# FRUCTAN SYNTHESIS USING WHOLE-CELL-BASED AND RECOMBINANT ENZYME SYSTEM


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**by**

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

***to the***


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***This research work is dedicated to my Family and Supervisors***

# Certificate

This is to certify that the thesis entitled "**Fructan synthesis using whole-cell-based and recombinant enzyme system"** being submitted by **Mr. Avijeet Singh Jaswal** to the Department of Biochemical Engineering and Biotechnology, 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 him, which has been prepared under our supervision and guidance 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.


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*Avijeet Singh Jaswal*

# Abstract

Fructan is a polymer of fructose and can be categorised into various types, such as inulin, levan, and agavin, and graminan, based on their linkage patterns and degree of polymerization. Owing to the physiological effects induced, these have been used as prebiotics, and in foods and beverages, pharmaceutical, and cosmetic industries. With increasing interest in both high molecular weight (HMW) and short-chain fructans, more efficient methods need to be developed for their production. In this study, we disclose the biosynthesis of high molecular weight (HMW) levan employing whole cells of *Microbacterium paraoxydans*. The structural characterization of the fructan produced extracellularly revealed predominant β-2,6 linkages between adjacent fructose units. Notably, over 90% of the fructan exhibited a molecular weight of approximately 2 x $10^3$ kDa, while the remaining 10% displayed a lower molecular weight of around 20 kDa. The optimization of factors influencing the conversion rate was conducted through a systematic One-Factor-At-a-Time (OFAT) analysis. The synergistic effects of these optimized conditions, namely an initial sucrose concentration of 400 g/L, 100 mM MOPS buffer (pH 7), a temperature of 37 °C, and the addition of 20 mM $CaCl_2$, resulted in the production of approximately 129 g/L of levan. The achieved yield was approximately 0.41 g/g sucrose consumed, against the theoretical yield of 0.47 g/g, accompanied by a volumetric productivity of 1.8 g/L/h.

For the production of 1-kestose enriched short chain-fructooligosaccharides (scFOS), a plant-derived recombinant fructosyltransferase was explored. High-level extracellular production of the recombinant sucrose: sucrose 1-fructosyltransferase (r1-SST, EC 2.4.1.99) from *Festuca arundinacea* was achieved in *Komagataella phaffii* (formerly *Pichia pastoris*) X-33 strain containing 3 copies of the codon-optimized *1-SST* gene. Continuous production of the r1-SST was carried out in a 2 L bioreactor. In a bid to overcome the redox imbalance and

hypoxic stress caused in bioreactor cultivation due to inadequate mixing and methanol uptake, a single-run chemostat-based strain characterization methodology was used to evaluate the effect of methanol-sorbitol co-feeding strategy and the specific growth rate (μ) on the cell-specific r1-SST productivity (Qp) and the cell-specific oxygen uptake rate (Qo). The co-feeding showed ~46% lower Qo for similar Qp than the methanol-alone feeding strategy. At an optimum dilution rate of 0.025 $h^{-1}$, a maximum Qp value of ~100 U/g dry cell weight/h was achieved at a steady-state biomass concentration of ~15 g/L. Finally, high-cell-density continuous cultivation demonstrated continuous production of the r1-SST for a sustained period of 15 days at 0.025 $h^{-1}$ achieving 78% of the maximum Qp value at a steady-state biomass concentration of ~64 g/L and volumetric productivity of ~5000 U/L/h. A kinetic model based on a combined approach of rapid equilibrium and steady-state kinetics was developed to predict the time profile of production of scFOS using purified r1-SST. The purified enzyme was shown to selectively convert sucrose into 1-kestose with a final yield of 0.66 g/g sucrose consumed. The model could predict the effect of substrate concentration on initial rate kinetics, half-saturation constant, and transferase to hydrolase ratio. For the known values of initial sucrose and enzyme concentration, the optimum reaction time and the required dilution rate were predicted for the development of a continuous process to produce 1-kestose enriched scFOS. A volumetric productivity of ~110 g/L/h of 1-kestose with 40% carbohydrate composition in the permeate flux was demonstrated.

