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Directed Evolution of a Thermophilic β-glucosidase for Cellulosic Bioethanol Production

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Abstract Characteristics that would make enzymes more desirable for industrial applications can be improved using directed evolution. We developed a directed evolution technique called random drift mutagenesis (RNDM). Mutant populations are screened and all functional mutants are collected and put forward into the next round of mutagenesis and screening. The goal of this technique is to evolve enzymes by rapidly accumulating mutations and exploring a greater sequence space by providing minimal selection pressure and high-throughput screening. The target enzyme was a β-glucosidase isolated from the thermophilic bacterium, *Caldicellulosiruptor saccharolyticus* that cleaves cellobiose resulting from endoglucanase hydrolysis of cellulose. Our screening method was fluorescence-activated cell sorting (FACS), an attractive method for assaying mutant enzyme libraries because individual cells can be screened, sorted into distinct populations and collected very rapidly. However, FACS screening poses several challenges, in particular, maintaining the link between genotype and phenotype because most enzyme substrates do not remain associated with the cells. We employed a technique where whole cells were encapsulated in cell-like structures along with the enzyme substrate. We used RNDM, in combination with whole cell encapsulation, to create and screen mutant β-glucosidase libraries. A mutant was isolated that, compared to the wild type, had higher specific and catalytic efficiencies (k_{cat}/K_M) with *p*-nitrophenol-glucopyranoside and -galactopyranoside, an increased catalytic turnover rate (k_{cat}) with cellobiose, an improvement

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in catalytic efficiency with lactose and reduced inhibition (K_i) with galactose and lactose. This mutant had three amino acid substitutions and one was located near the active site.

Keywords β-glucosidase · Directed evolution · Random drift mutagenesis · In vitro compartmentalisation · Fluorescence-activated cell sorting

Introduction

β-glucosidase enzymes catalyse the hydrolysis of β-D-glycosidic bonds and can display promiscuous activity towards various β-D-glucoside substrates [1]. They have several applications in industrial processes such as the hydrolysis of cellulose [2] and lactose [3]. β-glucosidase has a dual role in the depolymerisation of cellulose. First, it catalyses the final step in the reaction, hydrolysis of cellobiose to two glucose molecules which can then be fermented to biofuels such as ethanol [4]. Secondly, the cellulase enzymes that break cellulose down into cellobiose are highly end-product-inhibited by cellobiose [5]. Addition of a β-glucosidase has been shown to improve the overall rate of cellulose degradation by removing the inhibiting cellobiose [6]. Some β-glucosidase enzymes are also able to catalyse the hydrolysis of lactose to glucose and galactose. The hydrolysis of lactose has several important applications, namely in the production of milk products that are sweeter, more digestable and have a higher degree of solubility [7, 8].

Random drift mutagenesis (RNDM) has been developed to determine whether a phenotype that is derived from the interaction of multiple amino acids might require the accumulation and interaction of neutral mutations (neutral in isolation) and adaptive mutations [9]. The procedure uses iterative misincorporation mutagenesis but no screening for adaptive mutations occurs. Instead, screening is only done for retained ability (whether unchanged, improved or reduced) to catalyse the hydrolysis of a substrate. This procedure is intended to provide high-speed screening of mutants for retained activity without tedious assay procedures and to allow a comprehensive examination of sequence space for superior mutants. All positive recombinants are combined and used as template for a further round of mutagenesis for as many rounds as are necessary. In this manner, it is assumed that accumulation of multiple adaptive, neutral and harmful (but not inactivating) mutations occurs. Once generated, this library is then screened for recombinants with modified biochemical activity.

Similar directed enzyme evolution procedures have been explored by others [10–12]. They term the process ‘neutral drift’ with the same emphasis on the gradual accumulation of mutations to maintain the protein’s original function.

