TECHNISCHE UNIVERSITÄT DRESDEN

DRESDEN concept

Production of lysine for feed supplements from shrimp waste using metabolically engineered *Vibrio natriegens*

ScampiLys

A joint project of **Hanoi University of Science and Technology** and **TU Dresden**

Presented by Prof. Thomas Walther

Relevance of the project

Concept of ScampiLys

Optimization of shrimp waste hydrolysis methods

Metabolic engineering of a lysine-producing strain

Summary and additional project outcomes

Relevance of the project

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Summary and additional project outcomes

Relevance of the project

Shrimp production plays major role in Vietnamese Bioindustry

Total amount of shrimp produced: 1,000,000 t per year

Revenues: 32 Billion USD

Growth rate of market: 6 % per year

Sustainable growth supported by government programs

https://vietnamagriculture.nongnghiep.vn/vietnams-shrimp-production-can-reach-1-million-tons-this-year-d332904.html
https://easyhealthyrecipes.com/frozen-shrimp-air-fryer/

Relevance of the project

Large amounts of shrimp waste are produced

Total amount of shrimp waste produced: 350,000 t per year

Disposal generates environmental problems (not only in Vietnam)

Vietnamese government aims at reducing environmental cost of shrimp production

Current applications

Drying and milling -> shrimp meal -> replace fish meal in animal diets
BUT: low added value

Chitosan extraction provides high added value but market is too small (20,000 t world wide)

http://www.intprocon.com/Shrimp-waste.html
https://kimmyfarm.com/en/product/shrimp-waste-heads-and-shells-for-animal-feed
https://www.newindianexpress.com/states/odisha/2018/jun/26/odisha-illegal-dumping-of-shrimp-waste-continues-unabated-in-port-town-1833828.html

Relevance of the project

Problems during (bio)chemical upgrading of shrimp waste

Shrimp waste composition:
- 50 % protein
- **25 % chitin**
- 25 % minerals + other

Chitin is a very stable biopolymer
-> Harsh hydrolysis conditions (high acid content)
-> Causes high salt content in hydrolysis product (inhibits cell growth, waste disposal)

Hydrolysis products are
- N-acetylglucosamine (GlcNac, enzymatic hydrolysis)
- Glucosamine + acetate (GlcN, acid hydrolysis)

-> Microbial upgrading of these substrates difficult (not well studied)

Relevance of the project

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Summary and additional project outcomes

Concept of ScampiLys

Development of an integrated **shrimp waste biorefinery**

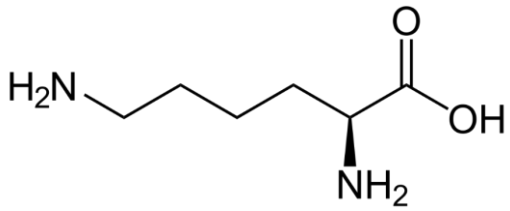
Separate chitin from protein fraction

Optimize acid and enzymatic **hydrolysis methods** to produce chitin monomers

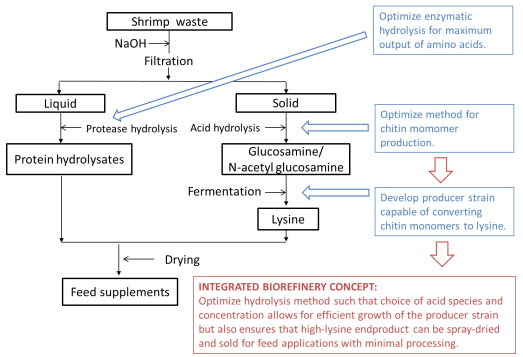
Use extremely fast-growing ***Vibrio natriegens*** for its
- High salt tolerance (more robust on salt-containing hydrolytic products)
- High growth rate on GlcNac, GlcN and acetate

Construct lysine-overproducing *V. natriegens* strain by metabolic engineering

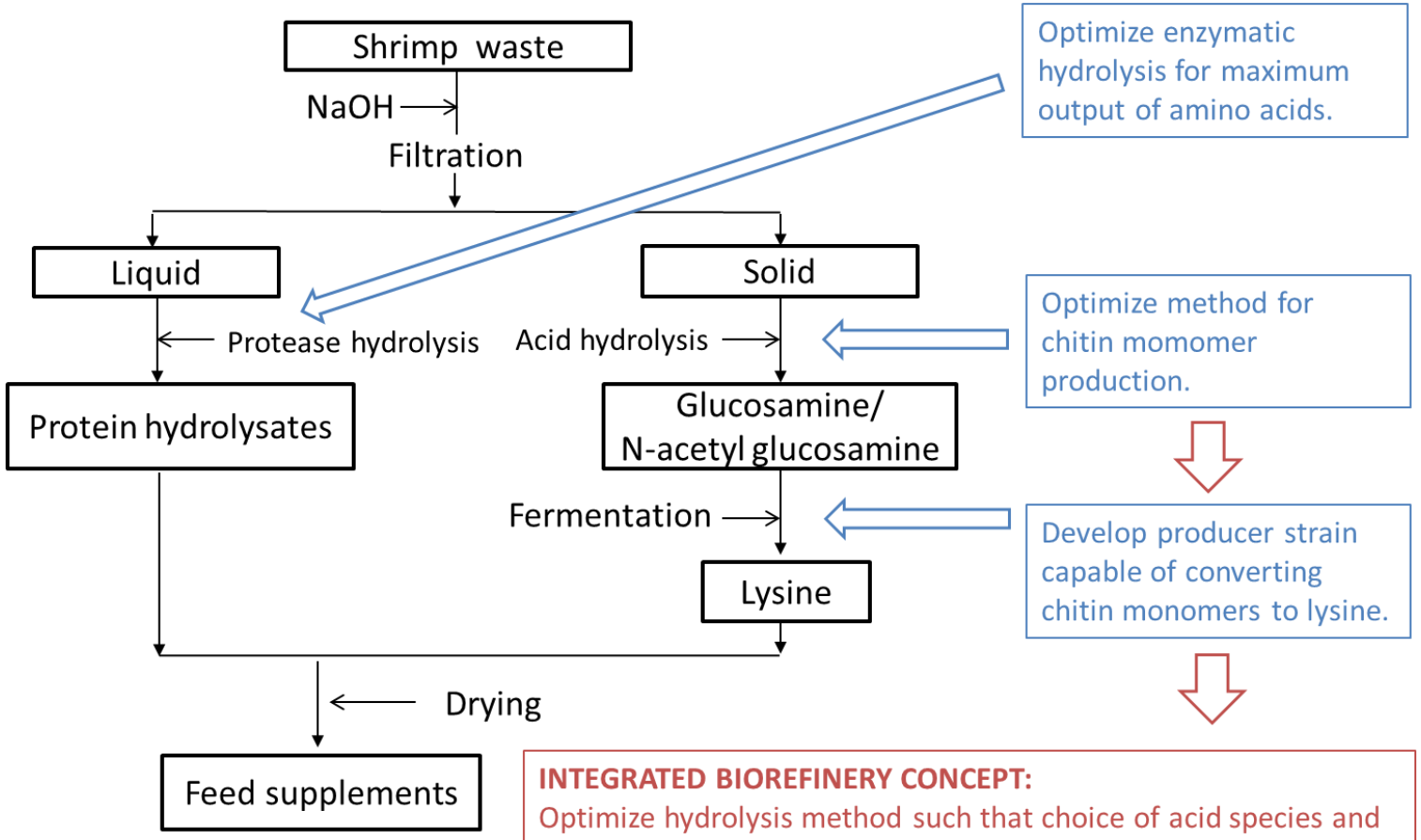
Fermentation product with high lysine content may be pooled with protein fraction to produce high-value feed product



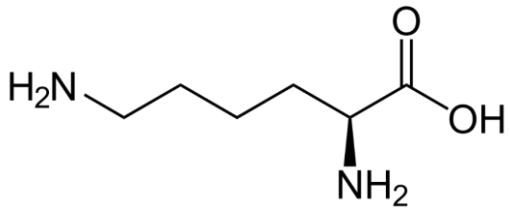
Shrimp waste biorefinery



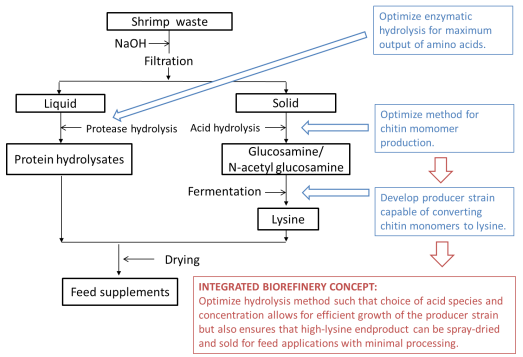
Concept of ScampiLys



INTEGRATED BIOREFINERY CONCEPT:
Optimize hydrolysis method such that choice of acid species and concentration allows for efficient growth of the producer strain but also ensures that high-lysine endproduct can be spray-dried and sold for feed applications with minimal processing.