# सार

फ्रुक्टेन फ्रुक्टोज का एक बहुलक है और इसे उनके लिंकेज पैटर्न और पोलीमराइजेशन की डिग्री के आधार पर विभिन्न प्रकारों में वर्गीकृत किया जा सकता है, जैसे कि इनुलिन, लेवन, और एगेविन और ग्रैमिनन। प्रेरित शारीरिक प्रभावों के कारण, इनका उपयोग प्रीबायोटिक्स के रूप में और खाद्य एवं पेय पदार्थ, फार्मास्युटिकल और कॉस्मेटिक उद्योगों में किया गया है। उच्च आणविक भार (एचएमडब्ल्यू) और लघु-श्रृंखला फ्रुक्टेन दोनों में बढ़ती रुचि के साथ, उनके उत्पादन के लिए अधिक कुशल तरीकों को विकसित करने की आवश्यकता है। इस अध्ययन में, हम माइक्रोबैक्टीरियम पैराऑक्सिडैन्स की संपूर्ण कोशिकाओं को नियोजित करके उच्च आणविक भार (एचएमडब्ल्यू) लेवन के जैवसंश्लेषण का खुलासा करते हैं। बाह्यकोशिकीय रूप से निर्मित फ्रुक्टेन के संरचनात्मक लक्षण वर्णन से आसन्न फ्रुक्टोज इकाइयों के बीच प्रमुख β-2,6 संबंधों का पता चला। विशेष रूप से, 90% से अधिक फ्रुक्टेन ने लगभग $2 \times 10^3$ kDa का आणविक भार प्रदर्शित किया, जबकि शेष 10% ने लगभग 20 kDa का आणविक भार प्रदर्शित किया। रूपांतरण दर को प्रभावित करने वाले कारकों का अनुकूलन एक व्यवस्थित वन-फैक्टर-ए-टाइम (ओएफएटी) विश्लेषण के माध्यम से किया गया था। इन अनुकूलित स्थितियों के सहक्रियात्मक प्रभाव, अर्थात् 400 ग्राम/लीटर की प्रारंभिक सुक्रोज सांद्रता, 100 mM एमओपीएस बफर (पीएच 7), 37 डिग्री सेल्सियस का तापमान, और 20 mM $CaCl_2$ को जोड़ने के परिणामस्वरूप लगभग 129 का उत्पादन हुआ। लेवन का जी/एल. प्राप्त उपज लगभग 0.41 ग्राम/ग्राम सुक्रोज की खपत थी, जबकि सैद्धांतिक उपज 0.47 ग्राम/ग्राम थी, साथ ही वॉल्यूमेट्रिक उत्पादकता 1.8 ग्राम/लीटर/घंटा थी।

1-केस्टोज़ समृद्ध लघु श्रृंखला-फ्रुक्टुलिगोसेकेराइड्स (एससीएफओएस) के उत्पादन के लिए, एक पौधे-व्युत्पन्न पुनः संयोजक फ्रुक्टोसिलट्रांसफेरेज़ का पता लगाया गया था। पुनः संयोजक सुक्रोज का उच्च-स्तरीय बाह्यकोशिकीय उत्पादन: फेस्टुका अरुंडिनेसिया से सुक्रोज 1-फ्रुक्टोसिलट्रांसफेरेज़ (आर1-एसएसटी, ईसी 2.4.1.99) कोमागाटेला फाफी (पूर्व में पिचिया पास्टोरिस) एक्स-33 स्ट्रेन में प्राप्त