Cell sorting has great potential for high-throughput enzyme screening since such instruments are capable of rapid multiparametric analysis of individual cells at rates of more than 10^7 cells screened per hour [13]. However, enzyme screening via fluorescence-activated cell sorting (FACS) poses several challenges—the biggest obstacle being the lack of suitable enzyme substrates that remain associated with individual cells. FACS can be used to screen libraries where the product of the enzyme reaction remains connected to the cells and there is a detectable change in fluorescence when the substrate is converted to product. Whilst many substrates can cross a cell membrane readily to act as a substrate for an expressed enzyme, the product can also diffuse out of the cell just as rapidly. Flow cytometry analyses and sorts individual cells so it is essential that the detectable product remains associated with the cell that produced it. Ideally, the enzyme reaction would produce an insoluble or non-diffusible fluorescent product. However, the lack of

availability of a wide range of such substrates has led researchers to develop alternative strategies to the problem of genotype–phenotype linkage.

In vitro compartmentalisation (IVC) is a technique that was developed to maintain the association of genotype and phenotype by creating cell-like structures where single genes are encapsulated in an artificial membrane along with the components required to transcribe and translate them, and any additional substances such as enzyme substrates [14, 15]. The original IVC method has been modified to allow flow cytometric screening and sorting of compartments [16] and the technique can be applied also to encapsulate whole cells, expressing the enzyme of interest, with substrate and the activity of the mutant enzymes screening using flow cytometry [15]. This type of screening for enzyme activity has been used to evolve the properties of thiolactonases [17].

The compartmentalisation procedure results in large numbers of empty compartments and others possessing multiple cells, reducing the efficiency of the screen and leading to the inclusion of false positives [16, 17]. One way that has been used successfully to enrich for compartments containing individual cells is the co-expression of a fluorescent protein with the enzyme of interest [10, 17]. Difficulties encountered with the expression of a fluorescent protein that was compatible with the fluorescent product of the enzyme reaction led us to investigate the potential for enriching compartments containing single cells based of the side and forward light scattering properties of individual compartments. IVC experiments where the gene, the machinery required to express the enzyme and the appropriate substrates are encapsulated together does not use a fluorescent marker to highlight droplets that contain genes. That process relies on the fact that on average, each droplet will contain one gene. Therefore, we determined also whether or not the number of compartments containing single cells could be improved by reducing the concentration of cells emulsified. Using a combination of flow-cytometry-based detection and 96-well-plate assays, we identified a mutant enzyme with altered kinetic parameters that could be employed as a basis for the modification of other characteristics such as thermostability.

Materials and Methods

Cloning and Expression of the β-glucosidase Gene

The β-glucosidase A (*bgl*A) gene was originally isolated from *Caldicellulosiruptor saccharolyticus* and was cloned into a modified version of the pProEXTHc plasmid (Invitrogen) carrying the gene for kanamycin resistance [9, 18]. Expression of the β-glucosidase enzyme and an associated His-tag for downstream purification was induced using IPTG.

PCR Conditions and Mutant Library Construction

Standard PCR conditions were as follows: 1× Buffer II (10 mM Tris–HCl, pH 8.3, 50 mM KCl), 2 mM $MgCl_2$, 200 μM dNTPs, 1 μM forward and reverse primers, 1 unit of AmpliTaq Gold® (Applied Biosystems), 1–10 ng of DNA template (plasmid) in MilliQ water to a total volume of 25 or 50 μl. All PCR programmes started with a heating step of 94 °C for 15 min, then 25–35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min per 1,000 bp.

Error-prone PCR was performed to create libraries of *bgl*A genes with random point mutations. The fidelity of the *Taq* polymerase was reduced by using a high concentration of

$MgCl_2$ (7 mM), a skewed ratio of dNTPs (0.2 mM adenine and guanine; 1 mM cytosine and thymine) and 0.1–0.5 mM $MnCl_2$ (final concentration), depending on the mutation frequency required [19].

The mutant libraries were expressed in *E. coli* TOP10F' strain (Invitrogen). This strain has a lac^- deletion and does not hydrolyse X-gal

