THE INDUSTRIAL POTENTIAL OF ENZYMES FROM EXTREMELY THERMOPHILIC BACTERIA

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The thermal regions of the central North Island of New Zealand are some of the most extensive in the world. In addition, they are readily accessible and contain a diversity of ecological habitats, including a large number at 100°C. These areas are regarded as an important tourist attraction, and as a source of geothermal power. It is now clear that they also contain an important and unique genetic resource.

Micro-organisms which survive and grow at elevated temperatures (above 45°C) are loosely termed thermophiles. A more rigid definition requires that their growth optimum should be above 50°C[1]. Extreme thermophiles have growth optima above 65°C, and this group is comprised exclusively of bacteria. These are the organisms which are the subject of our research at Waikato. Under currently used laboratory conditions no bacterium has been isolated with a growth optimum above 80°C, and little growth occurs above 85°C. Since many of these extreme thermophiles are isolated from hot springs at 100°C it is quite possible that both the growth optimum and the upper temperature at which growth occurs are appreciably higher under natural conditions than in the laboratory. There seems to us to be no good microbiological or biochemical reason why, providing sufficient free water is available, bacterial growth should not take place up to 100°C.

We have isolated a variety of extremely thermophilic bacteria from the Rotorua area. Apart from the factors enabling their growth and survival at high temperatures (e.g. protein stability, membrane melting point), as a group they exhibit no unusual features.

Enzyme Stability

An early suggestion that growth and survival at high temperatures were achieved by rapid enzyme turnover rather than stability[2,3] has now been discredited, and it has been shown that enzymes isolated from extreme thermophiles are in general stable at the optimum growth temperature of the organism even in the purified state[4]. A number of hypotheses have been advanced to account for such stability. One of the first thermostable proteins characterised, an α-amylase from *Bacillus stearothermophilus*, was found to have a low molecular weight and an unusual tertiary structure[5,6]. This led to predictions[7] that thermostability might be a product of small rigid molecular structures, where conformational flexibility was sacrificed for stability. Other hypotheses, based on increased disulphide bonding[8], hydrogen bonding[9], hydrophobic interactions[10], or salt bridging (ionic interactions)[11] being responsible for thermophilic stability, have since been proposed. The fact that many thermophilic enzymes, like their mesophilic counterparts, demonstrate allosterism suggests that molecular flexibility is not seriously curtailed[4]. Nor has any general reduction in the molecular size of thermophilic enzymes been observed[4]. Several detailed comparative studies of

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similar proteins from mesophiles and thermophiles have failed to find any consistent changes in amino acid composition, and in particular, no bias towards amino acid residues responsible for any one type of intramolecular bonding[12,13]. (This work is reviewed in refs 1, 12, 14-16).

Current evidence suggests that, since the net free energy of stabilisation of proteins is small, (the result of a delicate balance between the large destabilising forces due mainly to chain entropy and the large stabilising forces due mainly to hydrophobic interactions) one or more quite small changes in protein structure may result in large changes in thermostability[17]. For example, it has been shown that the substitution of a histidine for the arginine 96 of lysozyme from bacteriophage T4 lowers the melting temperature of the enzyme by 14°C[18]. A study of the X-ray electron density map of the enzyme at 0.24nm resolution showed that, apart from this substitution, no detectable changes in the tertiary structure had taken place.

Enzymes in Industry

There are a number of advantages in using enzymes as industrial catalysts. Firstly, they are usually highly specific, and the undesirable side-products sometimes encountered in chemically catalysed reactions are rarely a problem. Effluent from processes using enzymes is usually less toxic than that from alternative processes. Enzymes are generally more active and more efficient than chemical catalysts. Whereas conversion by chemical means will often be limited by the restraints of the equilibria laws, high levels of conversion can result from catalysis by enzymes with high substrate affinities.

The industrial conversion of glucose to fructose[19] provides an example of some of the advantages of enzymic over chemical catalysis. Alkaline conversion at high temperatures results in side reactions which form quantities of materials such as the ketohexose, psicose, and objectionable coloured by-products (which are costly to remove[20]). Consequently, chemical conversion of glucose to fructose has not been economically viable. Progress in enzyme isolation and characterisation since the late 1950's has yielded a number of glucose isomerase enzymes (GI's) from various micro-organisms. The more suitable of the GI's are capable of a 50% conversion of glucose to fructose in 95% glucose syrups, with little production of undesirable by-products. (Commercial processes such as this, however, utilise vast quantities of enzyme: 17-20 tons of immobilised GI consumed per 10,000 tons of 42% fructose syrup produced — one month's production[21]).