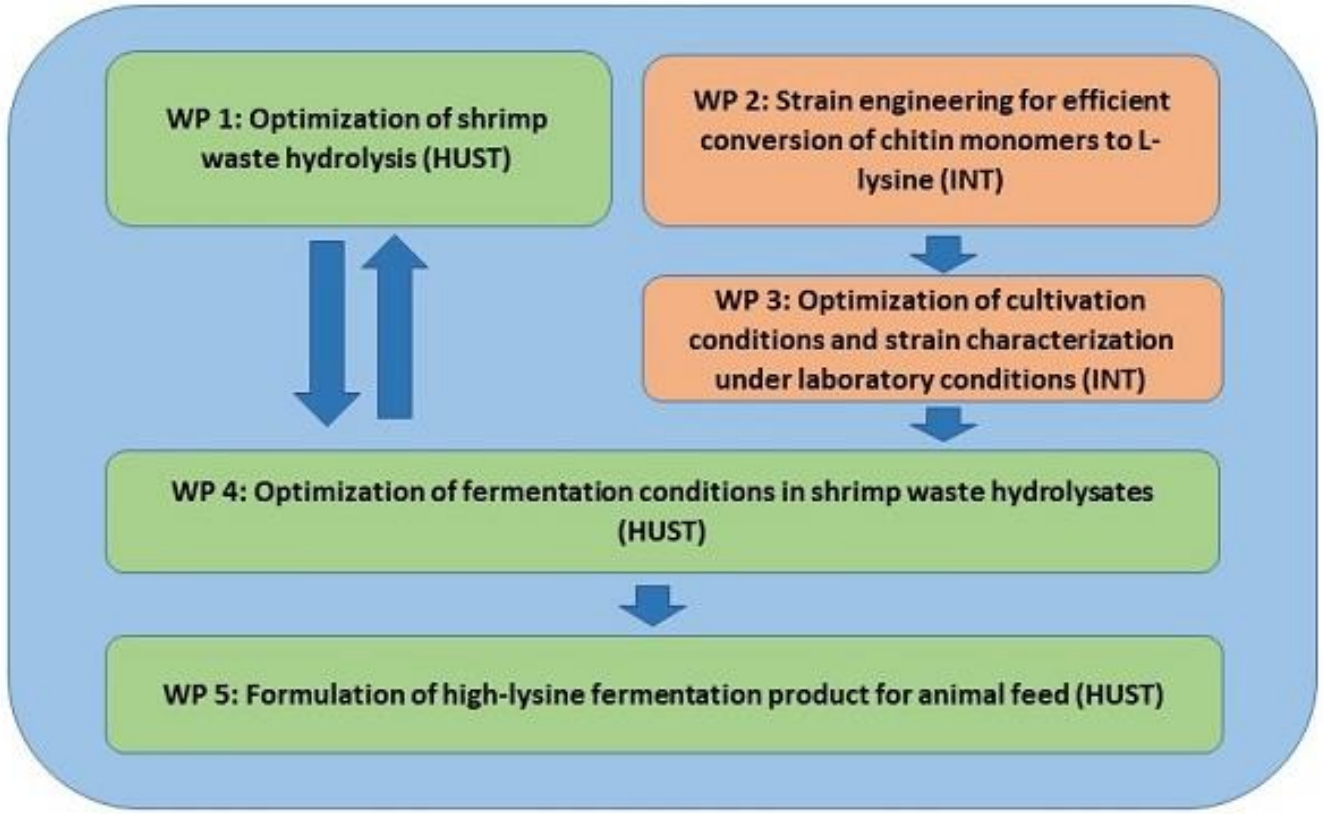


Shrimp waste biorefinery



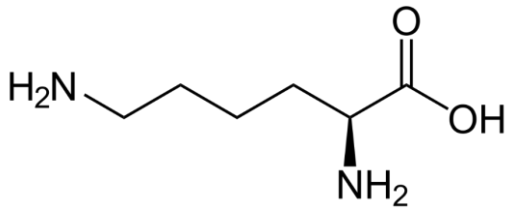
Concept of ScampiLys

Project outline

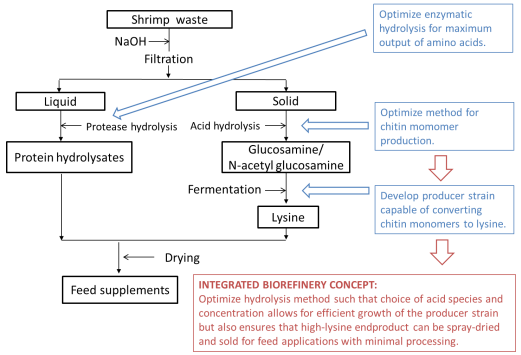


INT

HUST



Shrimp waste biorefinery



Relevance of the project

Concept of ScampiLys

Optimization of shrimp waste hydrolysis methods

Metabolic engineering of a lysine-producing strain

Summary and additional project outcomes

Optimization of shrimp waste hydrolysis methods

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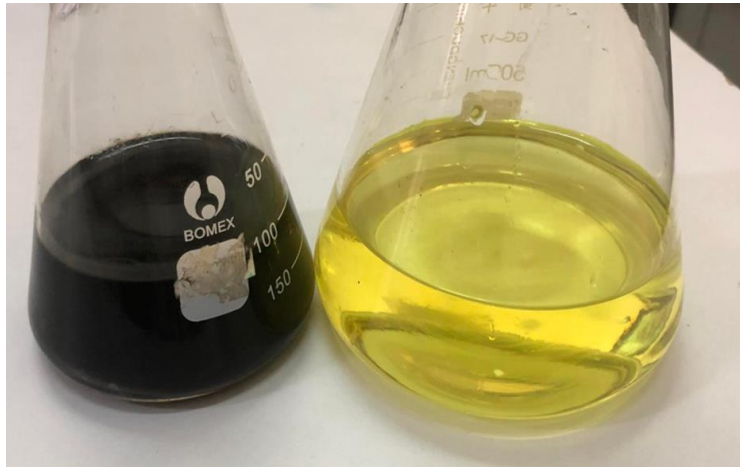
BÁCH KHOA HÀ NỘI
HANOI UNIVERSITY
OF SCIENCE AND TECHNOLOGY

Strategy

OBJECTIVE:
Obtain fermentable chitin hydrolysate

Optimize hydrolysis:
Optimize method to reduce HCl (=salt) content while still obtaining high chitin monomer yield



Identify robust strain:
Look for strains that grow at high salt content by phenotyping WT isolates

Optimization of shrimp waste hydrolysis methods

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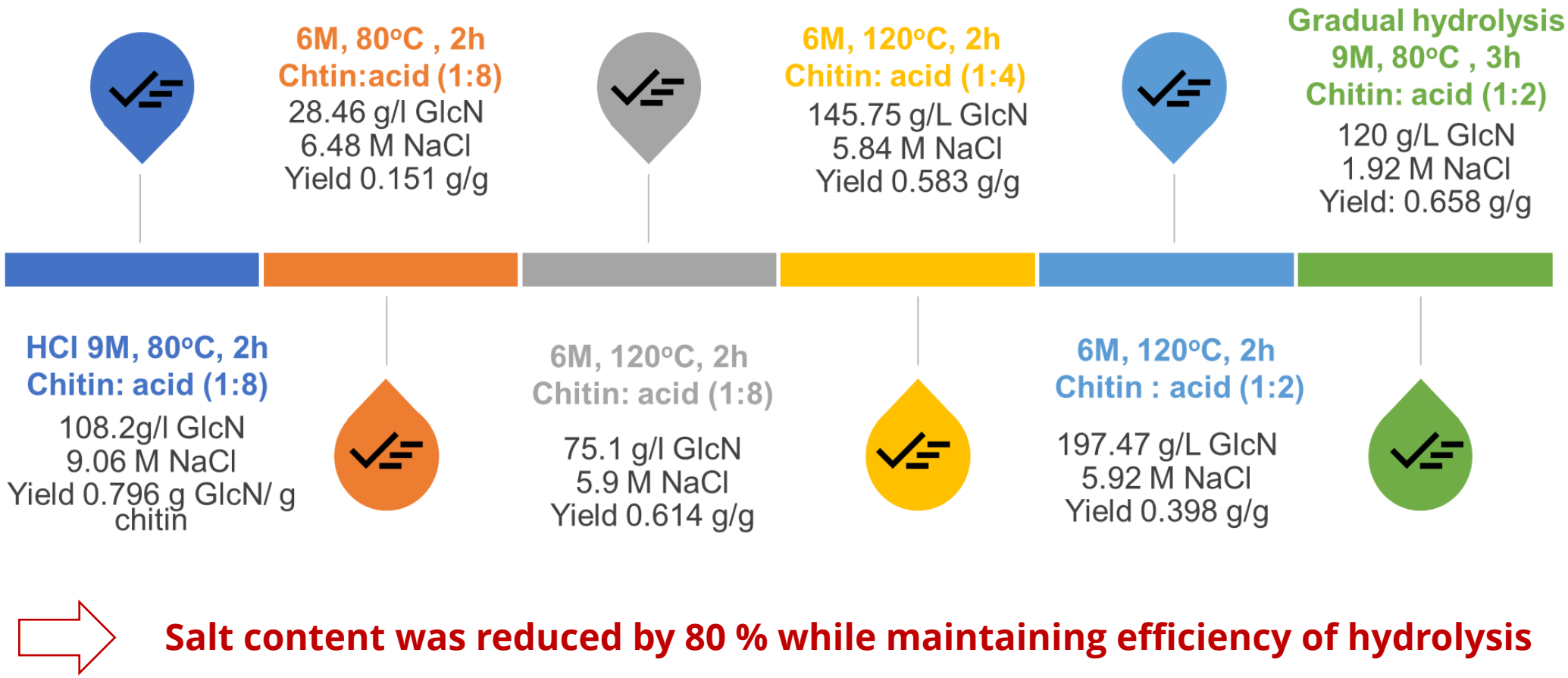
HANOI UNIVERSITY OF SCIENCE AND TECHNOLOGY

