Extracted from "Biologist", The Journal of the Institute of Biology Vol 53 No 1, February 2006-03-07

Indigo reduction in the woad vat: a medieval biotechnology revealed

Indigo, now best known as the dye responsible for the blue of denim jeans, has a long history. In the Middle Ages, cloth was dyed in a fermenting woad vat, which dissolved the indigo by reducing it to a soluble form. We showed that the basis of this medieval biotechnology was the activity of clostridia capable of reducing indigo, spores of which were revived from a 1000year-old Viking woad vat. How the bacteria reduce the insoluble indigo is not yet known.

Indigo has been one of man's favourite dyes since prehistoric times, and remains one of the most important textile dyes, largely due to the enduring popularity of denim jeans (Balfour-Paul, 1998). Before synthetic indigo became available a century ago, the dye was obtained from a variety of plants, with wad (*Isatis tinctoria* L.) being the major source outside the tropics (Hurry, 1930).

The woad vat

Indigo is insoluble, and to be used as a dye it has to be reduced to a soluble form, the colourless *leuco*-indigo. In the dyeing industry, alkaline sodium dithionite (also known as sodium metabisulphite) is used as the reductant. But before the introduction of chemical methods, indigo was reduced in a woad vat, an anaerobic fermentation of treated woad leaves. These provided both the source of indigo and a substrate for the fermentation. Even when indigo was imported from the tropics and replaced woad as a source of the dye for the European dyeing industry (from the 17th century onwards), the woad vat continued to be used as a means of reducing, and thus dissolving, the indigo.

Towards the end of the 19th century, however, the introduction of more reliable and controllable chemical reduction methods caused the woad vat to fall out of commercial use, and to become an extinct biotechnology. Its neglect by commerce was matched by a neglect from scientists, so that when we started our research about 10 years ago, virtually nothing was known of the chemistry or microbiology involved in its operation.

The woad plant is a biennial member of the brassica family. It is sown in the spring year; these are harvested during the summer. In the Middle Ages, harvested woad was prepared for the woad vat by a process that varied only in minor ways over a wide geographic range: the basic process may well have dated back to prehistoric times, one of the earliest records coming from an 8th century Irish law-text (Kelly, 1997).

As soon as possible after harvest, the woad leaves were crushed (the larger dyeing operations using a horse-driven mill) and the resulting paste was kneaded into woad balls about 10 cm in diameter, which were air-dried (Figure 1). The dried woad balls could be stored indefinitely, and dried woad balls were sufficiently stable for them to be shipped around Europe's coasts from the centres of production Before woad from a woad ball could be used for dyeing it had to be *couched* (Hurry, 1930). In this process, the crushed woad ball material was arranged in heaps, which were watered sufficiently to encourage an intense aerobic microbiall activity. The heat generated within the heaps was controlled, by careful turning of the heaps and judicious watering, in order to maintain a temperature from 60° to 80°C for about two weeks, after, which time the couched woad was allowed to dry. The couched woad was now ready for the woad vat. Hot water was added, along with bran, and an alkali in the form of wood ash or slaked lime. The vat was maintained at about 50°C by heating. During the initial stages of fermentation, aerobic bacteria exhaust the oxygen and the vat enters an anaerobic phase which allows a vigorous fermentation to develop.

Woad leaves are of course green; they contain no indigo. Instead, the indigo is formed from indoxyl precursors in the, leaves during the subsequent processing. In woad, the principal precursor was long thought to be indoxyl *keto*-gluconate (isatan B) with lesser amounts of indoxyl glucoside (indican). More recently, however, evidence has been obtained that there may also be other indoxyl compounds in woad leaves (Maugard et al, 2001; Oberthür et al, 2004). Whatever the actual precursors may be, when we reproduced the medieval process we found that conversion to indigo occurred in the formation of the woad balls; there were no indoxyl compounds present once the woad balls had dried (Kokubun *et al*, 1998). Nobody has studied the couching process, but it seems likely that during couching, cellulose and other complex plant polymers are broken down, and this allows the indigo to be more readily liberated in the woad vat.

About eight days after setting up the woad vat, we observed a vigorous and spontaneous fermentation that led to an evolution of gases, which we identified as carbon dioxide and hydrogen, and to the rapid acidification of the medium. This had to be neutralised by addition of alkali to allow fermentation to continue (Padden *et al*, 1998). At the same time, the indigo released from the couched woad could be seen to dissolve, with the vat turning a greenish-brown, and a blue layer of oxidised indigo being apparent on the surface.

