

Novel thermostable enzymes for industrial biotechnology

Project acronym: THERMOGENE

Project no: EIB.12.012

Name Prof Jennifer Littlechild

ERA-IB-2 final conference, Berlin, 16./17.02.2016

Project partners



Co-ordinator – Prof Jennifer Littlechild (Exeter, UK)
Prof Peter Schoenheit (Kiel, Germany)
Prof Nils-Kåre Birkeland (Bergen, Norway)

Molecular Technologies Ltd. SME (Moscow, Russia)
Industrial Advisor Dr Roland Wohlgemuth, Sigma–Aldrich/Merck.

Budget Exeter- 326,260 (80% funding), 362,000 (Kiel), 296,602 (Bergen), 306,378 (50% funding) TOTAL 1,291,240 euros

Industrial Relevance of Project

- The project aims to "Improve enzyme systems for new and more efficient bioprocesses".

- Use of enzymes for chemical processes is a route to lower energy consumption and reduced waste generation. In addition the selectivity of enzymatic processes reduces raw material costs and the safety issues surrounding the production of wasteful bi-products. Optimised enzyme production will lead to economically viable and cost effective, sustainable production.

- New thermostable transferase enzymes with enhanced performance and/or novel functionalities can provide savings in time, money and energy for industrial processes in the areas of high value chemical production and other "white" biotechnology applications.

Introduction

Objective -Identify and characterizise different classes of thermostable transferase enzymes – transaminases, transketolases, prenyl transferases and hydroxymethyl transferases with applications in biotechnology.

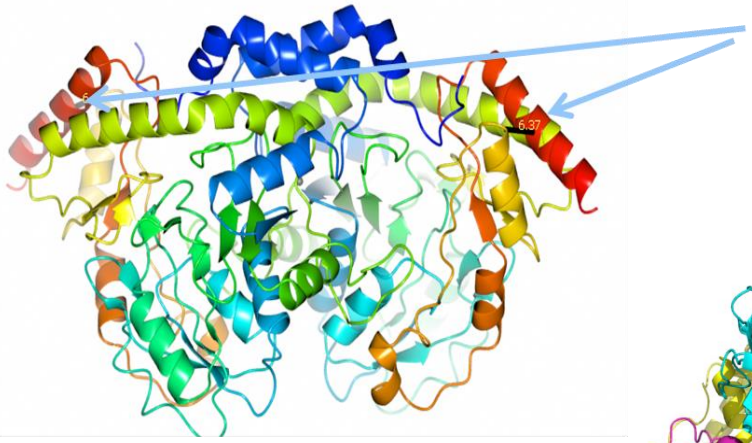
General Project Approach-The project uses natural thermophilic resources to isolate and enrich microorganisms with the desired enzymatic activities. The selected transferases have been identified from newly sequencing genomes and metagenomes by a bioinformatic approach and by screening for activity with selected substrates. Novel transferases of interest have been biochemically and structurally characterized with their potential industrial applications in mind.

Technical overview



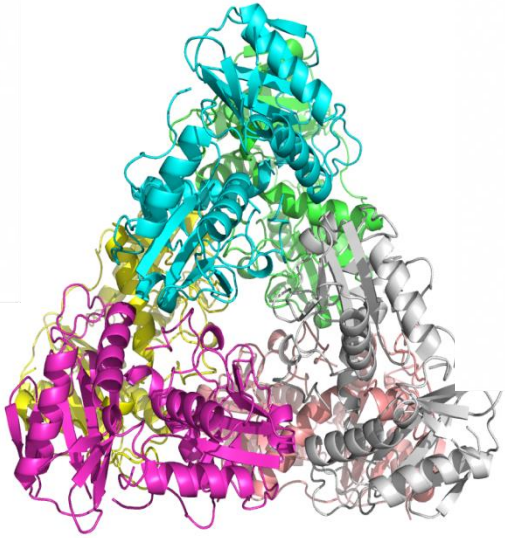
- Environmental sampling, enrichment of thermophilic microorganisms on selected enzyme substrates, analysis of microbial community structures and isolation of metagenomic DNA
- Identification of candidate enzymes through genome and metagenome sequencing and bioinformatic approaches
- Screening and initial characterization of novel thermostable transferase enzymes
- Structural characterization of selected novel transferase enzymes