Whole Cell Encapsulation

Induced cells expressing the β-glucosidase enzyme were encapsulated in a water-in-oil-in-water (w/o/w) emulsion [15]. All emulsification steps were carried-out on ice with pre-chilled buffers and reagents. Cell numbers and concentrations were determined by diluting cultures in 100 mM sodium phosphate buffer and counting cells in a haemocytometer (Neubauer). An 80-μl portion of cells at a concentration of 6×10^6 cells/μl in 100 mM sodium phosphate buffer (pH 6.3) was mixed with 2 μl of 20 mM fluorescein di-glucopyranoside (FDGlu; Molecular Biosystems) and then emulsified with 800 μl of 2.9% ABIL EM90 (Degussa) in mineral oil using an Ultra Turrax T-10 basic homogeniser (IKA) with a S 10 N-5 G dispersing element. The samples were mixed for 5 min at 9,500 rpm. A volume of 800 μl of 1.5% carboxy-methyl cellulose, 1% Triton-X 102 in 100 mM sodium phosphate buffer was added to the primary emulsion. The secondary emulsion was made by homogenising this mixture for 3 min at 8,000 rpm. Secondary emulsions were diluted one in ten in 100 mM sodium phosphate buffer prior to the heating step required for enzyme activity (5 min at 55 °C). The emulsions were diluted further in PBS and were stored on ice prior to analysis and sorting.

Flow Cytometry

Flow cytometry and sorting of compartments was performed using a FACSAria™ flow cytometer (BD Biosciences). Compartments were selected based on size and complexity (FSC vs SSC) to enrich for the compartments containing single cells. These compartments were analysed for enzyme activity based on the release of fluorescein by active enzymes and detected as increased fluorescence in the FITC filter (530/30). All fluorescent compartments and the most active 5% of variants were collected (10^6 and 1×10^4–5×10^4 compartments, respectively) in 1–2 ml of Luria broth. Cells were recovered by incubation for 1 h at 37 °C with 250 rpm shaking. The most active variants were plated on L Agar containing kanamycin, IPTG and X-gal to confirm enzyme activity. The enzyme has wide substrate specificity and is able to cleave X-gal to give indigo release and, hence, colour-positive colonies blue. The sample containing all active variants was grown overnight at 37 °C in L broth with kanamycin. The plasmids were extracted and used as template in an error-prone PCR for the next round of mutagenesis and screening.

Enzyme Activity Assay

A selection of colony-forming units from the most active 5% of the population from each round were analysed further in 96-well-plate-based assays. Colonies were picked and grown overnight in 96-well plates. A 20-μl portion of each of the overnight cultures was transferred to 180 μl of fresh broth (with antibiotics). The plates were sealed and incubated with shaking for 3 h. Protein expression was induced by the addition of 10 μl of 20 mM IPTG and cultures were grown for a further 3 h. Cells were lysed by transferring 20 μl of induced culture to 180 μl of 100 mM sodium phosphate buffer (pH 6.3) containing 10 mg/ml lysozyme. Approximately 20–

30 µl of lysate was added to 100 mM sodium phosphate buffer to a final volume of 50 µl. A volume of 50 µl of *p*-nitrophenol glucopyranonside (pNPGlu) substrate was added to each well to a final concentration of 2 mM. Assays were performed in sealed 96-well PCR plates in an Eppendorf Mastercycler®. The standard assays conditions were incubation at 70 °C for 5 min and reactions were terminated by the addition of 100 µl of 1 M sodium carbonate. The absorbance of the released *p*-nitrophenol (pNP) was measured at 405 nm and the activity of mutants was normalised to cell density and wild-type activity. The most active mutants compared to the wild-type enzyme were streaked out on Luria agar plates and rescreened in triplicate in plates to remove false positives.

Kinetic Parameters

Enzyme and substrate were incubated for 1 min at 70 °C to determine initial velocity (V_0). Michaelis–Menten plots were created and K_M, V_{max} and k_{cat} values calculated using non-linear regression (GraphPad Prism 5 software).

Substrate and Product Inhibition

To determine sensitivity to the presence of substrates and end products, 10 nM of each enzyme was incubated with increasing concentrations of substrates (cellobiose or lactose) or products (glucose or galactose), for 30 min at room temperature. The enzymes were then assayed with 0–2 mM pNPGlu under standard conditions. K_i values were calculated using GraphPad Prism 5 software.

Glucose Release

The activity of the enzymes with natural substrates (cellobiose and lactose) was determined using an Amplex®Red Glucose/Glucose Oxidase Assay Kit (Molecular Probes). 10 nM enzyme in 100 mM phosphate buffer (pH 6.3) was incubated with cellobiose or lactose (0–200 mM) at 70 °C for 1 minute and then cooled. Negative controls of cellobiose and lactose (0–200 mM) without enzyme also were included. A 10 µl portion of this assay mixture was transferred to a fresh 96-well microtitre plate and mixed with 40 µl of Amplex Red 1× reaction buffer. The assay was performed according to the manufacturer's instructions and fluorescence was measured using an excitation wavelength of 540 nm and an emission wavelength of 590 nm.