These advantages are reflected in the diversity and rapid growth of the enzyme industry. Enzymes are used in the food and beverage industry (a market currently worth nearly $100 million per year), in detergents, and in medical diagnostics. Growth of the industry as a whole is probably 10-15% per year, but as new processes are developed this could rise dramatically. As an example, the development of processes for producing high-fructose corn syrup from starch, using the enzymes amylase, amyloglucosidase, and glucose isomerase, was largely responsible for the sales of these enzymes in the U.S.A. rising from $26 million (36% of the U.S. enzyme market) in 1975 to about $76 million (55% of the U.S. enzyme market) in 1980. The most significant new developments are likely to be in the field of clinical diagnostic enzymes, in cellulose hydrolysis, and in the immobilisation of enzymes. In diagnostics, the absolute specificity of many enzymes is utilised to measure the concentration in biological fluids of important metabolites such as glucose, urea, cholesterol, and of other enzymes. One clinical kit used for assaying the level of creatine kinase, a muscle enzyme whose blood level rises after a heart attack, utilises two other enzymes, hexokinase and glucose-6-phospate dehydrogenase, so that the end product is $NADPH_2$, which can conveniently be measured with an ultraviolet spectrophotometer.

$$\text{ADP + Creatine Phosphate} \xrightarrow[\text{Creatine Kinase}]{} \text{Creatine + ATP}$$

$$\text{ATP + Glucose} \xrightarrow[\text{Hexokinase}]{} \text{ADP + Glucose-6-phosphate}$$

$$\text{Glucose-6-phosphate + NADP+} \xrightarrow[\text{Glucose-6-phosphate Dehydrogenase}]{} \text{6-phosphogluconate + NADPH + H+}$$

The kit contains the two enzymes, ADP, creatine phosphate, glucose and NADP and the rate of formation of $NADPH_2$ is proportional to the creatine kinase activity in the blood sample assayed. Several clinical kits and analytical systems utilise the almost complete specificity of glucose oxidase for D-glucose.

The second important growth area centres on the enzymic hydrolysis of cellulose to glucose. The exciting commercial feature of this research is the low, or even negative cost of the starting material (in the sense that the material may be a waste product which is currently costing money to dispose of), its ready availability, and its renewable nature. A major end use of such glucose might be ethanol or single cell protein production. However, although enzymes which digest cellulose have been much investigated, so far no cost-effective process has been developed.

The recent development of enzyme immobilisation technology (the linking of enzymes to insoluble, often inorganic, support matrices) is likely to further widen the application of enzymes to industrial catalytic processes. Immobilisation often increases the stability of the enzyme, and may modify the properties of the enzyme in other ways, such as shifting the pH optimum. Immobilised enzyme preparations are suitable for recovery and reuse in batch processes, and have permitted the development of highly efficient continuous-flow reactor systems.

But although enzymes have many advantages, those derived from mesophilic organisms are generally unstable, as illustrated by the consumption figures for glucose isomerase, and expensive. The cost of commercial enzyme production arises from low yields of enzyme per unit of biomass, the expense of extraction and purification, and by losses of active enzyme during purification, storage and handling. Running costs are high because of the short lifetime of most enzymes. Some improvement in the yields of many enzymes has resulted from the work of geneticists in isolating high-producing mutant organisms, and more recently from genetic engineering. In addition, the stability of enzymes can often be improved by chemical manipulations and in particular by immobilisation. However, it is in the area of enzyme stability that thermophilic (and more particularly extremely thermophilic) micro-organisms can provide a significant improvement to existing biotechnology.

Advantages Of Enzymes From Extreme Thermophiles

Some enzymes from mesophilic sources exhibit extraordinary thermostability, but as might be expected, as a class enzymes from extremely thermophilic bacteria are more thermostable than those from thermophiles, which are in turn more thermostable than those from mesophiles*The differences in thermostability are often so large that it is difficult to obtain strictly comparable data since for convenience enzyme thermostabilities are usually measured at temperatures which give half lives of 5-200 minutes: but the half-life of a typical enzyme from a mesophile will be of the order of seconds at 75°C, while the half-life of an enzyme from an extreme thermophile may be of the order of days. It would be much easier to compare data if the thermostabilities were expressed as the temperature at which an enzyme had a given half-life, say one hour, but of course this value would be much less convenient to obtain.

*** Table 1:**

Thermal stability of enzymes from mesophiles, thermophiles, and extreme thermophiles.

		Half-life (Hours)		
	Temperature (°C)	Mesophile	Thermophile	Extreme Thermophile
Asparaginase	55°C	0.3 (a)	1.4 (b)	-
	75°C	0.3 (d)	-	70 (c)
α-Amylase	90°C	0.005 (e)	0.4 (b)	0.3 (f)
β-Galactosidase	50°C	0.3 (g)	>3 (h)	-
	65°C	-	-	>> 750 (i)
	80°C	-	-	> 20 (i)
Protease	60°C	0.25 (i)	-	-
	75°C	-	1 (j)	> 150 (c)
	85°C	-	0.2 (k)	5 (c)

Organisms and growth temperatures:

(a) *Bacillus coagulans* (37°C);[23]
(b) *B. stearothermophilus* (55°C)[23]
(c) *Thermus* T-351 (75°C)[24 25]
(d) *Escherichia* coli (37°C)[24]
(e) *B. subtilis* (37°C)[23]
(f) *B. caldolyticus* (72°C)[26]
(g) *Neurospora crassa* (25°C)[27]
(h) *Mucor pusillus* (25°C)[28]
(i) *B. subtilis* (37°C)[29]
(j) *Malbreachea pulchella* (45°C)[30]
(k) *B. thermoproteolyticus* (55°C)[31]
(l) Thermophile *4-1A* (75°C)[33]

Arising from this thermostability, and from other factors, enzymes derived from extremely thermophilic bacteria have a number of economic advantages:

1. The large scale growth of thermophilic bacteria is likely to be cheaper than that of mesophilic bacteria. Part of this is as a result of a reduced capital outlay, since the heat exchange/refrigeration equipment required for the cooling of mesophilic cultures will not be needed. (The additional costs of insulation, and heating and heat recovery equipment are not likely to outweigh this saving). Furthermore much of the cost of mesophile growth vessels is associated with preventing or reducing microbial contamination. Since such contamination is not a problem above 70°C, much simpler and cheaper vessels can be used to grow thermophilic bacteria.

Running costs may also be lower, since refrigeration is unlikely to be required, and most of the heat needed can be supplied by the exothermic growth of the organism, and heat recovery. Procedures for the sterilisation of the vessel and of the growth media can be less strict, or possibly even omitted completely for growth at 75°C or above.

2. There is evidence that higher yields of purified enzymes can be obtained from isolation procedures when thermophilic micro-organisms are used as the source[22 23]. This is a function of the greater stability of their enzymes.
3. Being more stable, thermophilic enzymes have a longer useful life in industrial enzyme reactors than their mesophilic equivalents, increasing their cost-effectiveness.
4. Reactors using thermophilic enzymes can be operated at temperatures sufficiently high to prevent microbial contamination. This would permit reactors to operate for longer periods between sanitising procedures, and without the necessity of presterilisation or any addition of antimicrobial agents.
5. Our work suggests that some enzymes from extremely thermophilic bacteria are resistant to the denaturing effects of detergents and of organic solvents, compared with those from mesophiles. For example we have observed that the apparent activity of a proteolytic enzyme from an extreme thermophile increases tenfold in the presence of low concentrations of detergent because of an effect on the casein substrate, but that mesophilic proteases are denatured and inactivated by the detergent[32]. This stability could minimise losses during cleaning of reactors utilising immobilised enzymes. It might also enable the use of mixed solvent systems to improve the solubility of substrates or products.
6. In high-temperature bioreactors, volatilisation of reaction end products could occur. This could be valuable either as a recovery step or as a means of removing a potentially inhibitory end-product.
7. The activity of enzymes from extremely thermophilic bacteria at ambient temperature is usually less than 2% of that at the optimum temperature. Reactions can be easily stopped simply by cooling.
8. There are a number of general advantages in operating industrial processes at higher temperatures. Viscosity is reduced, solubilities are higher, diffusion is accelerated, and less effort need be taken to dissipate the waste heat inevitably generated during industrial processes.

Many of the advantages referred to here for thermophilic enzymes, and some additional ones, apply to the use of whole extremely thermophilic bacteria to replace mesophilic microorganisms in existing processes. The advantages of an ethanol fermentation carried out near the boiling point of ethanol are fairly obvious for example. Extreme thermophiles are of course non-pathogenic, since by definition they will not grow at body temperatures.

All the techniques, such as immobilisation and genetic engineering, currently being developed to enhance the usefulness of enzymes from mesophiles are also applicable to enzymes from extreme thermophiles. We have been able, for example, to further increase the stability and to modify the properties of an extracellular protease and an intracellular asparaginase from an extreme thermophile by immobilisation (Table 2). Because of the impossibility of extreme thermophiles being pathogenic, the use of recombinant DNA techniques carried none of the potential hazards normally associated with these.

Table 2:

Effect of Immobilisation on Enzymes from *Thermus* T-351.

	D-asparaginase[24]		Protease[32]	
Property	Free Enzyme	Immobilised to Sepharose 4B	Free Enzyme	Immobilised to Sepharose 4B
$t_{\frac{1}{2}}$ (minutes) at 85°C	30	600	360	1060
$t_{\frac{1}{2}}$ (minutes) at 95°C	-	-	28	125
pH optimum	No change		7.0	6.0
K_m (mM)	20.0	28.5	No change	
Substrate inhibition	-	-	Present	Absent

Furthermore, we have no evidence that the metabolic activities of extremely thermophilic bacteria are in any way limited, or that any enzyme currently found in a mesophilic bacterium will not also be available from an extreme thermophile.

Conclusion

There are few areas where the use of enzymes from extreme thermophiles will not be cheaper and more efficient. We believe that not only will they eventually replace most enzymes from mesophiles used in existing processes, but that their advantages will greatly widen and increase the industrial use of enzymes.

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