Enzyme Structures of Novel Transferases

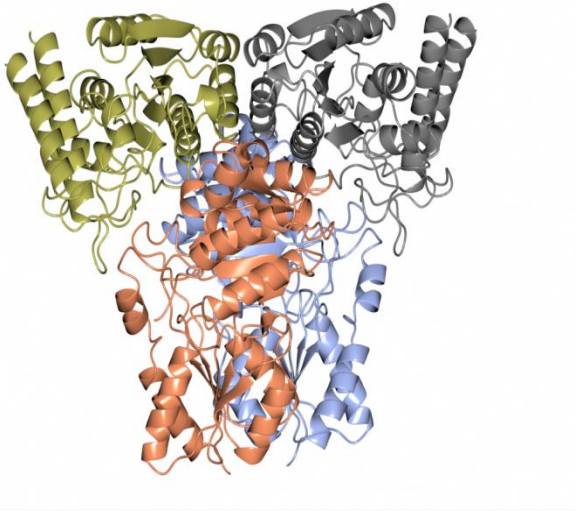


Disulfide bonds

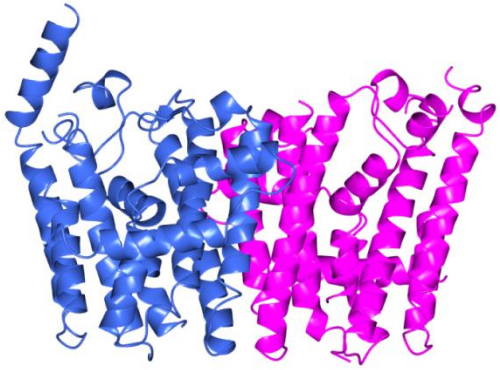
Sugar Transaminase



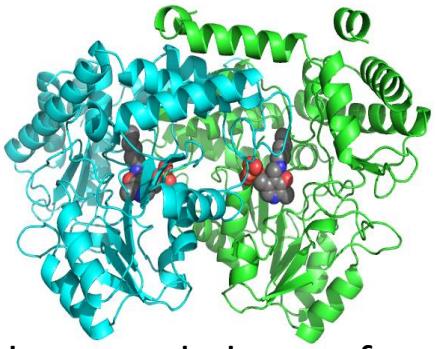
Branched chain Transaminase



'Split' transketolase



Prenyl transferase



Hydroxymethyl transferase

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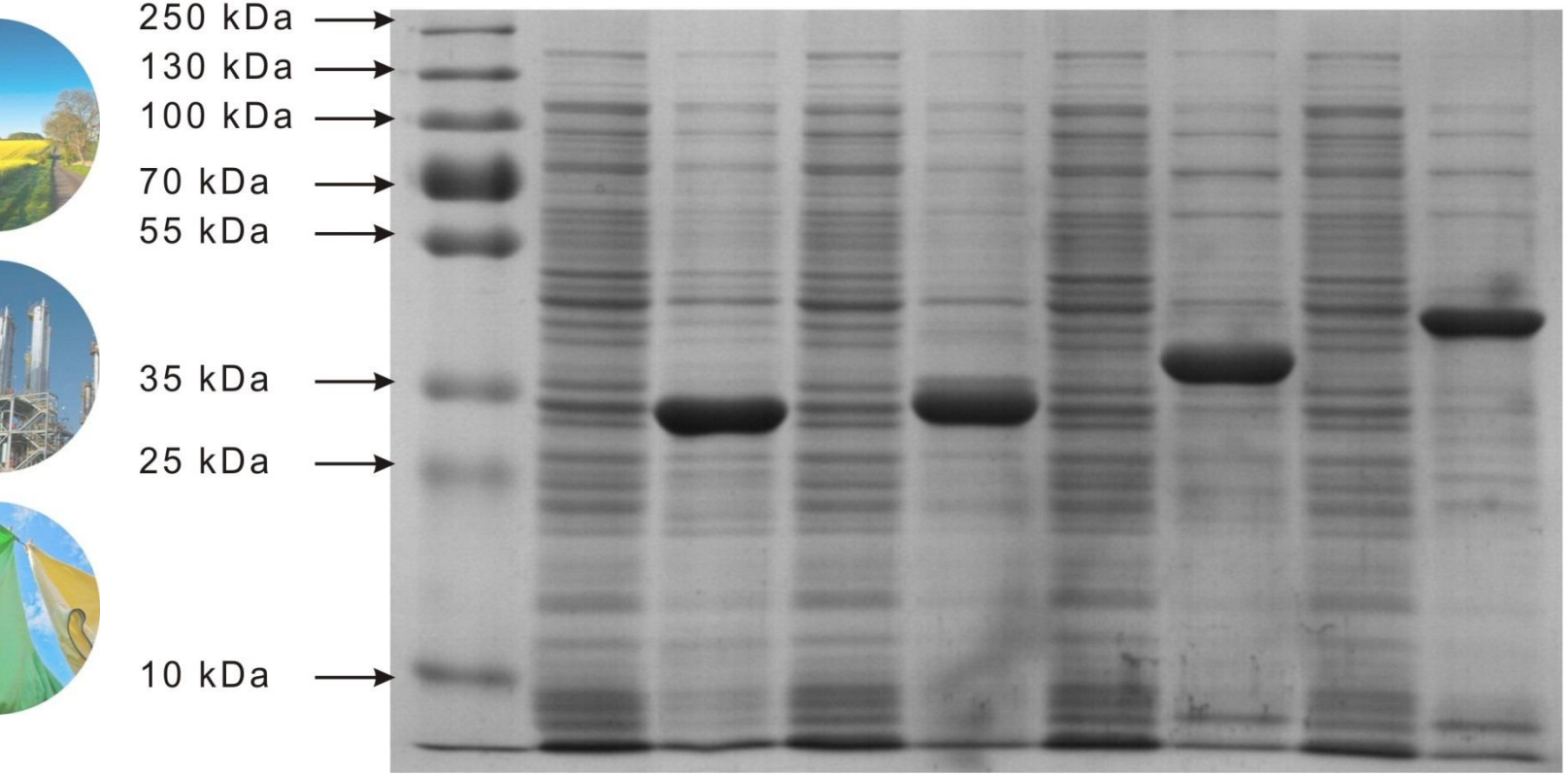
Transaminases

Transaminases can be used to synthesise chiral amines from pro-chiral ketones, D and L-amino acids and amino compounds from aldehydes. Amines are important as pharmacophores and impart biological activity to many compounds. Over 70% of pharmaceuticals are derivatives of chiral amines. This proposal has concentrated on the classes IV and VI, BCATs and and sugar transaminases, which are the least studied transaminase enzymes.

