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BIOTECHNOLOGY METHODS

Implementation of a biotechnological process for vat dyeing with woad

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Abstract The traditional process for vat dyeing with woad (*Isatis tinctoria* L.) basically relies on microbial reduction of indigo to its soluble form, leucoindigo, through a complex fermentative process. In the 19th century, cultivation of woad went into decline and use of synthetic indigo dye and chemical reduction agents was established, with a consequent negative impact on the environment due to the release of polluting wastewaters by the synthetic dyeing industry. Recently, the ever-growing demand for environmentally friendly dyeing technologies has led to renewed interest in ecological textile traditions. In this context, this study aims at developing an environmentally friendly biotechnological process for vat dyeing with woad to replace use of polluting chemical reduction agents. Two simple broth media, containing yeast extract or corn steep liquor (CSL), were comparatively evaluated for their capacity to sustain the growth and reducing activity of the strain *Clostridium isatidis* DSM 15098^T. Subsequently, the dyeing capacity of the CSL medium added with 140 g L^{-1} of woad powder, providing 2.4 g L^{-1} of indigo dye, was evaluated after fermentation in laboratory bioreactors under anaerobic or microaerophilic conditions. In all fermentations, a sufficiently negative oxidation/reduction potential for reduction of indigo was reached as early as 24 h and maintained up to the end of the monitoring period. However, clearly faster indigo dye reduction was seen in the broth cultures fermented under strict anaerobiosis, thus suggesting the suitability of the N_2 flushing strategy for enhancement of bacterial-driven indigo reduction.

Keywords Indigo · Dye-yielding plants · *Isatis tinctoria* L. · *Clostridium isatidis* · Batch fermentation

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Introduction

Indigo is one of the oldest dyes, used since the Neolithic era [8], and today is still among the industrial chemicals with the highest annual world consumption [4]. Throughout history, indigo has been obtained from a variety of plants from different geographical areas [2, 4, 25]. In Europe, *Isatis tinctoria* L., commonly referred to as woad, was the only source of blue dye from the Middle Ages until the end of the 17th century, when pigment from the tropics flooded the dye market and cultivation of woad rapidly went into decline [4]. The traditional method for dyeing with this dye-yielding plant basically relies on use of dye vats filled with limewater and couched (treated) woad [4]. During the couching of woad leaves, the colourless and water-soluble precursors to indigo, namely indican, isatan B and isatan A, are hydrolysed to indoxyl, and the latter is oxidised to indigotin by exposure to air [4]. Indigotin (commonly referred to as indigo dye) is practically insoluble in water, with no affinity at all for cellulosic or wool fibres [4], but in the presence of a reducing agent it undergoes a two-step reduction to its soluble form, leucoindigo. In the dye vat, leucoindigo is absorbed by the fabric fibres and is oxidised to the original insoluble form as the fabric is removed from the vat and exposed to air [4]. At this point the oxidised

insoluble indigo remains entrapped within the structure of the fibre, which explains the fast dyeing of cellulosic and wool fibres.

Since 1897, natural indigo has been replaced with synthetic dyes, and traditional methods have been abandoned in favour of the adoption of chemical reductants such as sodium dithionite ($Na_2S_2O_4$), in a process known as vatting. The toxic oxidised products of this chemical reduction, namely sodium sulphate (Na_2SO_4), sulphite ions (SO_3^{2-}) and thiosulphate ions ($S_2O_3^{2-}$), are the main pollutants in the wastewaters of modern dyeing industry [3, 5]. Considerable amounts of residual reducing agent may be also found in these wastewaters, with serious detrimental effects on the environment [5]. For these reasons, in the last decades, attempts have been made to replace the use of chemical reduction agents with more environmentally friendly alternatives, such as up-graded chemical approaches, electrochemical reduction techniques, biodegradable organic compound-based methods, ultrasonic energy, magnetic fields, ultraviolet (UV) light and microbial enzymes [5]. However, after years of research, no systems that use a biological approach to replace chemical reductants in vat dyeing are currently exploited by modern dye industry.