Strategy



Salt content was reduced by 80 % while maintaining efficiency of hydrolysis

Optimization of shrimp waste hydrolysis methods

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HANOI UNIVERSITY OF SCIENCE AND TECHNOLOGY

Identification of robust wild-type *V. natriegens* isolates
(based on 16S RNA sequence and physiological profile)

Number	Strain	Name	%ID
1	B01	*V. alginolyticus*	99.93%
2	4.1	*V. alginolyticus*	99.51%
3	6.3D	*Photobacterium ganghwense*	99.86%
4	B02	*V. proteolyticus*	99.51%
5	5.4	*V. alginolyticus*	99.73%
6	5.2S	*V. diabolycus*	99.86%
7	VS3	*V. alginolyticus*	99.65%
8	10.2T	*V. parahaemolyticus*	99.79%
9	7.2	*V. natriegens*	99.86%
9	10.3	*V. natriegens*	99.58%
10	N5.2	*Vibrio.sp (natriegens)*	99.58%
11	N5.3	*V. natriegens*	99.67%

⇨ ***V. natriegens* isolates have been identified**

Optimization of shrimp waste hydrolysis methods

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Phenotyping of wild-type *V. natriegens* isolates

***V. natriegens* strains** (15 g/L NaCl, 30°C)	**Growth rate μ (*1/h*)**			
	Glc	**GlcN**	**GlcNAc**	**Chitin hydrolysate**
5.3	1.212	0.813	1.271	1.165
10.3	1.202	0.535	0.935	0.822
DSM 759	1.102	0.517	0.840	0.807

Growth characteristics of WT isolates on synthetic carbon sources and chitin hydrolysate

WT *V. natriegens* isolate 5.3 grows grows faster on chitin hydrolysate than sequenced reference strain

Optimization of shrimp waste hydrolysis methods

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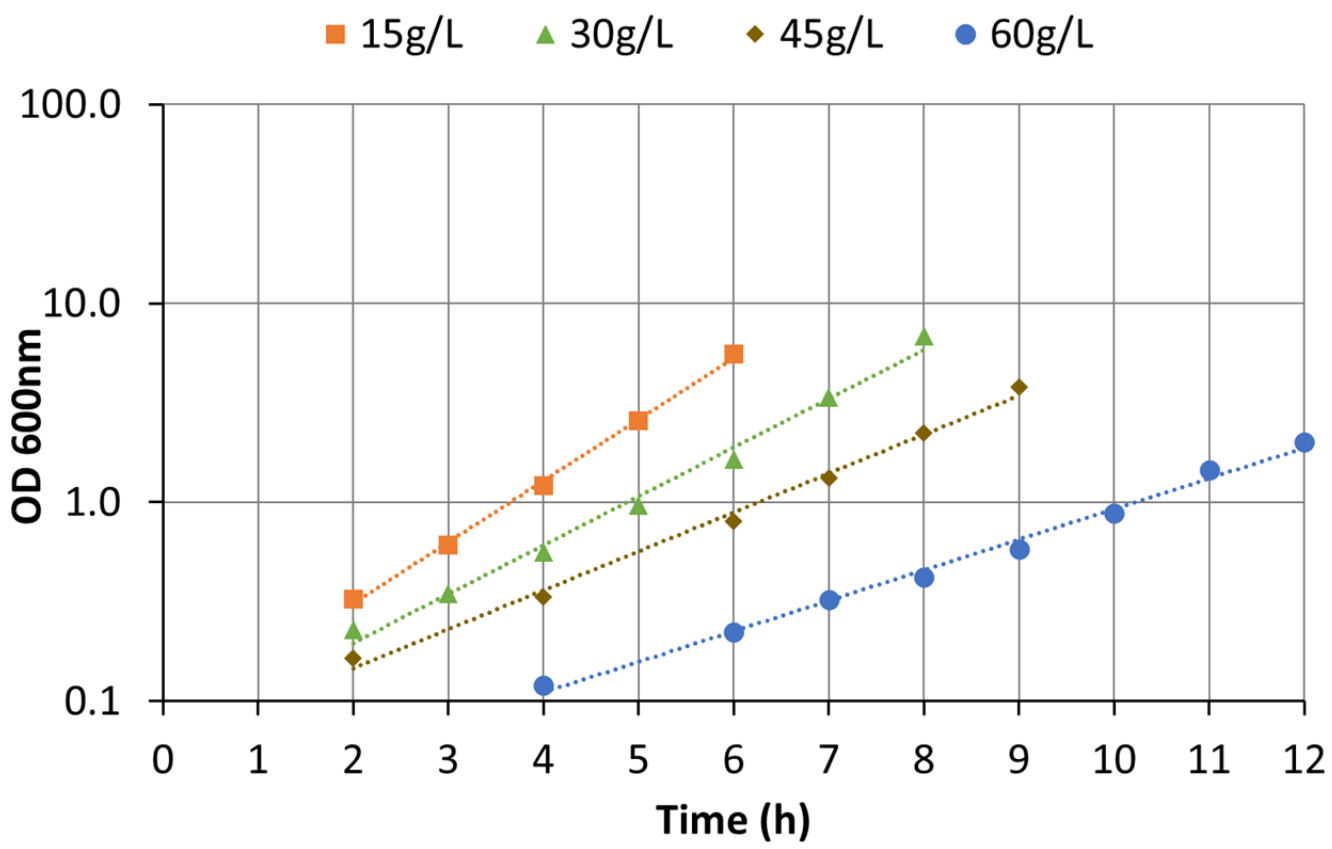

Growth characteristics of WT isolate Vn 5.3 at very high salt concentrations

WT *V. natriegens* isolate grows at extremely high salt concentration

Optimization of shrimp waste hydrolysis methods

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Phenotyping of wild-type *V. natriegens* isolates

NaCl	μ (1/h)	
g/L	*V. natriegens 10.3*	*V. natriegens 5.3*
0	No growth	-
15	0.505	0.710
30	0.366	0.565
45	0.236	0.473
60	0.182	0.354

Comparison of WT *V. natriegens* isolates regarding growth at extremely high salt concentrations

WT *V. natriegens* isolate 5.3 grows at extremely high salt concentrations

Optimization of shrimp waste hydrolysis methods

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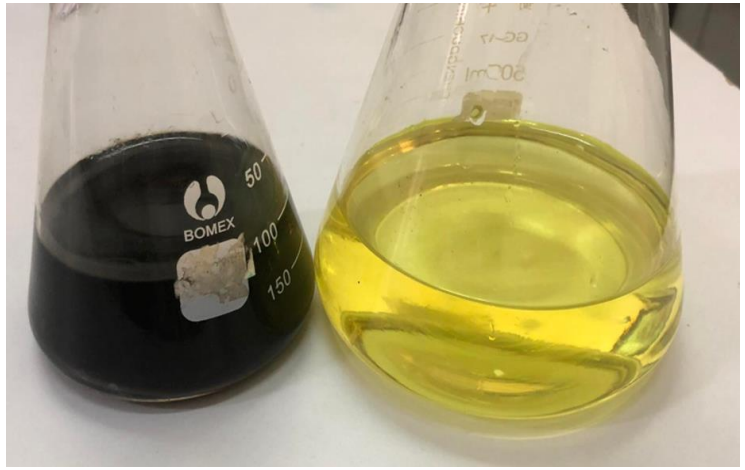
Strategy