The THERMOGENE project has identified and studied a range of Class IV BCAT transaminases - 10 of the most soluble enzymes have been characterised and three crystal structures are available to high resolution. Their substrate specificities have been determined with a view to industrial applications.

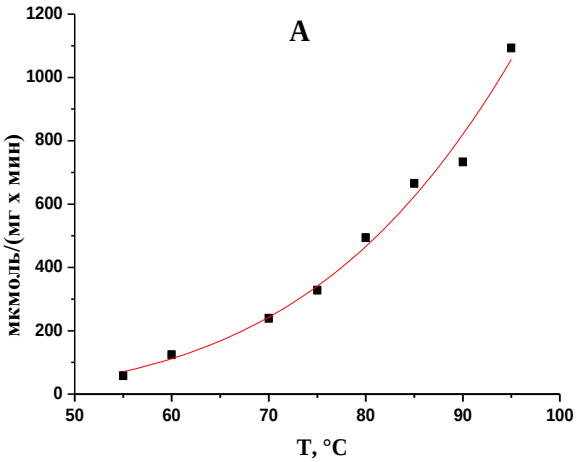
Also a range of sugar class VI enzymes have been identified and three crystallographic structures determined.

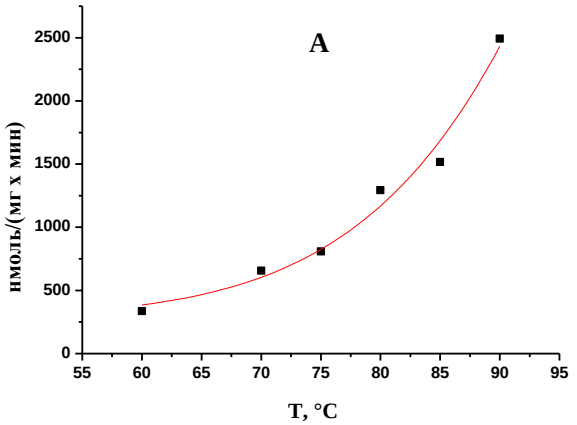
Transaminases IV–Over-expression



A SDS Gel showing a selection of novel thermophilic branched chain (BCAT) transaminases over-expressed in *E.coli* by the Russian partner

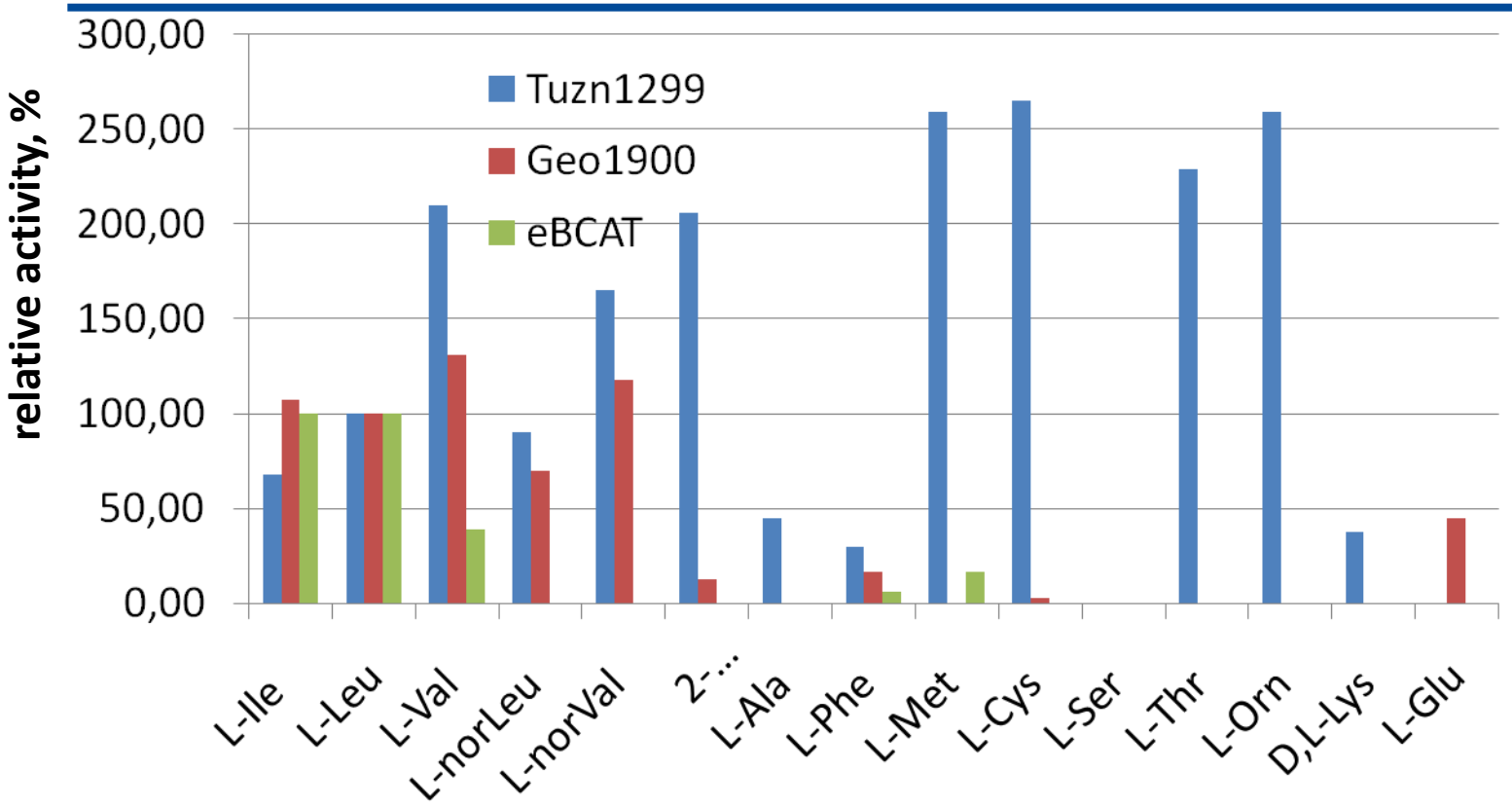
Thermophilicity of two new transaminases IV class (BCAT)