In 1999, an anaerobic moderate thermophilic bacterium (originally coded as Wv6^T and further deposited at various culture collections as CIP 107118^T, DSM 15098^T, NCFB 3071^T and NCIMB 703071^T) that is able to reduce indigo to its soluble form was isolated from a fermenting woad vat and proposed as the new species *Clostridium isatidis* [18], whose main features are summarised as follows: production of terminal endospores; fermentation of different carbohydrates, including either monosaccharides (fructose, glucose, galactose, glucose, maltose etc.) or starch; production of acetic, lactic and formic acids, ethanol, carbon dioxide and hydrogen in growth media containing glucose, peptone and yeast extract [19]. The uniqueness of the role of this microorganism in the vat dyeing process with either couched woad or other indigo-yielding plants, such as *Isatidis indigotica* (commonly referred to as Chinese woad), was further demonstrated by the constant isolation of the same species from a variety of sources, including a 10th century Viking dye vat [20]. Soon afterwards the bacterial mechanism for indigo reduction was elucidated [9, 17] and the main bacterial species involved in the fermentation of traditional vat woad were identified. They included both thermophilic, aerobic bacilli, such as *Geobacillus palidus*, *Ureibacillus thermosphaericus* and *Bacillus thermoamylovorans*, responsible for oxygen exhaustion through respiration, and fermentative, obligately anaerobic bacteria with indigo-reducing activity. More recently, the microbiota associated with reduction of indigo dye from *Polygonum tinctorium* have been investigated with a culture-dependent and culture-independent approach, and other indigo-reducing bacteria belonging to the genera *Amphibacillus* and *Oceanobacillus* have been isolated [1].

This study aims at developing an environmentally friendly biotechnological process for vat dyeing with woad, by exploiting the sole indigo-reducing activity of the type strain *C. isatidis* DSM 15098^T in 2-L working volume bioreactors under controlled fermentation conditions. To this end, two simple molasses-based media supplemented with either freshly prepared yeast extract (YE) or corn steep liquor (CSL) were preliminarily evaluated for their ability to sustain either the growth or the reducing activity of the strain used under standardised pH and temperature (T) conditions. Thereafter, the maturation of dye baths consisting of CSL broth added with 140 g L^{-1} of woad powder and fermented under either microaerophilic conditions or strict anaerobiosis was evaluated daily through viable counting, measuring of the oxidation/reduction potential (ORP), visual estimation of the level of bacterial-driven indigo reduction and blue dyeing of cotton fabrics and wool yarns. The choice to carry out batch fermentations under microaerophilic conditions in parallel with those under N_2 flushing, despite the anaerobic nature of the strain under study, was driven by the idea to try to develop a biotechnological process as close as possible to the traditional procedure for vat dyeing with woad, which involves use of open wooden vats. The success of such a biotechnological process would have been an advantage for exploitation at industrial level, due to the elimination of the costs associated with N_2 flushing.

Materials and methods

Microorganisms

The strain *C. isatidis* DSM 15098^T was obtained as a freeze-dried culture from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ GmbH, Braunschweig, Germany) and reconstituted on solid chopped meat medium with carbohydrates (DSMZ GmbH) as indicated by the culture supplier. Further subculturing was carried out using Clostridial Nutrient Medium (CNM, Fluka, Milan, Italy) at pH 6.8 ± 0.2 or adjusted to pH 9.0 ± 0.1 with sterile 3.0 M NaOH or KOH solution prior to sterilisation. Discrete colonies, grown overnight on solid chopped meat medium with carbohydrates were withdrawn with a sterile 0.01-mL calibrated loop and either resuspended in 15-mL polypropylene universal tubes with screw caps or streaked onto CNM plates. Agar streak plates were incubated at 37 and 49 °C for 24 and 48 h in polycarbonate jars with an anaerobic gas generating system (Oxoid,

Milan, Italy), whereas tubes were incubated at the same temperatures under either anaerobiosis or aerobiosis. The response of *C. isatidis* DSM 15098^T to the different conditions assayed was determined by visual evaluation of growth, coded as follows: +++, optimal growth; ++, good growth; +, weak growth; ± barely visible growth; −, no growth. The bacterial stock culture was stored at −80 °C in a mixture of glycerol and CNM broth adjusted to pH 9.0 with 3 M KOH, in 1:1 ratio (v v^{-1}).