OBJECTIVE:
Obtain fermentable chitin hydrolysate

Optimize hydrolysis:

High yield at 2 M NaCl



Identify robust strain:

Robust growth at 1 M NaCl

Forthcoming:
- Enzymatic hydrolysis (avoids salt entirely)
- Desalting by chromatography (partially already successful)

Relevance of the project

Concept of ScampiLys

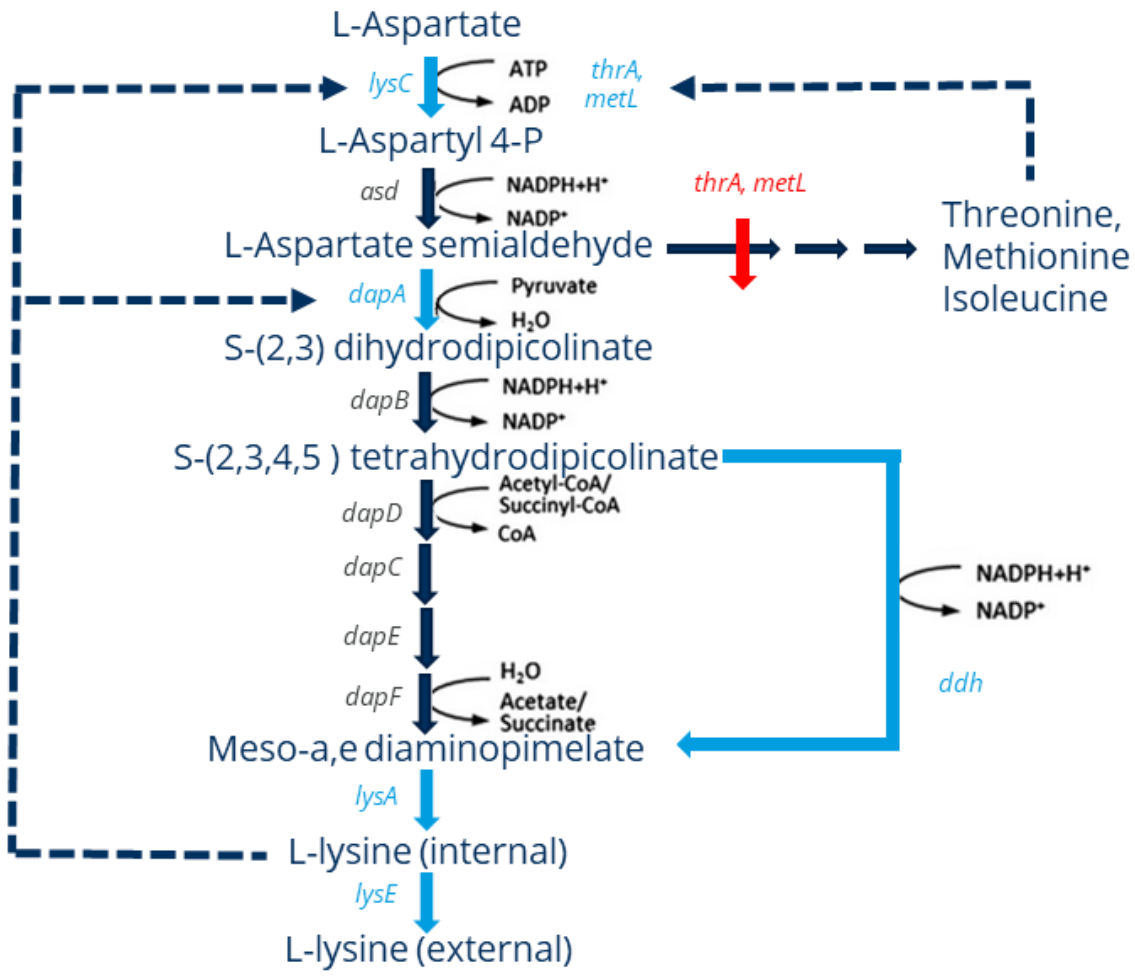
Optimization of shrimp waste hydrolysis methods

Metabolic engineering of a lysine-producing strain

Summary and additional project outcomes

Engineering of lysine-producing strain

Optimized design of lysine synthetic pathway



Lysine feedback inhibition on aspartate kinase (LysC) and tetrahydrodipicolinate synthase (DapA) must be removed

-> **Enzyme engineering**

Activity of key metabolic enzymes must be increased

-> **Strain/Metabolic engineering**

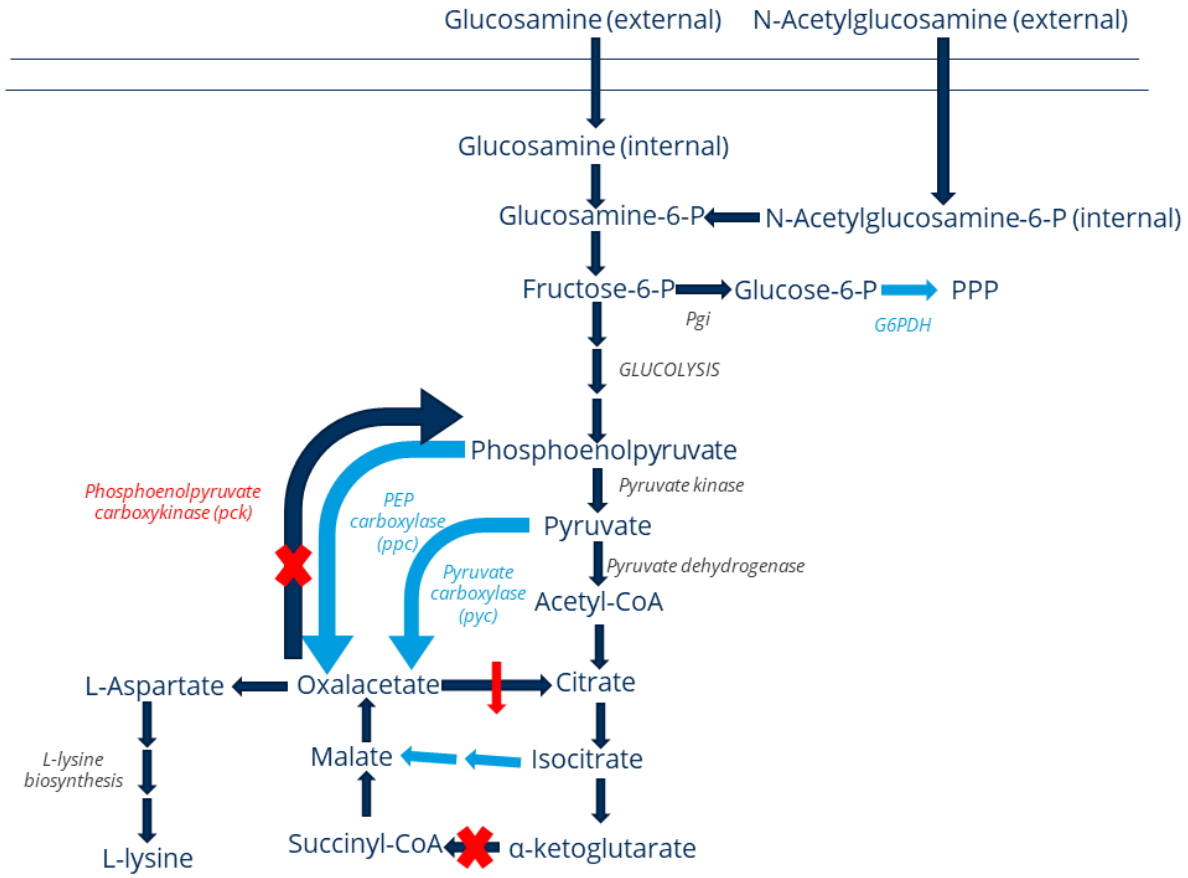
Allosteric feedback inhibition to remove

Activity to increase

Activity to decrease

Engineering of lysine-producing strain

Optimized design of lysine synthetic pathway



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Allosteric feedback inhibition to remove

Activity to increase

Activity to decrease

Engineering of lysine-producing strain

Workflow

Expected results

Identification and engineering of lysine pathway enzymes in *V. natriegens*
(Protein alignments, site directed mutagenesis, enzyme production and characterization)

→ Suitable pathway enzymes

↓

Strain engineering via plasmid-born expression of pathway genes
(Identification of plasmids, optimal enzyme activity via modulating strength of promoters and RBS sequences, deletion of competing reactions, cultivation experiments)