Thermostability of BCATs
from thermophilic genomes
5 h at 70 °C – residual activity 100%
24 h at 70 °C residual activity 62-65 %

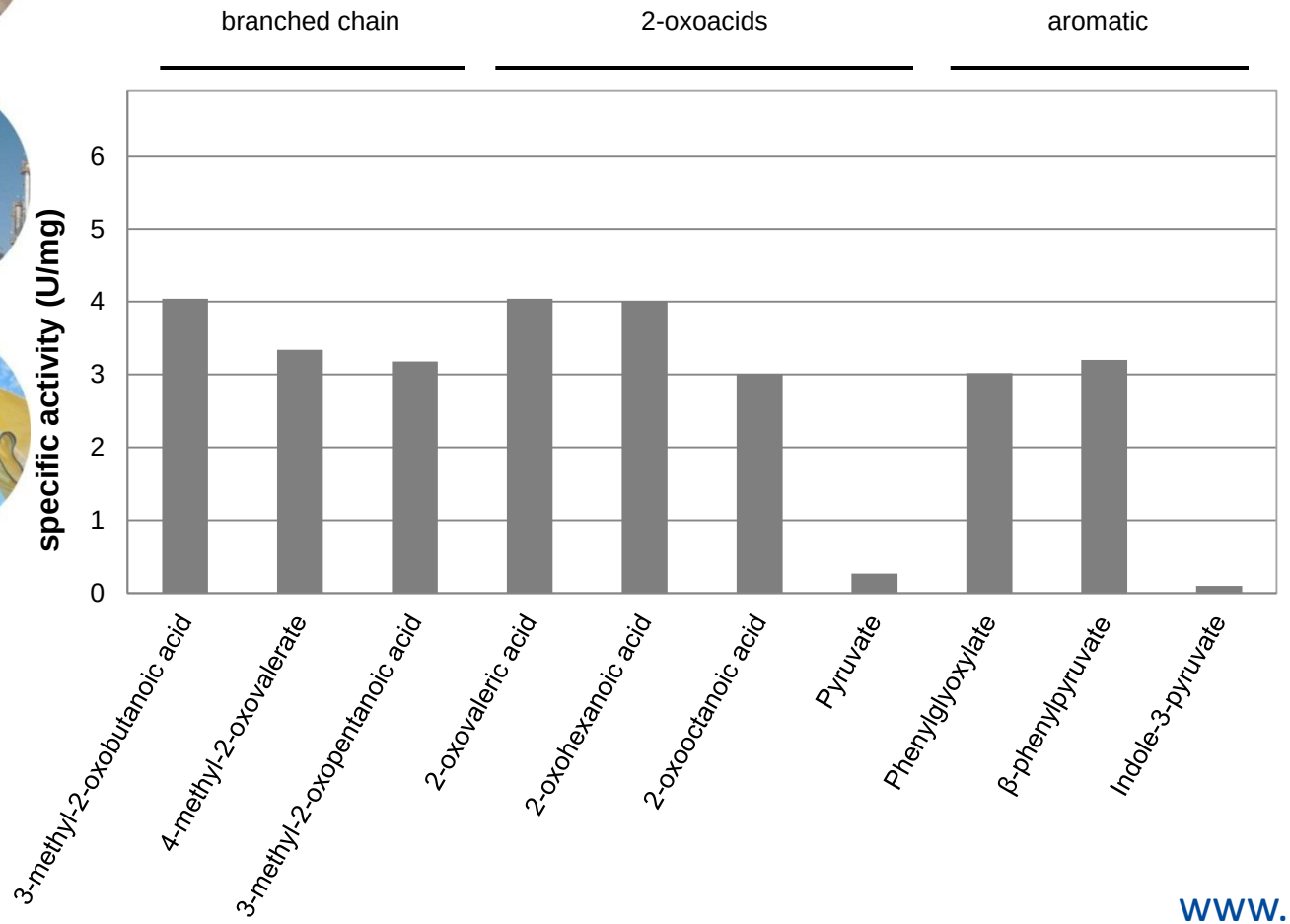
Substrate specificity to amino acids of new BCATs from *(Tuzn1299), (Geo1900)* in comparison *with BCAT from E.coli (eBCAT) Russian partner*