When synthetic indigo was added, it dissolved rapidly in the vat. The woad vat we set up in the laboratory had a noxious odour, for which the woad vat was notorious even in medieval times (Hurry, 1930). The organic headspace volatiles, analysed by Gas Chromatography/ Mass Spectrometry, were found to be predominantly methyl sulphides (Padden *et al*, 1998), which are characteristic of the anaerobic breakdown of brassica materials.

The midpoint potential of indigo is difficult to determine because of the insolubility of the oxidised form in water, but an indicative value of -474 mV vs SCE has been given (Vickerstaff, 1954), and in industrial practice a potential of -600 mV is regarded as a minimum for reduction. Initially, we considered whether the hydrogen produced might be the reductant, but we soon realised that hydrogen does not readily reduce indigo. Instead, we found that plating out

bacteria on nutrient agar containing indigo under anaerobic conditions gave us an unequivocal indication of isolates capable of reducing indigo (Padden *et al*, 1998). After incubation under anaerobic conditions, the agar plate became clear with the disappearance of the suspended indigo particles. Then, when oxygen was reintroduced to the culture, it turned blue due to the dissolved *leuco*-indigo being re-oxidised to a fine suspension of blue indigo.

The isolates that reduce indigo were rod-shaped bacteria, with oval terminal endospores, and scored negative for catalase. These are all characteristics of clostridia. We found these isolates to be a new species, related to C. *carnis* (Padden *et al*, 1999) using 16S rRNA gene sequence analysis. From its origin in the woad vat, we named the indigo-reducing bacterium C. isatidis.

The growth requirements of C isatidis matched those specified by the woad vat 'operating instructions' indicated by medieval manuscripts. But of course this is not surprising since we had isolated C. *isatidis* from such a recreated woad vat. However it became clear that the operators of the woad vat in a pre-scientific age without an understanding of bacterial fermentation, redox or pH, ran the vat in such a way as to optimise indigo reduction. For example, *C. isatidis* grows optimally at pH 7.0-7.5, while indigo reduction requires a pH above pH 9: thus the woad vat was allowed to acidify naturally from the acids produced by anaerobic fermentation, and then the pH was increased by the periodic addition of woad ash or other source of alkali. In adding these materials, care would have been necessary to allow a period near a neutral pH for bacterial growth, as well as a more alkaline pH for indigo reduction. Again, C. *isatidis* is a moderate thermophile, with a growth optimum at around 50T, which was the sort of temperature at which the woad vat was maintained.

On the basis that we could see indigo reduction by pure catures of C. *isatidis* (Figure 2), we concluded that *C. isatidis* was the organism that enabled indigo to be used as a dye in an age before chemical reductants were available. However, the question arose as to the uniqueness of the role of C. *isatidis:* was it the organism that actually reduced indigo in the woad vats centuries ago, or was it fortuitously enriched by us?

In resolving this question, luck was on our side. Archaeologists had discovered material they interpreted as dyebath waste from 10th century Anglo-Scandinavian (Viking) occupation deposits in York. The woad material, identified by anatomical

features of cell structure, had survived under conditions of anoxic waterlogging, Aseptic isolation of material from the centre of the archaeological material yielded isolates that we identified as C. *isatidis* by 16S rRNA sequencing (Padden *et al,* 2000). Thus we feel confident that *C isatidis* had a unique role in the traditional woad vat.

Madder

The medieval recipes for setting up a woad vat invariably called for the addition of madder (Hurry, 1930), a dried powder obtained by grinding the roots of *Rubia tinctorum,* and much used in the past as a red dye. Madder is rich in anthroquinones, notably alizirin. In pure culture *C. isatidis* readily reduced indigo without the addition of madder, but did madder have any effect on indigo reduction by pure cultures?

Recently, we found that madder (Figure 2), like the soluble anthroquinone-2,6disulphonate or preparations of humic: acid containing quinines, stimulates indigo reduction by pure cultures of *C. isatidis* (Nicholson and John, 2004; 2005). Anthroquinones have been shown to stimulate the electrochemical reduction of indigo (Bechtold *et al,* 1999). Quinones in general can act as mediators in bacterial respiration where insoluble electron acceptors such as Fe (Ill) are used. However, their redox potentials appear to be too positive for them to act in this way in indigo reduction, and how they stimulate indigo reduction by C. *isatidis* remains unknown.