Results

Random Drift Screening

The RNDM for directed evolution is summarised in Fig. 1, where FACS can be used to collect all functional mutants for further rounds of mutagenesis and screening. The idea behind RNDM is to evolve proteins by the accumulation and combination of neutral as well as beneficial mutations. A traditional directed evolution approach would discard neutral mutations at each round of mutagenesis. By mutating whole populations of functional mutants at each round, we hoped to isolate mutants with unforeseen and unique combinations of mutations that would not have been discovered using a traditional mutagenesis/screening approaches.

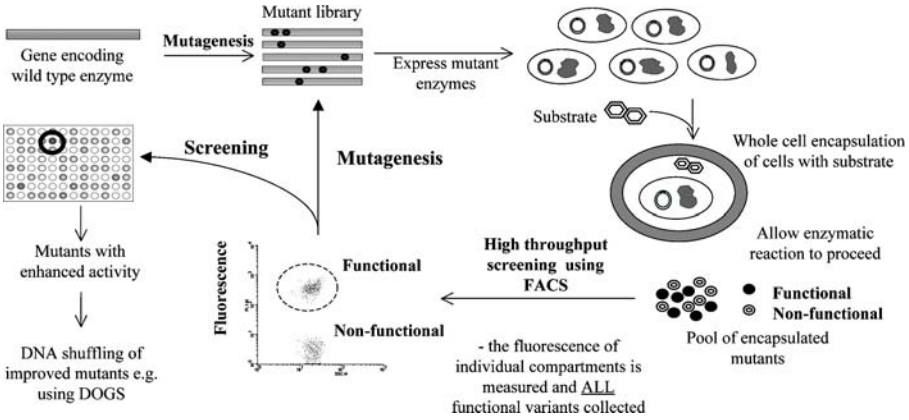

Fig. 1 Overview of the random drift mutagenesis process. Random point mutations are introduced into the gene encoding the enzyme of interest. The mutant library is transformed into *E. coli* and encapsulated with substrate. The encapsulated cells are incubated and then sorted using FACS into functional and non-functional populations based on the amount of fluorescence produced by the hydrolysed product. All mutants possessing a functional version of the enzyme are collected and used as template for the next round of mutagenesis. The mutagenesis frequency is kept low at approximately one to two amino acid substitutions per round. The most active mutants are also collected and further screened in 96-well-plate assays

Four rounds of RNDM were performed on the *C. saccharolyticus* β-glucosidase enzyme. A sample of mutants was sequenced to monitor the accumulation of mutations at each round of mutagenesis and screening. We observed an increase in the average number of nucleotide substitutions per mutant per round and a corresponding increase in amino acid substitutions as shown in Table 1. By the fourth round of mutagenesis, variants carried an average of five amino acid substitutions. In addition, the most active mutants identified by FACS were collected and screened in 96-well-plate format to identify any variants showing enhanced β-glucosidase activity compared to the wild-type enzyme.

Whole cell encapsulation allows cells and any substrate to be encapsulated together in a synthetic membrane that is robust enough for analysis and sorting using FACS (Fig. 2). We chose to encapsulate whole cells as opposed to in vitro compartmentalisation for several reasons. We knew that *E. coli* cultures carrying the plasmid with the β-glucosidase insertion produced sufficient levels of functional enzyme to detect activity via FACS and for initial experiments, we considered that it was simpler to encapsulate whole cells than establish an in vitro system where the levels of transcription/translation, and hence, protein expression may be affected by the compartment size—which can be variable [20].

Compartments containing single cells were enriched from 60% of droplets containing cells to almost 90% using the original number of cells (5×10^8 cells) suggested by Miller et

Table 1 Average number of nucleotide and amino acid substitutions at each round of mutagenesis compared to the wild-type enzyme/gene (±standard deviation).