Substrate specificity of BCAT from Kiel partner



L-glutamate + 2-oxoacid $\xrightarrow{TA}$ α-ketoglutarate + aminoacid



70 °C
0.1 mM PLP
1 mM substrate
10 mM L-glutamate
2.5 µg Aminotransferase

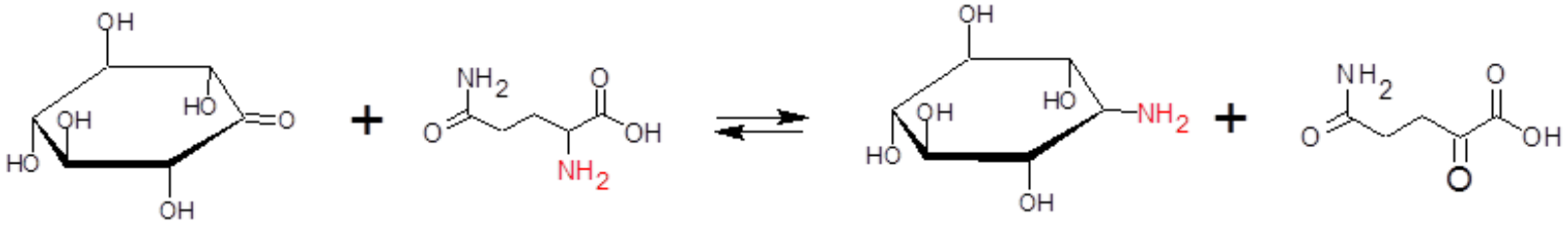
Sugar Transaminases class VI

- Relatively understudied
- Structurally type 1 pyridoxal phosphate cofactor fold.
- Novel motif based on the active site lysine region.
- Found in pathways for antibiotic pathways in *Bacillus* species
- Elaborate different carbohydrates
- Commercial applications for new antibiotics
- Also some of these enzymes recognizing sugar nucleotides

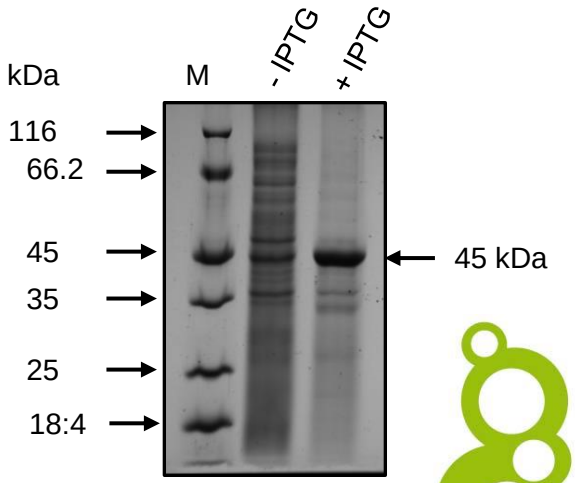
Sugar Transaminase



Class VI Sugar transaminase – from the Kiel partner



Proposed reaction



- Cloned in pET28a, expressed in *E. coli* Rosetta (DE3) pLysS
- Calculated MW. 41.7 kDa (+ 2 kDa His-Tag)

Sugar Transaminase

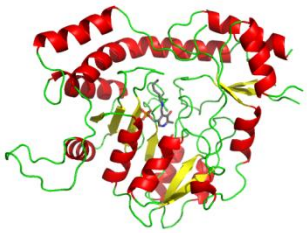
Sugar transaminase Bergen and Exeter partners

Enzyme purified and crystallized

Most related to GDP-Perosamine Synthase 39% amino acid sequence identity

GDP-perosamine synthase is a PLP-dependent enzyme that transfers a nitrogen from glutamate to the 4-keto position of GDP-4-keto-6-deoxymannose during the biosynthesis of GDP-perosamine produced by some bacteria such as *Vibrio* species. Also involved in the synthesis of the antibiotic perimycin.