In order to identify the features of C. *isatidis* that allow it to reduce indigo, we have carried out a comparative study using three related clostridia: C. aurantibutyricum; C. celatum; and C. papyrosol*vens. None* of these three species reduces indigo, but C. *papyrosolvens,* like C. isatidis, can reduce indigo carmine, a soluble derivative of indigo, and it shows a limited ability to reduce indigo when anthroquinone-2,6-disulphonate is present (Nicholson and John, 2005). We have also seen that the supernatant of the C. isatidis culture, unlike that of the other bacteria, is able to decrease the size of indigo particles from 35 µm to 3 µm. Thus we have concluded that C. isatidis uniquely reduces indigo because it can both decrease indigo particle size and generate a sufficiently reducing potential (Nicholson and John, 2005).

Direct or indirect?

A question we have been asking for some time is whether indigo is reduced by direct contact with the bacteria or indirectly via a redox mediator. When C. isatidis cells were allowed to colonise a basal plane pyrolytic graphite electrode, evidence was obtained which was interpreted as the ability of bacteria to couple directly to the insoluble electrode surface (Compton et al, 2000). However, for a Gram-positive bacterium such as C. isatidis there is no biochemical mechanism available to account for a transfer of electrons from the interior of the cell to the solid external electron acceptor.

While it is clear that C. isatidis plays the predominant role in indigo reduction in the woad vat, there are other bacterial species present, and in order to understand better the ecology and functioning of the woad vat we have identified the other species present, using 16S rRNA gene sequence analysis (Lawson et al, in press). The three most common species were: Geobacillus (Bacillus) pallidus; Urefflacillus (Bacillus) *thermosphaericus; and Bacillus thermoamylovorans.* G. pallidus and U *thermosphaericus are* moderately thermophilic strict aerobes, the former a characteristic species of hot composts. Thus the populations of these species in the woad vat probably originated from the highly aerobic couching procedure undergone by the woad material before its inclusion in the woad vat. *B.*

thermoamylovorans, on the other hand, is a facultative anaerobe but again moderately thermophilic and populations probably grow up in the woad vat during. the initial aerobic phase. Being a facultative anaerobic, it can continue to grow into the anaerobic phase. Here it could help to ' consume whatever oxygen may have entered the vat. This would be essential to the growth of the strictly anaerobic C. *isatidis,* and be essential to prevent re-oxidation of the leuco-indigo.

The future

The chemical reduction of indigo for denim dyeing results in the annual production of thousands of tonnes of sulphur waste. Bacterial reduction of indigo is unlikely to replace this process, as it not sufficiently fast or controllable to meet modern production demands. Instead, as a more sustainable alternative to chemical methods of indigo reduction, electrochemical methods are being investigated. However electrochemistry is limited - compared with solution chemistry - by the relatively poor contact between the electrodes and the dispersed indigo particles, as well as the low current efficiency (Roessler and Jin, 2003). In our present work we are investigating whether aspects of bacterial indigo reduction can help enhance the electrochemical technology: in effect harnessing the biology of the medieval woad vat for a cleaner technology for the future.

Figure 2. Reduction of indigo in an anaerobic fermenter by a pure culture of Clostridium isatidis. The medium clarifies as the insoluble indigo is reduced by the bacteria. Traces of blue unreduced indigo are evident around the stirrer and suspended above the medium.

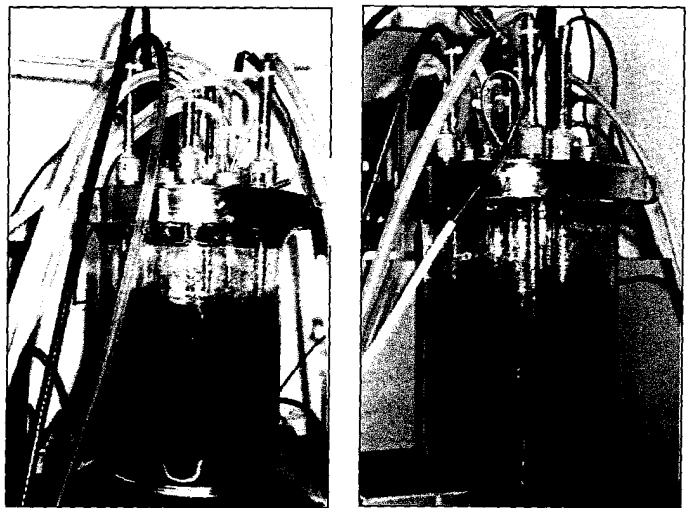

Figure 3. Addition of madder stimulates indigo reduction by Clostildum isatidis. The anaerobic cultures containing 0.01% indigo suspension were incubated for 5 days at 501C, in the absence

(controls) and presence of madder added at 0.3 mg.1-1 (top) and 1 mg.1-1 (bottom). The cultures cleared as the indigo was reduced to feuco-indigo (See Nicholson and John, 2004).