Round of mutagenesis	Amino acid substitutions	Nucleotide substitutions
1	0.8±0.8	1.1±1.1
2	2.2±1.6	3.9±1.7
3	3.7±1.7	4.8±1.9
4	5.0±2.3	6.6±2.8

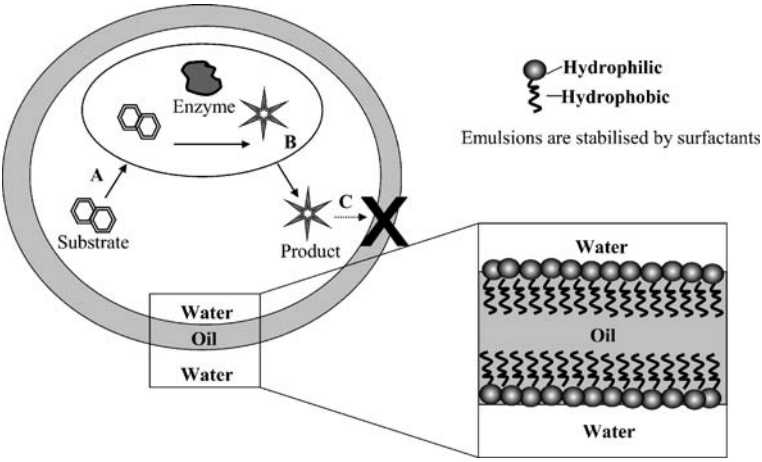

Fig. 2 Cell expressing enzyme encapsulated with substrate in water-in-oil-in-water emulsion. **a** The substrate readily crosses the cell membrane; **b** a detectable fluorescent product is released upon hydrolysis; **c** the product remains associated with the cell. Cells and substrates are encapsulated in FACS compatible water-in-oil-in-water emulsions

al. [15]. Lower concentrations of cells had more droplets containing single cells prior to sorting. Starting with a population of 2.5×10^8 cells resulted in a pre-sort population with almost 90% of cell-containing compartments with single cells and a final population post-sorting of 95% of droplets containing one cell. Further reduction of the cell concentration did not enhance the number of the compartments containing one cell. This reduction also was not desirable because reducing the concentration of cells would reduce the efficiency of the screen by having to analyse and sort more compartments to obtain sufficient numbers for further mutagenesis (Fig. 3).

Enrichment of Cells Expressing β-glucosidase

A population of $BglA^-$ and $BglA^+$ cells were grown separately, counted and mixed in equal proportions prior to encapsulation with substrate to determine whether or not cells expressing functional β-glucosidase enzymes could be enriched over non-functional mutants using this method. A portion of the mixed cells were plated on X-gal to confirm the ratio of positive to negative cells prior to sorting and enrichment using FACS. The mixed cultures were encapsulated with FDGlu and sorted based on the FSC and SSC (P1) and product fluorescence (FITC released from positive cells). FITC-positive-cells were collected and plated onto X-gal plates. The numbers of blue ($BglA^+$) and white ($BglA^-$) colony-forming units were counted after overnight incubation of the sorted and unsorted cells. In the original, unsorted population, 50% of colony-forming units (CFU) were blue and hence possessed a functional version of the β-glucosidase enzyme. When this population was sorted using encapsulation and FACS, the $BglA^+$ population was enriched and over 90% of the population expressed the wild-type gene (Fig. 4).

Enzyme Kinetic Parameters

A sample of mutants was sequenced to monitor the accumulation of mutations at each round of mutagenesis and screening. In addition, the most active mutants identified by