Batch fermentations

Culture media

Two culture media were comparatively used, both containing 1 g L^{-1} of molasses, which was purchased from local sugar beet refining industry (Eridania Sadam, Jesi, Ancona, Italy) and supplemented with two different sources of nitrogen compounds, salts and vitamins, namely freshly prepared yeast extract (YE) or corn steep liquor (CSL), which is a cheap by-product of corn wet-milling. The YE medium contained 150 mL L^{-1} of yeast extract, which was prepared as follows: a 20 % (w v^{-1}) aqueous suspension of commercial compressed baker's yeast was autoclaved for 15 min at 121 °C, allowed to settle overnight at 4 °C and decanted. The supernatant was further clarified by centrifugation at 6,000*g* for 3 min and used immediately. The CSL medium contained 10 g L^{-1} of commercial corn steep liquor (Sigma-Aldrich, Milan, Italy). For both media, the pH was adjusted to 9.0 ± 0.1 with sterile 3.0 M KOH aqueous solution, prior to sterilisation at 121 °C for 15 min.

Inoculum preparation

Three different conditions for inoculum preparation were preliminarily assayed. The surface of a frozen culture of *C. isatidis* DSM 15098^T was scraped with a sterile loop and streaked onto CNM agar plates (pH 9.0 ± 0.1). A loop of the bacterial patina grown after 24 h of incubation at 49 °C under anaerobic conditions was inoculated into Erlenmeyer flasks sealed with cotton stoppers and containing 100 mL of: (1) CNM broth adjusted to pH 9.0 ± 0.1 with 3.0 M KOH; (2) CNM broth made in 0.2 M glycine-NaOH buffer, pH 9.3 ± 0.1; (3) CNM broth made in 0.1 M Tris–HCl buffer, pH 8.8 ± 0.1. All the media were sterilised at 121 °C for 15 min prior to inoculation. Broth cultures were incubated at 49 °C under anaerobiosis and static conditions; after 1–4, 6, 12 and 24 h of incubation, they were subjected to pH measurement and viable counting of vegetative cells. On the basis of the results obtained, a further procedure for inoculum preparation was evaluated. The bacterial patina grown on two CNM agar plates (pH 9.0 ± 0.1) after incubation at 49 °C for 24 h under anaerobic conditions was harvested with a sterile spatula and suspended in 15 mL sterile saline solution (NaCl 0.9 %, w vol^{-1}). The turbidity of 1-mL aliquots of this bacterial suspension was adjusted with McFarland standard 0.5, 1, 3, 4 and 5. For each cell suspension, the vegetative cells were enumerated on CNM agar (pH 9.0 ± 0.1) incubated at 49 °C for 24 h under anaerobiosis.

Aliquots (20 mL) of the bacterial suspension adjusted to match a 5 McFarland turbidity standard were used as an inoculum; these were transferred into 500-mL glass flasks and then introduced into the 2-L working volume bioreactors containing sterilised CSL or YE broth with peristaltic pumps and sample inlets.

Equipment and culture conditions

All the batch fermentations with YE and CSL broths were carried out in stirred tank BIOSTAT-B reactors (B-Braun, Biotech International, Melsungen, Germany), equipped with a jacketed borosilicate glass vessel, two six-bladed Rushton turbines, four vertical baffles, a stainless-steel temperature sensor, and electrodes for control/measurement of pH and pO_2 (Mettler-Toledo, Novate Milanese, Milan, Italy). Oxidation/reduction potential (ORP) was measured with a Liq-GlassORP electrode (reference system Ag/AgCl) (Hamilton Company, Reno, Nevada, USA). Prior to each measurement, the ORP electrode was rinsed with distilled water and further calibrated with the opportune solution (Hamilton Redox buffer 475 ± 5 mV). Measurement of ORP was performed, avoiding any contamination, using a sterile bioreactor sampling system (B-Braun).

Continuous control of pH (set at 9.0 ± 1.0) was achieved by supplying sterile 3 M KOH aqueous solution with a peristaltic pump, whereas maintenance of temperature at 49 ± 1 °C was ensured with the help of a thermostat and a pump for circulation of heated water through the bioreactor jacket.

The effects of microaerophilic and strict anaerobic conditions on both the growth and the reducing activity of the strain under study were comparatively evaluated by performing two series of batch fermentations. In the first series, the bioreactor was operated without air input, applying slow stirring (15 rev min^{-1}) to keep the fermentation broth homogeneous. In the second series of batch fermentations, sterile oxygen-free N_2 was flushed into the bioreactors; in this case the broth cultures were subjected to the sole pneumatic agitation due to the flow of N_2, which was kept at a constant flow rate of 0.1 volume per volume per minute (VVM) throughout the fermentation process.