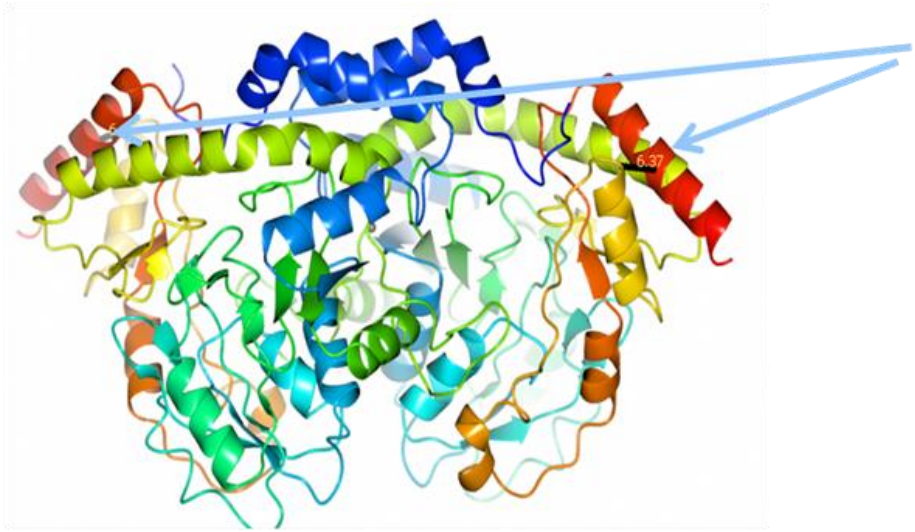
Refined to 1.5 Å resolution



Sugar Transaminase from Thermophilic archaeon



Exeter partner



Structure at 1.4 Å resolution. Enzyme cloned and over-expressed in *E.coli*
Enzyme very thermostable and stabilised by disulfide bonds

Prenyl Transferase from archaeon

A geranylgeranyl diphosphate synthase catalyses condensations of isopentenyl diphosphate with allylic diphosphates to produce an important precursor of ether lipids and has been found in most archaeal genomes that have been sequenced. Three genes have been cloned and over-expressed in a soluble form and one has been subjected to crystallographic studies and initial biochemical characterisation by the Russian partner.

Biochemical characteristics:

Active at 65 °C

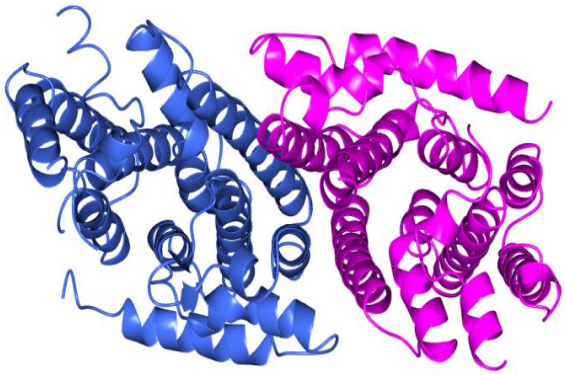
Substrate specificity:

GPP + IPP – FPP (minor) +GGPP (major)

DMAPP + IPP – GGPP

FPP + IPP -- GGPP

C25 is not detected



Resolution of collected data is 2.7 Å.

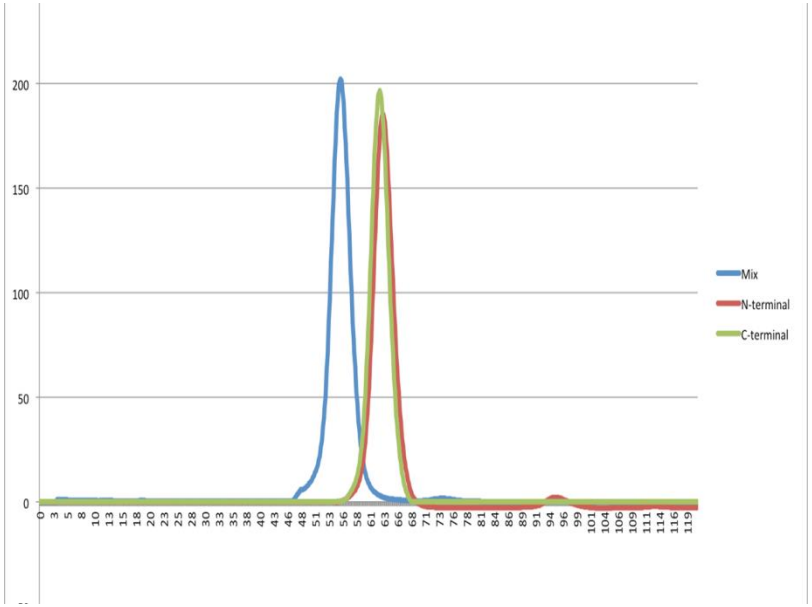
Transketolase



Transketolases are able to form a new carbon-carbon bond and transfer a 2-carbon unit to an acceptor sugar in the pentose phosphate pathway. The reaction can be made irreversible using β-hydroxypyruvate as the 2-carbon ketol donor and glycoaldehyde as an acceptor with the production of L-erythrulose and carbon dioxide which is released from the reaction. Thermophilic archaea and deep branching bacteria have both novel 'split' transketolase genes and a full length gene.