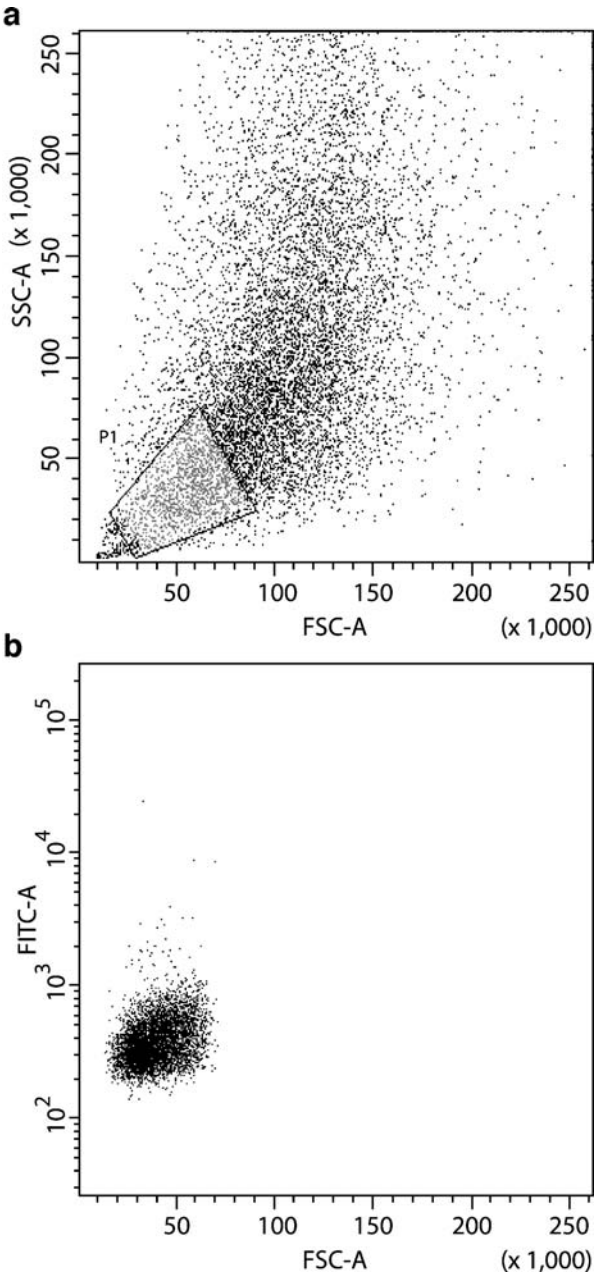

Fig. 3 Flow cytometry dot plots of encapsulated bacteria. **a** Compartments were first selected based on the level of fluorescence, followed by size (FSC) and complexity (SSC). Medium sized compartments (P1) were collected, grown overnight and the plasmid extracted for the next round of mutagenesis and screening. **b** Enzyme activity of P1 compartments was analysed by detecting product fluorescence using the FITC filter. Highly fluorescent compartments were collected and analysed in more detail using 96-well plates

FACS were collected and screened in a 96-well-plate format to identify any variants showing enhanced glucosidase activity compared to the wild-type enzyme. The most active mutant, E1, isolated in round 1, was His-tag purified along with the wild-type enzyme and their kinetic parameters were determined. E1 was found to have a higher specificity (k_{cat}/K_M) than the wild-type enzyme for the pNPGlu and pNPGal substrate analogues. The catalytic turnover rate (k_{cat}) for E1 with cellobiose was increased

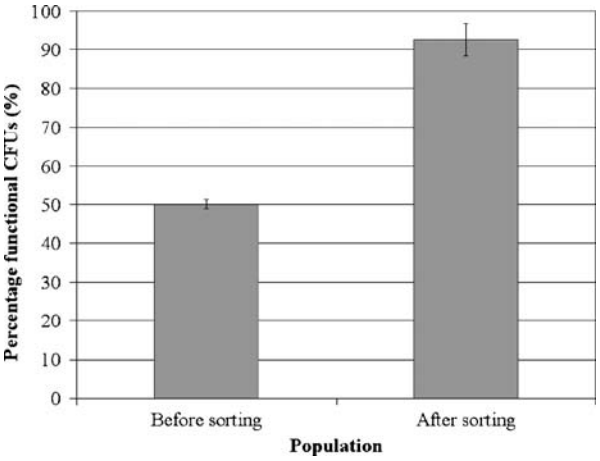

Fig. 4 Enrichment of $BglA^+$ cells after cell encapsulation and FACS. Cells expressing the wild-type enzyme ($BglA^+$) were mixed with cells carrying the same plasmid without the insertion ($BglA^-$) in a 1:1 ratio, encapsulated with substrate and sorted. The ratio of $BglA^+$ to $BglA^-$ CFUs increased significantly after sorting by FACS

compared to the wild-type and the mutant enzyme showed an almost twofold increase in specificity for lactose than the parent enzyme (Table 2). Inhibition by the reaction products of cellobiose and lactose was investigated as the activity of both enzymes is affected adversely by the presence of high concentrations of these sugars (Table 3). Cellobiose is hydrolysed to two glucose monomers and lactose to glucose and galactose. Whilst both enzymes showed similar inhibition by glucose, the mutant enzyme was much less inhibited by galactose than the wild type (Table 3). Similarly, both enzymes displayed comparable inhibition by cellobiose but the mutant was less inhibited by lactose (Table 3).