Micro-MFCS software (B-Braun, Biotech International GmbH, Melsungen, Germany) for Windows XP was used for monitoring and controlling process parameters during fermentation. Three repetitions for each condition were performed, and the results were expressed as average ± standard deviation.

Vegetative cell and spore counting

Aliquots (10 mL) of YE and CSL broth cultures were withdrawn daily from the bioreactors under aseptic conditions starting from 24 h up to 15 days of fermentation. For enumeration of heat-resistant spores, one aliquot of each sample was preliminarily treated at 80 °C for 15 min and then rapidly cooled on ice to inactivate the vegetative cells and induce spore germination. Vegetative cells and spores were counted on CNM (Fluka) agar (pH 9.0 ± 0.1) incubated at 49 °C for 24 h under anaerobiosis.

Laboratory dyeing trials

Preparation of woad powder

Woad powder was prepared according to the traditional procedure [14] using leaves from indigo plants grown at La Campana Soc. Coop. Agricola (Montefiore dell'Aso, Ascoli Piceno, Italy) in 2010. Leaves from mature woad plants harvested at full rosette expansion were steeped in a vat filled with hot water and maintained at about 50 °C for a period of 2 h 30 min. The aqueous extract was strained off and added with a further aliquot of extract obtained by squeezing the indigo leaves. The resulting liquid mass was roughly filtered, transferred into a new vat, added with potash to reach final pH of 9.5–10, and vigorously stirred for about 3 h to allow intense contact with atmospheric oxygen to be accomplished. Insoluble indigo was allowed to precipitate as a blue mud, which was collected, washed twice with tap water, roughly filtered, dried in an oven at about 85 °C for 72 h and finally ground to obtain a fine deep-blue powder.

Assessment of indigo dye content in woad powder

Synthetic indigo dye having 95 % purity (Sigma-Aldrich, Milan, Italy) was used to build a calibration curve. Five working solutions with concentrations ranging from 1 to 50 mg L^{-1} were prepared by accurately dissolving woad powder in glacial acetic acid at 90 °C for 5 min. The absorbance of the working solutions was measured at 664 nm (A_{664}) using a Shimadzu UV-1800 spectrophotometer (Shimadzu, Milan, Italy). The indigo dye content of the woad powder prepared as described in the previous paragraph was determined after repeatedly washing it in deionised water until clear washing water was obtained. The washed woad powder was dried again in an oven at 80 °C for 24 h, after which a 1-mg aliquot was dissolved in 5 mL glacial acetic acid at 90 °C for 5 min and A_{664} was measured. The indigo dye content was finally determined by referring the obtained value to the calibration curve.

Indigo dye baths

Indigo dye baths were prepared by fermentation, under either microaerophilic or strict anaerobic conditions (as described in "Equipment and culture conditions" section), of the CSL medium adjusted to pH 9.0 ± 0.1 with 3 M KOH and added with 140 g L^{-1} of woad powder prior to sterilisation at 121 °C for 15 min. Dye reduction was monitored daily by visual evaluation over a period of 15 days. Since it is acknowledged that spectrometric determination of dye reduction in broth cultures is not reliable due to interference by oxidised indigo particles in suspension, the variable light scattering from the bacterial growth and the formation of a surface film [11, 17], the extent of indigo reduction was estimated either by visually checking the proportion of the culture cleared of dye or by staining cotton fabrics and wool yarns. Laboratory dyeing trials were performed as follows: aliquots of broth culture (100–150 mL) were transferred from the bioreactor into a 200-mL flask using a sterile syringe under aseptic conditions. Samples of cotton fabrics (5 × 5 cm) and wool yarns (~5 g) were wetted out, carefully washed with the aid of a detergent, then rinsed and gently squeezed. Each sample was dipped in a dye bath while gently shaking the flask to favour contact between the dye and the fibres. After about 15–20 s, the samples were withdrawn, exposed to air for 5 min to fix the indigo and then washed again, rinsed, squeezed and air-dried. The amount of reduced indigo absorbed by the cotton fabrics and re-oxidised after exposure to the air was estimated using the method described by [1] with slight modifications. Briefly, pieces (1.0 × 1.0 cm) of the cotton fabrics were transferred into glass tubes, added with 800 μL glacial acetic acid, and kept at 90 °C for 5 min, to allow the indigo to dissolve completely. The absorbance of the indigo solutions was assessed as described in "Assessment of indigo dye content in woad powder" section, and the amount of oxidised indigo was expressed as mg cm^{-2} of cotton fabric.