Members of THERMOGENE have cloned, over-expressed and characterised novel transketolase enzymes. The structure of one full length thermophilic bacterial enzyme has been determined and one novel 'split' archaeal transketolase, These have been compared with the commonly used *E.coli* transketolase and other members of the transketolase family with a view to using the cheaper starting substrate, pyruvate rather than hydroxypyruvate.

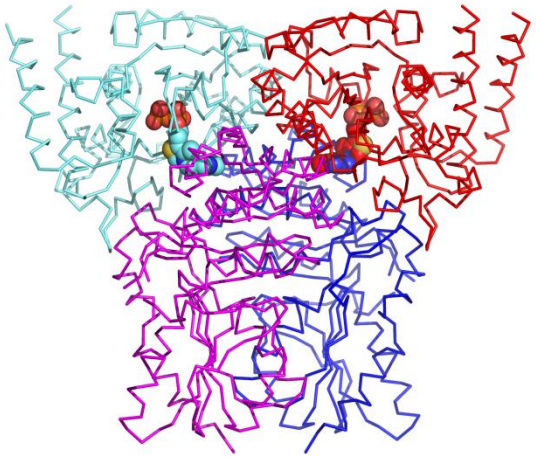
Novel ‘Split’ Archaeal Transketolase



Gel Chromatography

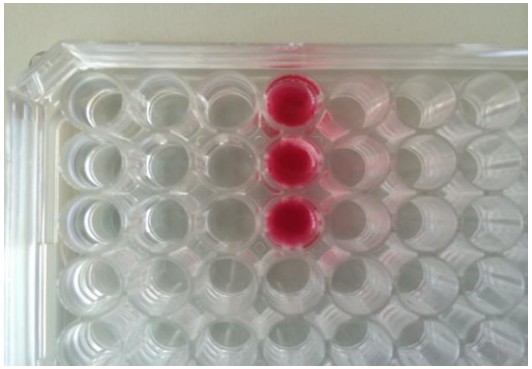
Exeter partner

Reconstituted ‘split’ transketolase



Tetrazolium assay -N and C terminal domains individually which showed no activity

An equimolar mix of N and C terminal domains produced a red colour



Hydroxymethyl transferase



Methyl transferase and the **hydroxyl methyl transferases** have both been considered a target for commercial biotransformations. A cascade reaction between a glutamate- 3 methyl transferase has been described with a branched chain transaminase. These enzymes have been used in a cascade reaction with transaminase enzymes.

These enzymes have potential for applications as a biocatalyst for the stereo-selective synthesis of β-hydroxy-α-amino acids.

A thermophilic hydroxymethyl transferase enzyme has been identified, cloned and crystallised in the Bergen group and its structure determined by the Exeter Group.

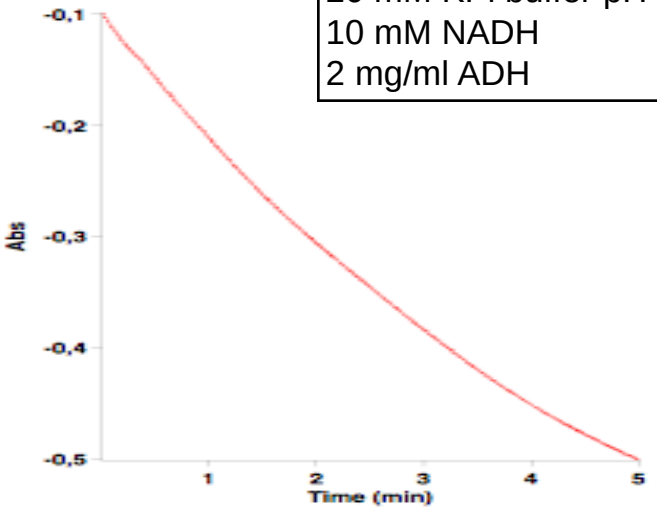
Hydroxymethyl Transferase

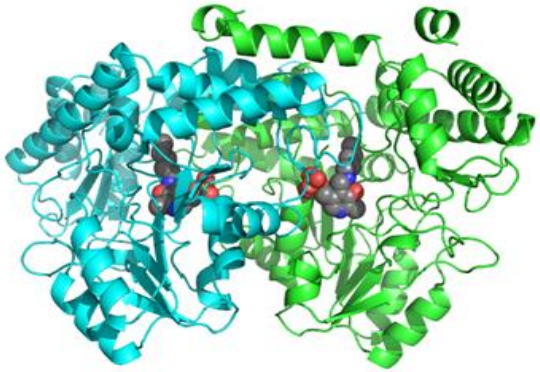


Bergen and Exeter partners

20 mM KPi buffer pH 7.5
20 mM L-threonine
50 uM PLP

20 mM KPi buffer pH 7.5
10 mM NADH
2 mg/ml ADH





Summary



- The proposed objectives of THERMOGENE have all been met.
- A large selection of new novel stable transferase enzymes with representatives from Transaminases IV and VI, Transketolases, Prenyltransferases, Hydroxymethyl Transferases have been identified.
- Many of these enzymes have been cloned and over-expressed in *Escherichia coli* which has allowed them to be characterised biochemically for their stability and substrate specificities with relevance for industrial applications.
- Representatives from all these transferase enzymes have been characterised by X-ray crystallography to allow a greater understanding for their enzymatic mechanism, stability and substrate specificity.
- These enzymes will be made available to the wider biocatalysis community by commercialisation through related SMEs
- Applications to partners home country funding bodies. Application to Horizon 2020 Innovation in SMEs.