→ Lysine-producing strain with optimized carbon flux (yield) **suitable for lab-scale** investigations

↓

Strain engineering via chromosome-born expression of pathway enzymes
(Chromosomal integration of expression cassettes, adaptation of copy number)

→ Lysine-producing strain with high yield **suitable for large scale** applications

Engineering of lysine-producing strain

Enzyme identification and engineering

Protein alignments (blastp) using *E. coli* aspartate kinases (Ec.LysC, Ec.MetL, Ec.ThrA) as template

-> Identification of 2 bi-functional aspartate kinases (with AK + HOM-DH activities)

Vn.MetL
Vn.ThrA

- Verify activity
- Possibly: reduce expression in producer strain

-> Identification of 3 potential mono-functional aspartate kinases

Vn.LysC1
Vn.LysC2
Vn.LysC3

- Verify and characterize activity
- **E. coli contains only one mono-functional AK!!!**

Engineering of lysine-producing strain

Enzyme identification and engineering

3 out of 5 *V. natriegens* candidate enzymes could be expressed and purified

Enzyme (AK)	Vmax (U/mg)	K_{05} (mM)	Hill coefficient
Ec.LysC	11,97 +- 1.65	17.11 +- 4.09	0.82 +- 0.13
Vn.LysC1	14,76 +- 2.74	11.06 +- 4.24	1.19 +- 0.44
Vn.LysC2	6.96 ± 0.3	9.01 ± 0.95	0.95 ± 0.04

Ec.lysC as control of the assay

Enzyme (AK-HD)	Vmax (U/mg)	K_{05} (mM)	Hill coefficient
Vn.ThrA	1.60 +- 0.23	20 +- 0	0.72 +- 0.02

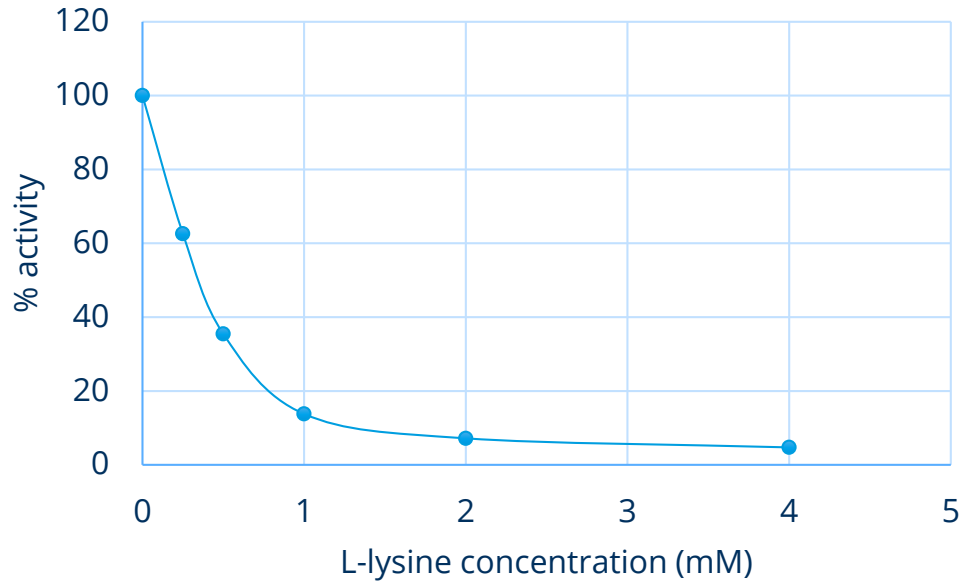
Vn.AK enzymes successfully identified

Vn contains at least one additional monofunctional AK – What for???