Gene Sequence of Mutant E1

The mutant β-glucosidase gene was found to have three nucleotide changes compared to the wild-type gene, each of which resulted in an amino acid change. The co-ordinates correlate with the N-terminal methionine prior to the beginning of the His-tag in the pProEXTHcKan vector (Table 4). It could be significant that mutation Thr389Ser is located near to one of the proposed catalytic residues but systematic site-directed mutagenesis would be necessary to establish that only a single amino acid is involved.

Table 2 Summary of kinetic parameters of wild-type and mutant E1.

Substrate	Enzyme	K_M(mM)	$k_{cat}(s^{-1})$	k_{cat}/K_M
pNPGlu	WT	0.40±0.03	154±2	384
	E1	0.25±0.02	177±3	710
pNPGal	WT	1.92±0.12	181±4	94
	E1	1.76±0.07	203±3	115
Cellobiose	WT	7.2±0.8	275±12	39
	E1	7.3±0.8	311±13	44
Lactose	WT	101±12	742±52	7
	E1	83±6	985±38	12

Table 3 Inhibition constants (K_i) for wild-type and mutant E1 enzymes.

Enzyme	K_i(mM)			
	Products		Substrate	
	Glucose	Galactose	Cellobiose	Lactose
WT	75±4	92±5	27±2	39±5
E1	69±4	153±10	31±3	92±13

Inhibition constants were determined for the substrates, cellobiose and lactose and the products released from hydrolysis of these substrates, glucose and galactose

Discussion

The goal of this research was to develop RNDM, a directed evolution technique for the development of mutant enzymes with potentially commercial and industrially important applications. RNDM was designed to address two main problems associated with traditional directed evolution approaches:

First, screening, as utilisation of FACS as a high-throughput screening tool would enable more mutants to be examined, thus increasing the chances of finding the rare variants with mutations that enhance activity; and secondly, diversity as functional mutants, with neutral phenotypes, generally would be discarded in a traditional directed evolution approach. The use of FACS as a screening tool allows all functional mutants to be quickly and easily collected for further rounds of mutagenesis. Hence, more sequence space can be explored and unique combinations of mutations discovered that are beneficial in conjunction but separately neutral. The most active mutant was isolated after round 1 but we were able to generate libraries with an accumulation of mutations. It is possible that the accumulation of neutral mutations may be better applied to the evolution of alternative enzyme properties, such as changes in substrate specificity and the evolution of novel functions [10, 12].

Cloned genes for directed evolution generally are expressed in *Escherichia coli*, which has a complex cell membrane [21, 22]. Initially, we investigated a lipophilic derivative of fluorescein digalactoside that was non-fluorescent until cleaved but the hydrolysis product was prone to leaching out of the cells [9]. Over time, the amount of fluorescence observed in the positive cells dropped and the cells were probably losing product. A substrate that could be used directly to screen enzyme libraries via FACS would have allowed a straightforward means to separate functional mutants and non-functional variants. In its absence, we utilised a method that compartmentalises the cells in water-in-oil-in-water emulsions [14, 17]. This modification allowed the incubation of cells at high temperatures, the use of a more cell-permeable substrate and overcame the problem of substrate leakage.

The 3D structure of the *C. saccharolyticus* β-glucosidase protein has not been solved so it is difficult to determine the potential impact of the individual and the combination of

Table 4 DNA and amino acid substitutions present in E1 mutant β-glucosidase compared to the wild type.