General Evaluation

- This project has benefited from the International collaboration and could not be carried out without the partners complimentary expertise and specialised techniques to successfully obtain the project goals.
- General dissemination of this ERA-net THERMOGENE project has been carried out by poster presentations at local and International Scientific Meetings and the publication of scientific papers some of which have already been submitted to high impact journals and others that have been submitted or are in preparation.
- Exchange visits of postdoctoral and graduate students between partners laboratories and annual meetings have been both scientifically beneficial and have offered a culturally experience. Annual meetings in Exeter,UK, Bergen, Norway and Moscow, Russia between partners, the industrial advisor, associated postdoctoral fellows and students have been very productive and have built on 3 monthly web conferences between the consortium members during the project.

Meetings of THERMOGENE Group

Exeter UK

Bergen , Norway

Moscow, Russia

Public Dissemination

Blog – Forum A blog under UiB's server was created (http://thermogene.b.uib.no/). Description of the project as well as the consortium is explained in the blog. It includes a forum plugin with restricted access to the Thermogene community for scientific discussions in different threads.

Twitter -The twitter account @biointex (https://twitter.com/biointex) was created for dissemination of different news related to the Thermogene project.

Film - (https://www.youtube.com/watch?v=CgzKVIYNaeA) The video is titled "The Hidden World" and some of the outcomes from the Thermogene project are shown

Web Site:-
https://www.exeter.ac.uk/biocatalysis/research/THERMOGENE

Conference Dissemination



International Thermophile Meeting, 2015 Chile Oral Presentations Novel thermophilic enzymes for industrial biocatalysis. Littlechild. New archaeal branch-chain transaminases: high thermostability, typical structure and untypical substrate specificity. Bezsudnova, Stekhanova, Mardanov, Rakitin, Nikolaeva, Boyko, Popov. **Poster** Structural studies, substrate identification and activity of sugar aminotransferase enzymes from thermophilic (meta)genomes Karki, García-Moyano, Sayer, Isupov, Littlechild and Birkeland

SIMB Meeting, Philadelphia, USA, 2015, USA Oral Presentation Thermophilic Enzymes and Applications as Industrial Biocatalysts, Littlechild

International Extremophile Meeting, 2014 St Petersburg, Moscow Oral Presentation. A novel Archaeal 'Split Transketolase' Enzyme: reconstitution, structural and evolutionary perspectives Littlechild **Poster** THERMOGENE, All partners

ProStab Meeting 2014 Stressa, Italy Oral Presentation, Thermostable Enzymes for Industrial Biocatalysis. Sayer and Littlechild

Conference Dissemination



International Thermophile Meeting, 2013 Regensburg, Germany **Oral** Presentation Thermophilic Enzymes for Industrial Biocatalysis. Littlechild. **Poster** THERMOGENE, All partners
Biotrans Meeting 2015, Vienna, Austria Oral Presentation Thermogene- Novel Thermostable Transfer Enzymes for Biocatalysis, Littlechild
TRANSAM Meeting Greitswald, Germany 2015. Oral Presentation Structural Studies on Transaminase Enzymes and Applications in Biocatalysis, Littlechild. **Poster** Sayer, James, Isupov and Littlechild
BBSRC BIOCATNET meeting, London, 2014 Oral presentation Novel Thermostable Transfer Enzymes for Industrial Biocatalysis.
Biocatalysis and Application Moscow, Russia, **2015 Poster** Why typical branch-chain transaminases have untypical substrate specificity? Bezsudnova, Stekhanova, Mardanov, Rakitin, Nikolaeva, Boyko, Popov.

Scientific Papers



Papers

First structure of archaeal branched-chain amino acid aminotransferase from *Thermoproteus uzoniensis* specific for L-amino acids and R-amines Konstantin M. Boyko, Tatiana N. Stekhanova, Alena Yu. Nikolaeva, Andrey V. Mardanov, Andrey L. Rakitin, Nikolai V. Ravin, Ekaterina Yu. Bezsudnova and Vladimir O. Popov (2016) (doi:10.1007/s00792-016-0816-z)

Structural Characterisation of the first archaeal 'split' transketolase and comparison with other members of the transketolase family enzymes, James, Sayer, Isupov and Littlechild 2016 manuscript in preparation.

The biochemical and structural characterisation of thermophilic archaeal branched chain transaminases James, Sutter, Schmidt, Sayer, Isupov, Littlechild, Schoenheit 2016 Manuscript in preparation

The thermophilic hydroxymethyltransferase from the bacterial *Thermovirgo* species, Garcia-Moyano, Sayer, Isupov, Littlechild, Birkeland 2016 Manuscript in preparation

The first structures of thermophilic archaeal class 6 sugar transaminases from the Thermogene project. 2016 All partners, manuscript in preparation

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