Statistical analysis

One-way analysis of variance (ANOVA) was carried out, together with the Tukey–Kramer honestly significant difference (HSD) test, based on three replicates, using JMP software (SAS Institute Inc., Cary, NC, USA) in order to assess differences in: (1) vegetative cell and spore counts

Table 1 Response of *C. isatidis* DSM 15098^T to different growth conditions

Temperature	Incubation	pH 6.8		pH 9.0			
		Aerobiosis	Anaerobiosis	Aerobiosis		Anaerobiosis	
				NaOH	KOH	NaOH	KOH
CNM agar (°C)							
37	24 h	n.d.	±	n.d.	n.d.	+/−	+/−
	48 h	n.d.	+	n.d.	n.d.	+++	+
49	24 h	n.d	+	n.d.	n.d	+	+
	48 h	n.d	++	n.d.	n.d	+++	++
CNM broth (°C)							
37	24 h	–	++	±	±	+++	+++
	48 h	–	++	+++	+++	+++	+++
49	24 h	–	++	+++	+++	+++	+++
	48 h	–	++	+++	+++	+++	+++

CNM Clostridial Nutrient Medium (Oxoid, Milan, Italy)

n.d. not determined; +++, optimal growth; ++, good growth; +, weak growth; ±, barely visible growth; −, no growth

Table 2 Viable counts of bacterial suspensions adjusted to progressive standards of the McFarland scale

McFarland standard	Log cfu mL^{-1}
0.5	3.96 ± 0.20
1	4.45 ± 0.23
3	4.71 ± 0.34
4	4.94 ± 0.28
5	5.38 ± 0.25

Values reported as mean ± standard deviation

and ORP values of YE and CSL broth cultures; (2) viable counts and ORP values of CSL broth cultures added with woad powder and maintained under microaerophilic and strict anaerobic conditions. In all analyses, significance of differences was defined at $P < 0.05$.

Results

Preliminary assessment of culture conditions

The response of *C. isatidis* DSM 15098^T to the different growth conditions assayed is presented in Table 1. When streaked on CNM agar plates, optimal growth was obtained by adjusting the pH of the medium to 9.0 ± 0.1 with 3 M NaOH and by incubating at either 37 or 49 °C for 48 h. In CNM broth, the highest increase in turbidity was detected when the pH of the medium was adjusted to 9.0 ± 0.1 with either NaOH or KOH, and the tubes were incubated at 49 °C for 24 h or 37 °C for 48 h, irrespective of the oxygen availability during incubation. By contrast, a positive impact of incubation under anaerosis was found for growth in CNM broth at pH 6.8 ± 0.2, irrespective of the temperature or duration of incubation.

Inoculum standardisation

As far as inoculum standardisation is concerned, no growth was detected in CNM broth made in either glycine-NaOH or Tris–HCl buffer, while an early but modest increase in viable counting was detected in CNM broth adjusted to pH 9.0 ± 0.1 with 3 M KOH. After 4 h of fermentation, vegetative cells reached 3.60 ± 0.04 Log cfu mL^{-1}, but as early as 2 h afterwards, they became undetectable; at this time point (6 h of fermentation) pH values dropped to below 6.0. Based on these results, an alternative procedure for inoculum standardisation was developed, with the aid of the McFarland turbidity scale. As expected, increasing values of vegetative cells were found in the bacterial suspensions adjusted to progressive standards of the McFarland scale (Table 2), up to a maximum of two orders of magnitude higher than the value obtained in CNM broth.

Comparative evaluation of YE and CSL media

As shown in Table 3, the two media developed in this study were found to sustain both the growth and the reducing activity of *C. isatidis* DSM 15098^T. However, higher or comparable counts of vegetative cells and lower ORP values were reached in CSL broth. This medium was therefore chosen for further batch fermentations with woad powder.