किया गया था जिसमें कोडन-अनुकूलित 1 की 3 प्रतियां शामिल थीं। -एसएसटी जीन. आर1-एसएसटी का निरंतर उत्पादन 2 एल बायोरिएक्टर में किया गया। अपर्याप्त मिश्रण और मेथनॉल ग्रहण के कारण बायोरिएक्टर खेती में होने वाले रेडॉक्स असंतुलन और हाइपोक्सिक तनाव को दूर करने के लिए, मेथनॉल-सोर्बिटोल सह-फीडिंग रणनीति और विशिष्ट के प्रभाव का मूल्यांकन करने के लिए एकल-रन केमोस्टेट-आधारित तनाव लक्षण वर्णन पद्धति का उपयोग किया गया था। कोशिका-विशिष्ट r1-SST उत्पादकता (Qp) और कोशिका-विशिष्ट ऑक्सीजन ग्रहण दर (Qo) पर वृद्धि दर (μ)। मेथनॉल-अलोन फीडिंग रणनीति की तुलना में सह-फीडिंग ने समान क्यूपी के लिए ~46% कम क्यूओ दिखाया। 0.025 h-1 की इष्टतम कमजोर पड़ने की दर पर, ~15 g/L की स्थिर-अवस्था बायोमास सांद्रता पर ~100 U/g शुष्क सेल वजन/h का अधिकतम Qp मान प्राप्त किया गया था। अंत में, उच्च-कोशिका-घनत्व निरंतर खेती ने 0.025 एच-1 पर 15 दिनों की निरंतर अवधि के लिए आर1-एसएसटी के निरंतर उत्पादन का प्रदर्शन किया, जिससे ~64 ग्राम/लीटर की स्थिर-अवस्था बायोमास एकाग्रता पर अधिकतम क्यूपी मूल्य का 78% प्राप्त हुआ। और ~5000 यू/एल/घंटा की वॉल्यूमेट्रिक उत्पादकता। शुद्ध आर1-एसएसटी का उपयोग करके एससीएफओएस के उत्पादन की समय प्रोफ़ाइल की भविष्यवाणी करने के लिए तीव्र संतुलन और स्थिर-अवस्था कैनेटीक्स के संयुक्त दृष्टिकोण पर आधारित एक गतिज मॉडल विकसित किया गया था। शुद्ध किए गए एंजाइम को सुक्रोज को चुनिंदा रूप से 1-केस्टोस में परिवर्तित करने के लिए दिखाया गया था, जिसकी अंतिम उपज 0.66 ग्राम/ग्राम सुक्रोज की खपत थी। मॉडल प्रारंभिक दर कैनेटीक्स, अर्ध-संतृप्ति स्थिरांक, और ट्रांसफ़रेज़ से हाइड्रॉलेज़ अनुपात पर सब्सट्रेट एकाग्रता के प्रभाव की भविष्यवाणी कर सकता है। प्रारंभिक सुक्रोज और एंजाइम एकाग्रता के ज्ञात मूल्यों के लिए, 1-केस्टोस समृद्ध एससीएफओएस का उत्पादन करने के लिए एक सतत प्रक्रिया के विकास के लिए इष्टतम प्रतिक्रिया समय और आवश्यक कमजोर पड़ने की दर की भविष्यवाणी की गई थी। पर्मेट फ़्लक्स में 40% कार्बोहाइड्रेट संरचना के साथ 1-केस्टोज़ की ~110 ग्राम/लीटर/घंटा की वॉल्यूमेट्रिक उत्पादकता प्रदर्शित की गई।

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# Abbreviations

| | |
|---|---|
| μ | specific growth rate |
| 1-FFT | fructan:fructan 1-fructosyltransferase |
| 1-SST | sucrose:sucrose 1-fructosyltransferase |
| AOX1 | alcohol oxidase 1 |
| DP | degree of polymerization |
| E | free enzyme |
| E'G | glycosylated-enzyme-glucose complex |
| EK | enzyme-kestose complex |
| E'K | glycosylated-enzyme-kestose complex |
| EMR | enzyme membrane reactor |
| EN | enzyme-1,1-kestotetraose complex |
| E'N | glycosylated-enzyme-1,1-kestotetraose complex |
| EPSs | Exopolysaccharides |
| ES | enzyme-sucrose complex |
| E'S | glycosylated-enzyme-sucrose complex |
| Et | Total enzyme used |
| F | Fructose |
| Ffase | fructofuranosidases |
| Ftase | fructosyltransferases |
| FT-IR | Fourier Transform InfraRed |
| G | Glucose |
| GH | glycoside hydrolase |
| GRAS | generally regarded as safe |
| HAD | hydrogen bond-mediated aglycone delivery |
| HMW | high molecular weight |
| HPLC | High-performance liquid chromatography |
| ISC | inulosucrase |
| K | 1-kestose |
| Kek | dissociation constant of EK |
| Ken | dissociation constant of EN |
| Kes | dissociation constant of ES |
| Kfg | dissociation constant of E'G |
| Kfk | dissociation constant of E'K |
| Kfn | dissociation constant of E'N |
| Kfs | dissociation constant of E'S |
| LSC | levansucrase |
| MOPS | 3-(N-morpholino) propane sulfonic acid |
| N | 1,1-kestotetraose |
| NMR | Nuclear Magnetic Resonance |
| NN | 1,1,1-kestopentaose |

| | |
|---|---|
| Qo | cell-specific oxygen uptake rate |
| Qp | specific r1-SST productivity |
| ROS | reactive oxygen species |
| S | Sucrose |
| scFOS | short-chain fructooligosaccharides |
| SCFA | Short chain fatty acids |
| SPOS | solid-phase oligosaccharide synthesis |