Engineering of lysine-producing strain

Enzyme identification and engineering

Lysine-sensitivity of Vn.LysC1



Vn.LysC1 is inhibited at physiological lysine concentrations

Vn.LysC1 must be engineered

Engineering of lysine-producing strain

Enzyme identification and engineering

```
Vn_lysC3        --MTFTVEKIGGTSMTAFDAV---LDNIILRPKTPYNRVFVVSAYGGMTDALLECKKTSK
Ec_lysC         -MSEIVVSKFGGTSVADFDAMNRSADIVL---SDANVRLVVLSASAGITNLLVALAE---
Vn_lysC1        -VSAFNVAKFGGTSVANFEAMSRCAAIIE---NNPNTRLVVSSACSGVTNLLVELAN---
Vn_lysC2        VKKPLIVQKFGGTSVGSIERIHQVAEHIIKAKNDGNQVVVVVSAMSGETNRLMDLAK---
Cg_lysC         --MALVVQKYGGSSLESAERIRNVAERIVATKKAGNDVVVVCSAMGDTTDELLELAA---
Bs_lysC         --MGLIVQKFGGTSVGSVEKIQNAANRAIAEKQKGHQVVVVVSAMGKSTDELVSLAK---
                   : * * **:*:   : :          .    :.* ** .  *: *:

Vn_lysC3        AGVYQLVAKRDDSWEEALAYVENRMLLTNENIFADPMNRMRADKFIRSRISEAKNCIANI
Ec_lysC         -GLEPGE-RFE-------------KLDAIRNIQFAILERLRYPNVIREEIERLLENITVL
Vn_lysC1        -GVQDQEQRAE-------------LLRKLAEIHDDILSQLRDAAEASAEVYAILDTVTSL
Vn_lysC2        -QVDSVPTARE-------------L----------------------------------
Cg_lysC         -AVNPVPPARE-------------M----------------------------------
Bs_lysC         -AISDQPSKRE-------------M----------------------------------
                  :      :

Vn_lysC3        LETCQYGQFSLRHYLPQIREFLSSIGEAHSAYNTALKLKNMGINAKFVDLSGWD----TT
Ec_lysC         AEAAALATS------PALTDELVSHGELMSTLLFVEILRERDVQA--QWFDVRKV-MRTN
Vn_lysC1        AEAASIQAS------SKLTDHLVACGELMSTHILAQLMRERGINA--VRFDIRDV-LRTD
Vn_lysC2        ------------------DVLLSAGEQVSMALLAMTLNKMGHPA--RSLTGAQANIVTD
Cg_lysC         ------------------DMLLTAGERISNALVAMAIESLGAEA--QSFTGSQAGVLTT
Bs_lysC         ------------------DMLLATGEQVTISLLSMALQEKGYDA--VSYTGWQAGIRTE
                                  : * : **  :      : . .  *        .    *

Vn_lysC3        EPKSLDESISEAFADIDVSKELP--------IVTGY-AYCKEG-LMHTYDRGYSEMTFSR
Ec_lysC         DRFGRAEPDIAALAELAALQLLPRLNEG-LVITQGFIGSENKG-RTTTLGRGGSDYTAAL
Vn_lysC1        DNFGRAEPNVEAISQLAQEKLVPLCQES-VVITQGFIGSDEEG-NTTTLGRGGSDYSAAL
Vn_lysC2        NQHNDA-----TIKHIDTTRVMALLEQEHVVIVAGFQGVNENG-DITTLGRGGSDTSAVT
Cg_lysC         ERHGNA-----RIVDVTPGRVREALDEGKICIVAGFQGVNKETRDVTTLGRGGSDTTAVA
Bs_lysC         AIHGNA-----RITDIDTSVLADQLEKGKIVIVAGFQGMTEDC-EITTLGRGGSDTTAVA
                   .        : :               *. *: .  :.     * .** *. :
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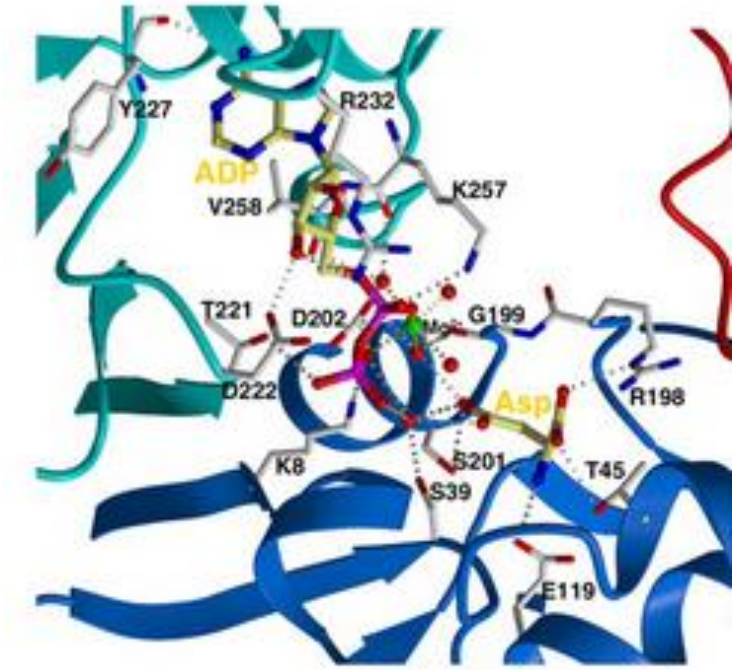
Vn – *Vibrio natriegens*
Ec – *Escherichia coli*
Cg – *Corynebacterium glutamicum*
Bs – *Bacillus subtilis*

Active site residues

Experimentally verified active site residues in Ec.LysC:

T45 E119 RGGS 198-201



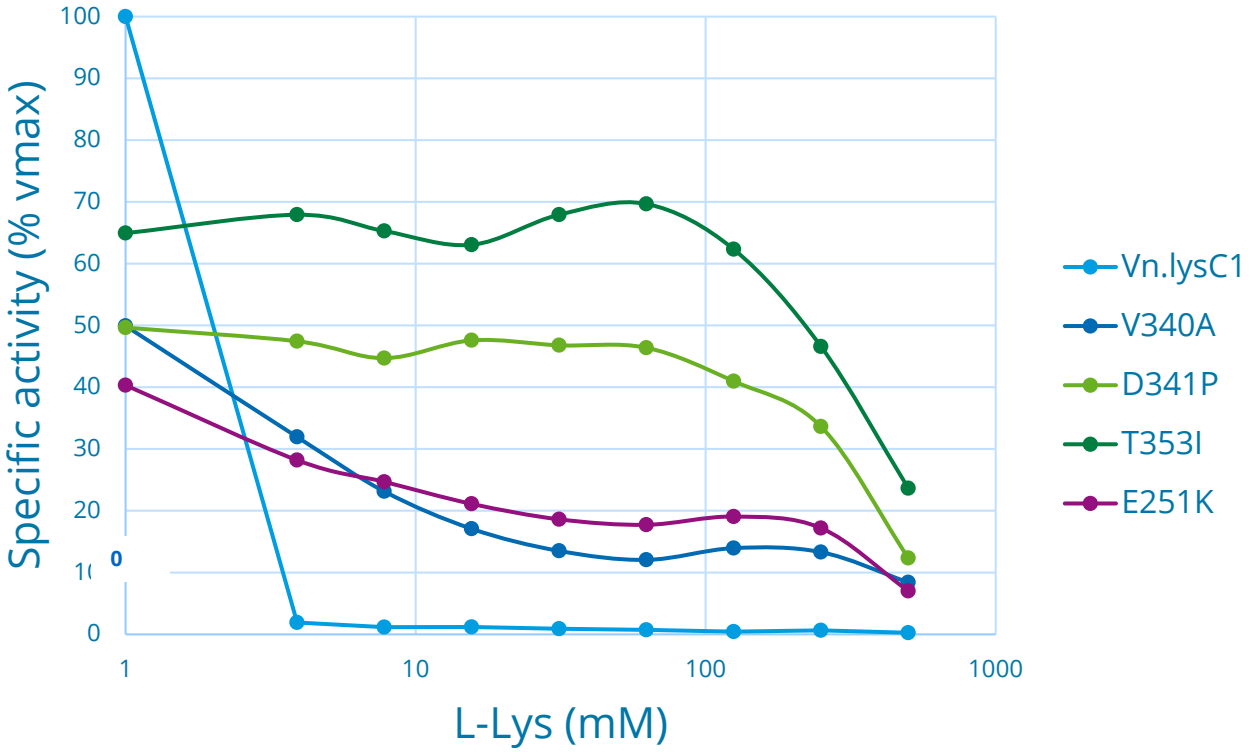
Candidate positions to alleviate lysine feedback inhibition

Ec.LysC (Literature)	Vn.LysC1 (Homologous residue)
E250K (Kikuchi et al. 1999)	E251K
V339A (Chen et al. 2011)	V340A
D340P	D341P
T352I (Kikuchi et al. 1999)	T353I

Engineering of lysine-producing strain

Enzyme identification and engineering

Engineering of Vn.LysC1



All mutants escape lysine feedback inhibition