Nucleotide changes	Amino acid changes
A710C	N237T
T1041A	F347L
A1165T	T389S

mutational changes. However, a structure exists for a β-glucosidase from the thermophilic organism *Thermotoga maritima* similar to the wild-type *C. saccharolyticus* enzyme (47% similarity at the amino acid level). The proposed catalytic residues involved in the hydrolysis of the β-1,4-glycosidic bond are two glutamate residues—one acts as the nucleophile and the other as the acid/base [23]. These residues are highly conserved in this enzyme family, supporting their proposed catalytic role and the probable catalytic residues of the *C. saccharolyticus* β-glucosidase would be the Glu192 (acid/base) and Glu390 (nucleophile). The threonine to serine mutation (Thr389) lies adjacent to Glu390 and this mutation may be responsible for the change in activity of the mutant enzyme compared to the wild type. This threonine residue is highly conserved amongst β-glucosidase enzymes. Both serine and threonine are polar, uncharged molecules and possess a very similar structure. The presence of the smaller serine residue possibly may create greater flexibility in the active site or allow greater access to the substrates.

The specific activities of the wild-type enzyme and mutant E1 were examined and compared under a variety of conditions. The mutant enzyme displayed enhanced catalytic activity compared to the wild-type enzyme on two different pNP-glycoside substrates—pNPGlu and pNPGal. These substrates mimic disaccharide substrates that the enzyme would encounter in nature or industry and are useful in screening enzyme libraries for activity because the amount of substrate broken down can be determined directly by measuring the absorbance of the released pNP. However, they are analogues of the substrates that the enzyme would be required to act upon in an industrial environment and therefore, it is important to assess the activity of the mutant with natural disaccharide substrates. β-glucosidases have potential applications in the hydrolysis of cellobiose, a glucose disaccharide released during cellulose degradation and in the hydrolysis of lactose to produce low-lactose milk products. Although E1 showed enhanced activity compared to the wild type with the cellobiose analogue pNPGlu, its ability to hydrolyse the natural substrate cellobiose was only marginally improved due to an increase in the turnover number. However, E1 showed a marked preference for lactose as a substrate compared to the wild type.

The effects of the E1 mutations on other properties of the enzyme were also investigated. Whilst the pH profile of the mutant and wild-type enzymes remained relatively unchanged, the thermostability of the mutant was slightly reduced, but it retained almost 50% activity after incubation 70 °C for 4 h (data not shown). Mutant E1 showed a slight improvement in catalytic activity over the wild-type enzyme in the hydrolysis of cellobiose and hence has potential for application as part of an enzymatic cellulose hydrolysis system. Although it has slightly lower thermostability, it may be more versatile than the wild type as it retained higher activities at temperatures below 70 °C.

The RNDM concept was designed to survey large amounts of sequence space that would be normally be ignored, for example, combinations of seemingly neutral mutations, and, in order to maximise the potential of finding these combinations, a large number of mutants need to be screened. Generally, this approach is limited by the number of individual mutants that it is feasible to screen, i.e. using 384 and 96 well formats and robotics. Using FACS, it is possible to screen populations of 10^7 mutants in a day (compared to days or weeks using the former method) and highly active mutants can be quickly enriched from the general population. However, even though FACS facilitates the screening of orders of magnitude more variants than a typical plate-based screen, the enzymes are expressed in *E. coli* and therefore, the amount of diversity searched is limited by the transformation efficiencies of the bacteria. Accordingly, only a small portion of the variety available is expressed and screened. This problem will be compounded in subsequent rounds of mutagenesis and screening as neutral or beneficial mutations may combine with deleterious mutations and be lost.

In order to increase significantly the number of mutants that could be screened, it would be necessary to move into an in vitro system. In vitro compartmentalisation involves the encapsulation of a single gene and an in vitro transcriptional/translational cocktail [14]. The main advantage of this system is that transformation is unnecessary and hence permits the production and screening of a far greater array of mutant enzymes. Since this approach does not require a live host, enzyme reactions can be carried-out at temperatures appropriate for thermophilic enzymes. Furthermore, inhibitors and other components can be added to the compartments and have direct access to the enzyme without exclusion by the bacterial cell wall.

Flow cytometry is a powerful technique that has been used extensively in health bioscience-related applications, particularly with antibodies. The technique is versatile and has applications in other areas such as molecular biology and genetics. It is exquisitely sensitive in being able to allow cell sorting at high speed of defined populations or individual cells. It has applications in taxonomy and bioprospecting when coupled to appropriate culturing techniques for rare or novel microorganisms [24].

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