HPLC	High-performance liquid chromatography
LA	Lactic acid
LB	Luria Bertani
LDH	Lactate dehydrogenase
NAD^+	Nicotinamide adenine dinucleotide

$NADP^+$	Nicotinamide adenine dinucleotide phosphate
OD	Optical density
PP	Pentose phosphate
PCR	Polymerase chain reaction
RBS	Ribosome binding site
RI	Refractive index
TCA	Tricarboxylic acid cycle
YE	Yeast extract

*Gene names are written in lowercase and italicised. Protein names are written in uppercase.