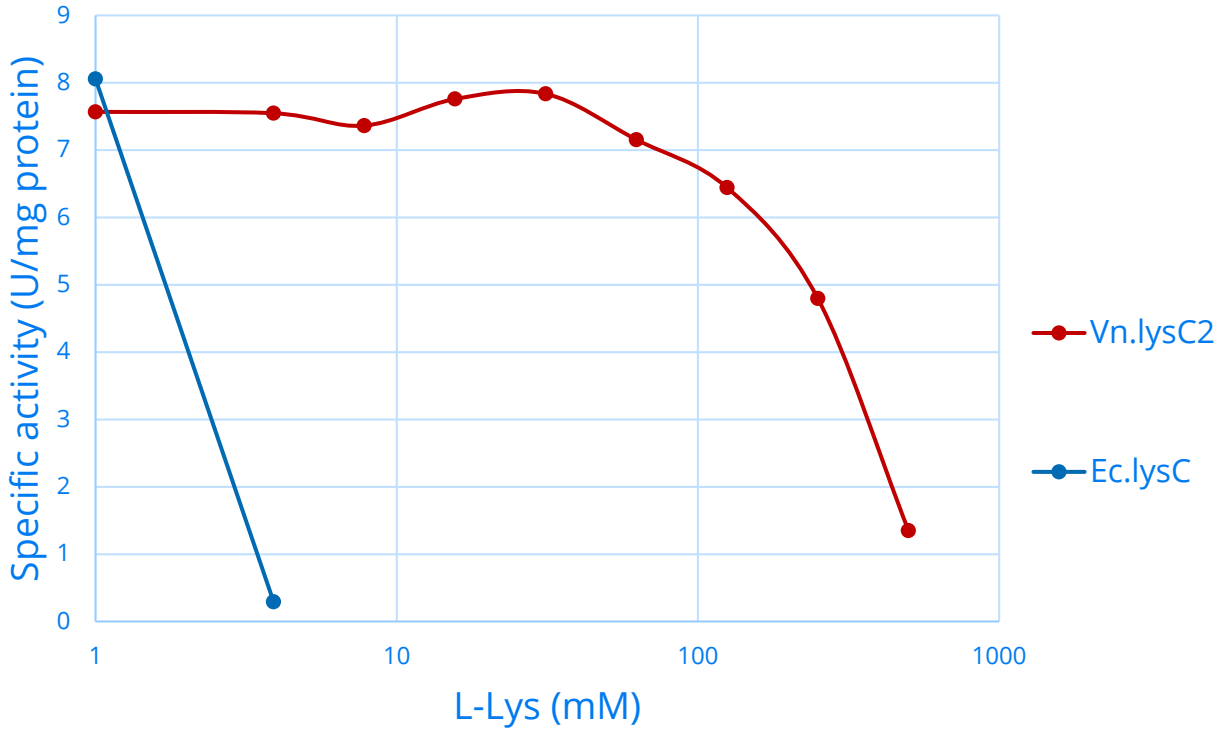
Vn.lysC1 T353I is lysine resitant and retains highest activity

Use Vn.LysC1 T353I variant for metabolic engineering

Engineering of lysine-producing strain

Enzyme identification and engineering

Properties of Vn.LysC2



Vn.LysC2 is not subject to lysine feedback inhibition

Test Vn.LysC2 for metabolic engineering

Engineering of lysine-producing strain

Strain engineering

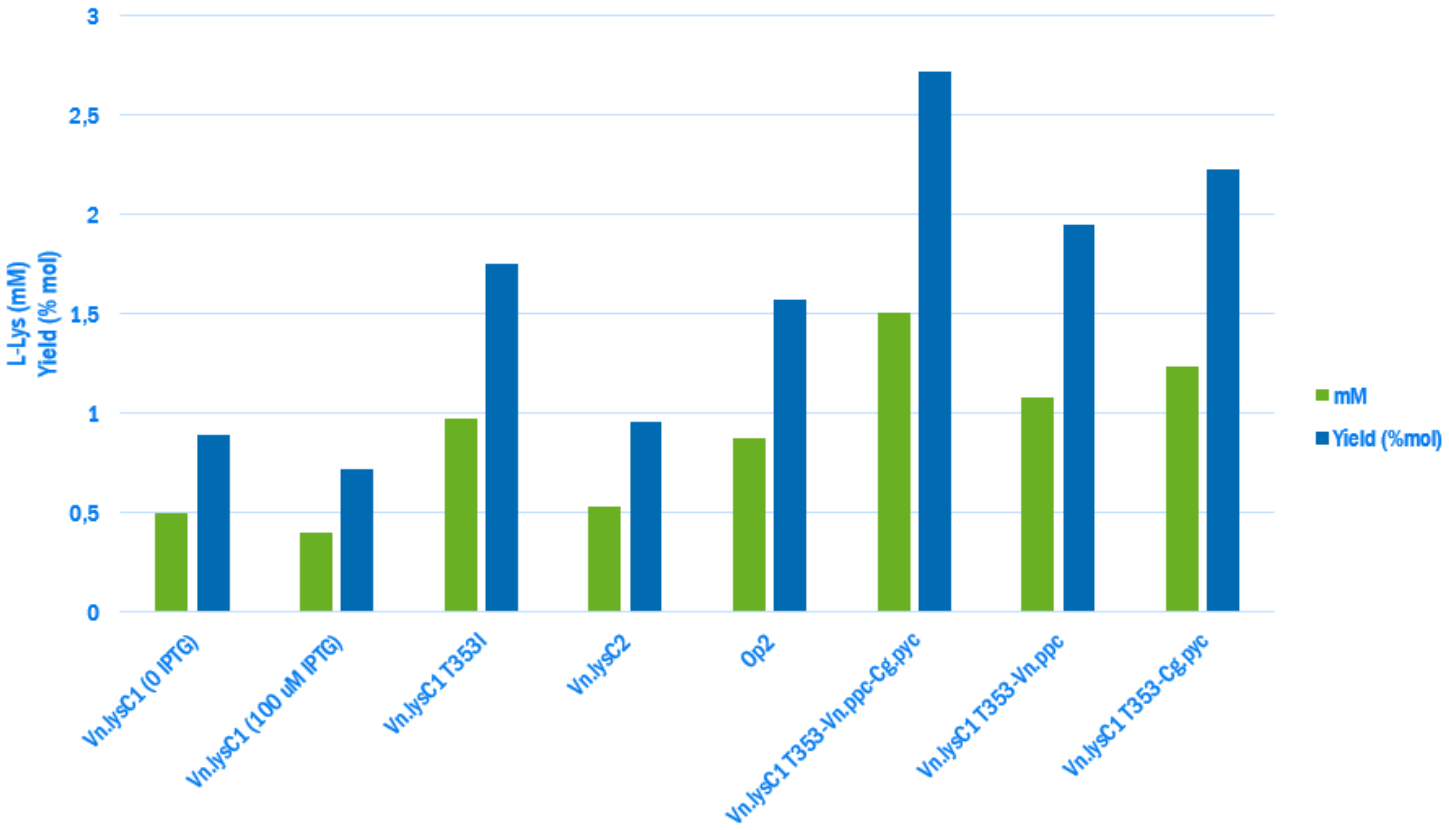
Constructed strains express AK variants and anaplerotic enzymes

Vector	Genes on plasmids
pTrc99A	**wt enzyme as reference** Vn.lysC1 -> **no expression of enzyme**
pSB-MC	**wt enzymes as reference** Vn.lysC1 -> **successful expression verified by enzymatic assay** Vn.lysC2 **Mutant AK enzymes with candidate anaplerotic enzymes** Vn.lysC1 T353I Vn.lysC1 T353I-Vn.ppc-Cg.pyc Vn.lysC1 T353I-Vn.ppc Vn.lysC1 T353I-Cg.pyc **Mutant AK enzymes with anaplerotic and lysine pathway enzymes** Vn.lysC1 T353I-Vn.dapA-Vn-lysA-Vn.lysE-Cg.ddh (Operon 1, not yet obtained) Vn.lysC2-Vn.dapA-Vn-lysA-Vn.lysE-Cg.ddh (Operon 2)

Engineering of lysine-producing strain

Strain engineering

Effect of overexpressing lysine pathway genes (shake flask experiments)



Expression of engineered pathway enzyme increases lysine production

Lysine accumulation is still comparatively small

Optimize medium to impose growth arrest

Co-express other feedback-resistant lysine pathway enzymes (DapA)

Relevance of the project

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Summary

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BÁCH KHOA HÀ NỘI
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Optimization of hydrolysis conditions has reduced salt content by 80 % while largely maintaining efficiency

Chromatography further reduces salt content

Wild-type *V. natriegens* isolate shows increased salt tolerance and better assimilation rates for chitin monomers

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DRESDEN

Aspartate kinases have been identified in *V. natriegens* and were rendered lysine-resistant by enzyme engineering

Suitable plasmid for metabolic engineering of *V. natriegens* has been identified

Overexpression of lysine-resistant aspartate kinase and anaplerotic enzymes results in increased lysine production

Lysine yields are still very small -> further engineering steps are straightforward

Additional project outcomes

Internships of Vietnamese students at INT
(2 Bachelor and 1 Master student, each for 3 months)

Online course in Systems Biotechnology (fall 2021) for students from HUST

Under discussion: Integration of specific biotech courses into new ELITECH program at HUST

Plenty of ideas how to continue the collaboration

Thanks for your attention

Cảm ơn đã quan tâm

Danke für Ihre Aufmerksamkeit

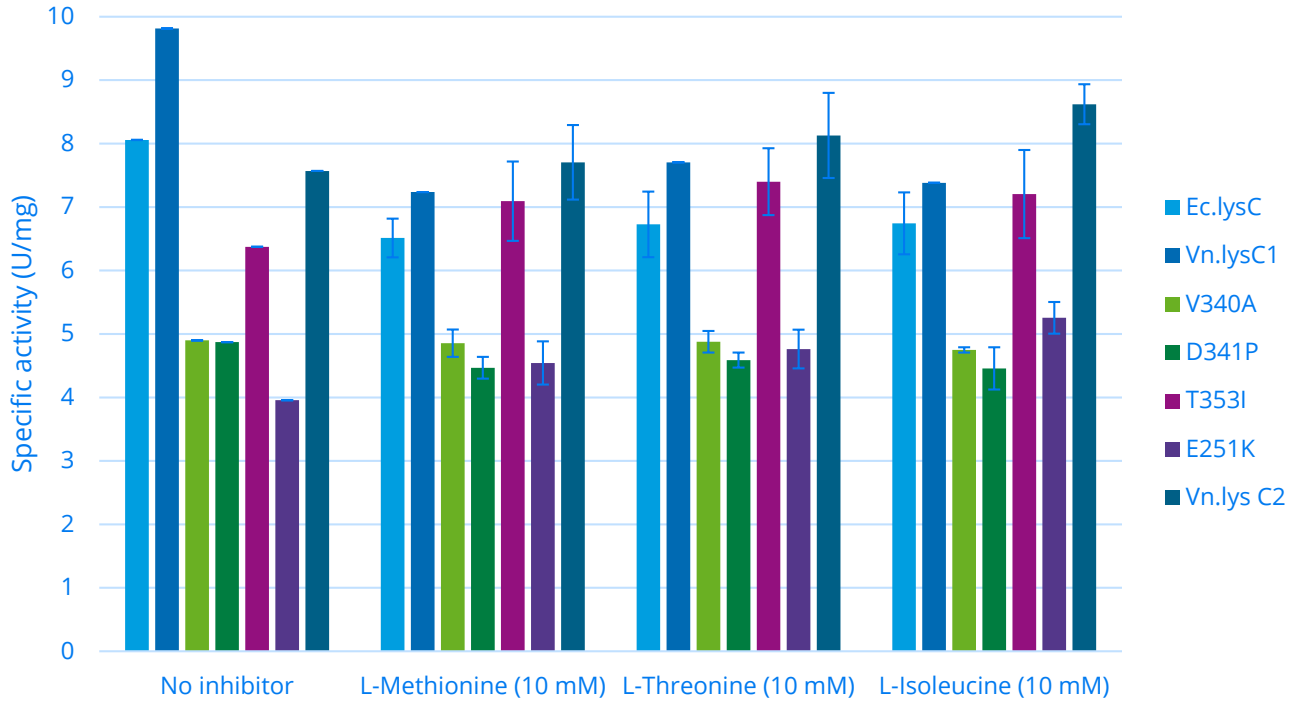
»Wissen schafft Brücken.«

BackUp slights

Engineering of lysine-producing strain

Enzyme identification and engineering

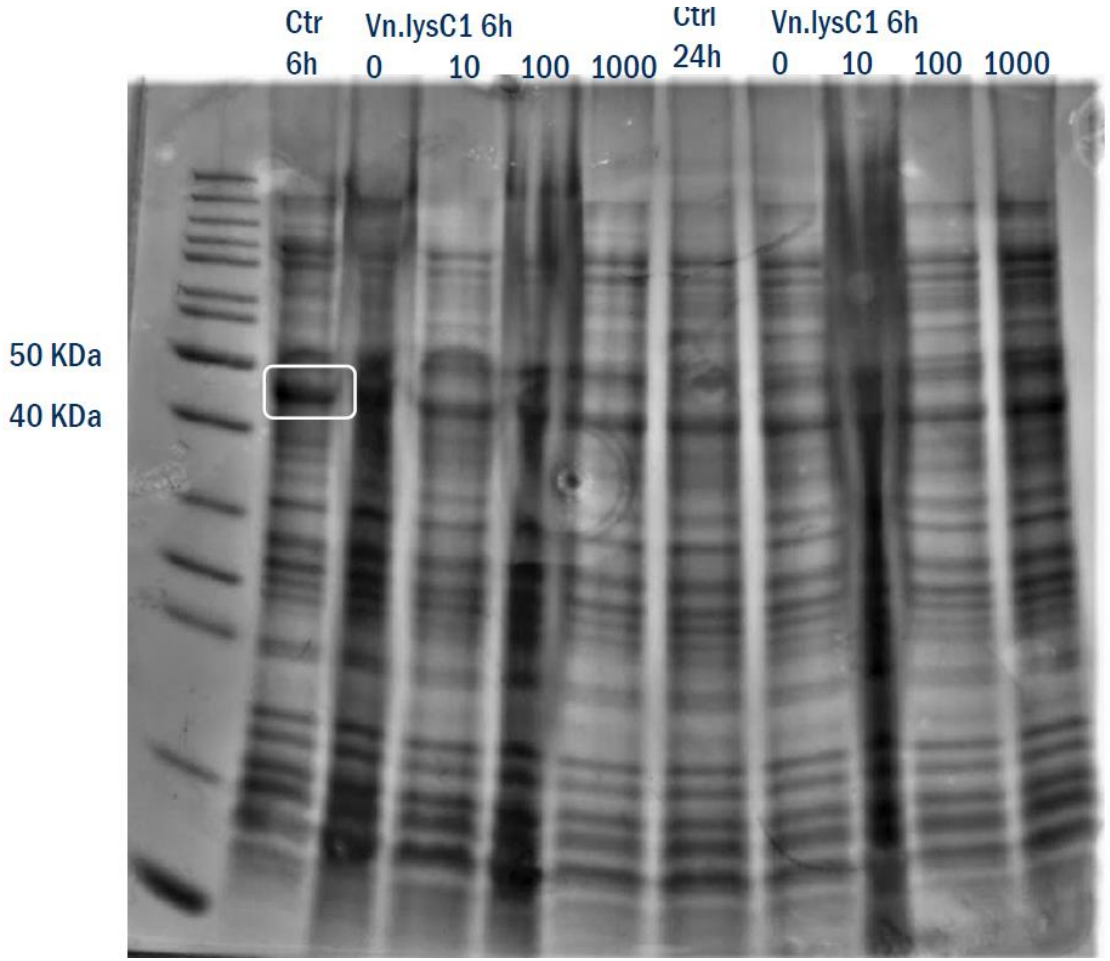
Sensitivity of Vn.LysC wild-type and mutant enzymes to other amino acids



Vn.LysC1 mutant enzymes and Vn.LysC2 are not sensitve to other relevant amino acids

Engineering of lysine-producing strain

Strain engineering – Verification of protein expression from different plasmids



C05
No notable SDS-PAGE gel band at 45 Kda

Enzymatic assay of AK activity (NADH consumption based) shows no activity on 24h crude extract

-Not even native NADH consumtion?

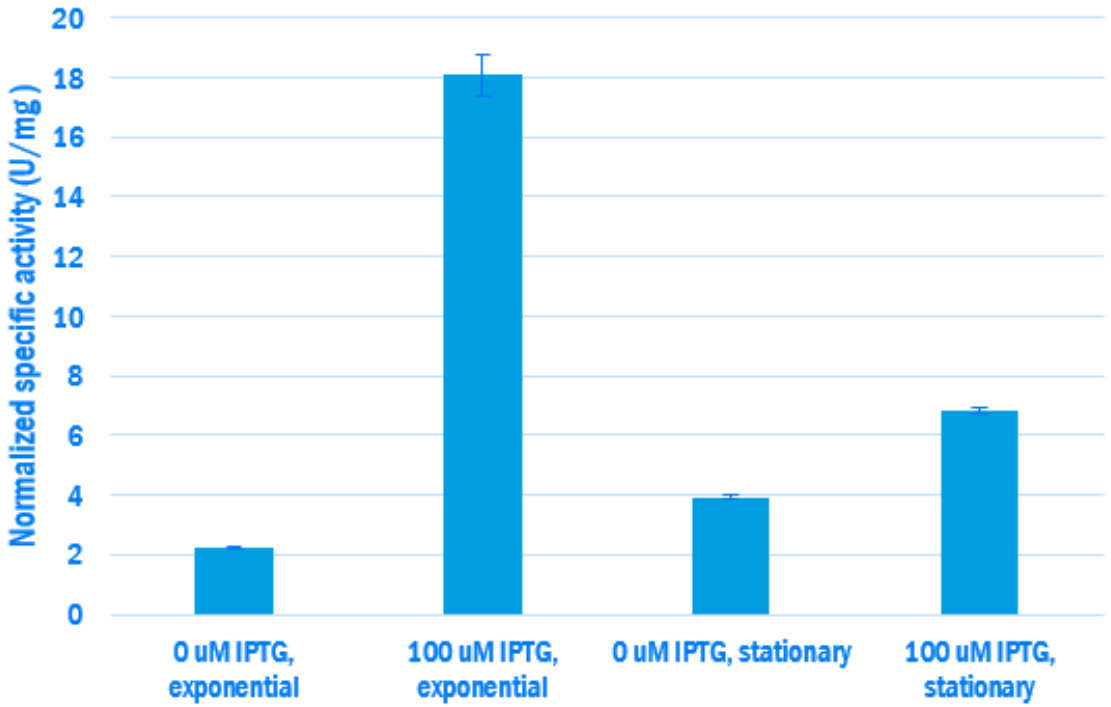
Induction conditions in *V. natriegens* could not be confirmed

Vn.LysC1 cannot be expressed from pTRC99 plasmid

Engineering of lysine-producing strain

Strain engineering – Verification of protein expression from different plasmids

Expression of Vn.LysC1 from pSB-MC plasmid



AK enzymatic assay on crude protein extract:

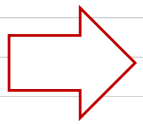
- AK activity is confirmed to be increased
- Expression of Vn.lysC1 is efficient at exponential phase and still present after 24h culture
- WT *V. natriegens* crude extract should be also assayed to separate background from leaky expression (0 IPTG)

Vn.LysC1 can be expressed from pSB-MC plasmid

Engineering of lysine-producing strain

Strain engineering – Plasmids tested for metabolic engineering

Vector	Insert	Transformed	Comment
pACYC184	-	Yes	Stable and easy to transform. Used as control for transformation efficiency
pZS	-	No	Transformation did not yield any cells after several attempts
pZA	-	No	Transformation did not yield any cells after several attempts
pZE	-	No	Transformation did not yield any cells after several attempts
pACT3	-	Yes	Low efficiency of transformation. Transformed cells showed a growth defect phenotype
	Ec.lysC E250K		Not transformed into *V. natriegens*
pTrc99a	-	Yes	Low efficiency of transformation. Transformed cells showed a small growth defect phenotype
	Vn.lysC1	Yes	No expression of Vn.lysC1 detectable by SDS gel and enzymatic assay
	Vn.lysC1 V340A	Yes	No change in metabolism in *V. natriegens* after induction in minimal media
	Vn.lysC1 D341P	Yes	No change in metabolism in *V. natriegens* after induction in minimal media
	Vn.lysC1 T353I	Yes	No change in metabolism in *V. natriegens* after induction in minimal media
	Vn.lysC1 E251K	No	Could not be transformed in *V. natriegens* after several attempts
pSB-MC	-	Yes	Severe growth defect on minimal media
pSB-HC	-	Yes	Severe growth defect on minimal media after induction unless using reduced concentration of IPTG
pSB-MC	-	Yes	Transformation was efficient, and not growth defect was observed
	Vn.lysC1	Yes	High expression of Vn.lysC1 was confirmed by enzymatic assay on crude extract.
	Vn.lysC1 T353I	Yes	Testing culture conditions for optimal Lys production
	Vn.lysC2	Yes	Testing culture conditions for optimal Lys production
	Vn.lysC1 T353I-Vn.dapA-	No	plasmid not yet obtained
	Vn.lysC2-Vn.dapA-Vn.lys	Yes	Testing culture conditions for optimal Lys production
	Vn.lysC1 T353I-Vn.ppc	Yes	Testing culture conditions for optimal Lys production
	Vn.lysC1 T353I-Cg.pyc	Yes	Testing culture conditions for optimal Lys production
	Vn.lysC1 T353I-Vn.ppc-C	Yes	Testing culture conditions for optimal Lys production



pSB-MC plasmid was suitable for metabolic engineering