Table 3 Response of *C. isatidis* DSM 15098^T to different subculturing conditions in corn steep liquor (CLS) or yeast extract (YE) after 48 h of incubation

Medium	1 Day of fermentation			2 Days of fermentation			4 Days of fermentation		
	Viable counts (Log cfu mL^{-1})		ORP (mV)	Viable counts (Log cfu mL^{-1})		ORP (mV)	Viable counts (Log cfu mL^{-1})		ORP (mV)
	Vegetative cells	Spores		Vegetative cells	Spores		Vegetative cells	Spores	
CSL broth	6.74 ± 0.53 (a)	3.61 ± 0.61 (a)	−663.50 ± 14.85 (a)	7.02 ± 0.33 (a)	4.77 ± 0.18 (a)	−473.50 ± 41.72 (a)	6.61 ± 0.38 (a)	5.03 ± 0.07 (a)	−558.00 ± 65.05 (a)
YE broth	5.79 ± 0.09 (b)	3.31 ± 0.12 (a)	−472.50 ± 10.61 (b)	5.98 ± 0.14 (b)	3.96 ± 0.17 (b)	−496.00 ± 19.80 (a)	5.78 ± 0.15 (b)	3.85 ± 0.19 (b)	−512.00 ± 4.24 (a)

Within each column, different letters in parenthesis refer to significantly different values ($P < 0.05$) as determined by one-way analysis of variance (ANOVA)

ORP oxidation/reduction potential

Batch fermentations with CSL broth added with woad powder

By fitting a linear line to the indigo dye calibration curve, a linear equation $y = 101.1x - 0.0159$ with regression coefficient of $R^2 = 0.9934$ was obtained (Fig. 1). With reference to this equation, the indigo dye content of the woad powder, prepared as described in "Preparation of woad powder" section, was estimated as 3.4 % (w w^{-1}). On the basis of this result, a woad powder concentration of 140 g L^{-1}, providing 4.8 g of indigo dye (corresponding to 2.4 g L^{-1}), was used.

Figures 2 and 3 show the viable counts and ORP values determined during the fermentation of CSL broth added with woad powder under either microaerophilic or strict anaerobic conditions. The two graphs indicate a steep drop in ORP in both cases; values notably lower than −470 mV but always higher than −600 mV versus Ag/AgCl were reached at as early as 24 h of fermentation and maintained up to the end of the monitoring period (9 days). A similar pattern was seen for the viable counts of vegetative cells when batch fermentations carried out under microaerophilic and strict anaerobic conditions were compared. In the first case, the highest values (7.60 ± 0.16 Log cfu mL^{-1}) were reached after 3 days of fermentation; thereafter, a progressive decrease was seen up to day 7, when viable counts reached 4.63 ± 0.46 Log cfu mL^{-1} and remained almost stable until the end of the monitoring period, when 4.59 ± 0.02 Log cfu mL^{-1} were counted. When sterile oxygen-free N_2 was flushed into the culture medium, the maximum number of vegetative cells (7.80 ± 0.21 Log cfu mL^{-1}) was reached at as early as 2 days of fermentation, but a progressive decrease was again recorded up to day 7, when 6.29 ± 0.36 Log cfu mL^{-1} were reached and almost stably maintained up to day 9, when 6.26 ± 0.12 Log cfu mL^{-1} were counted.

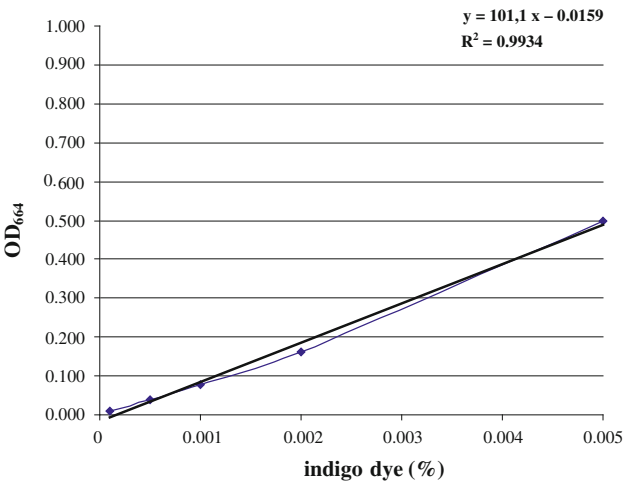

Fig. 1 Indigo dye calibration curve

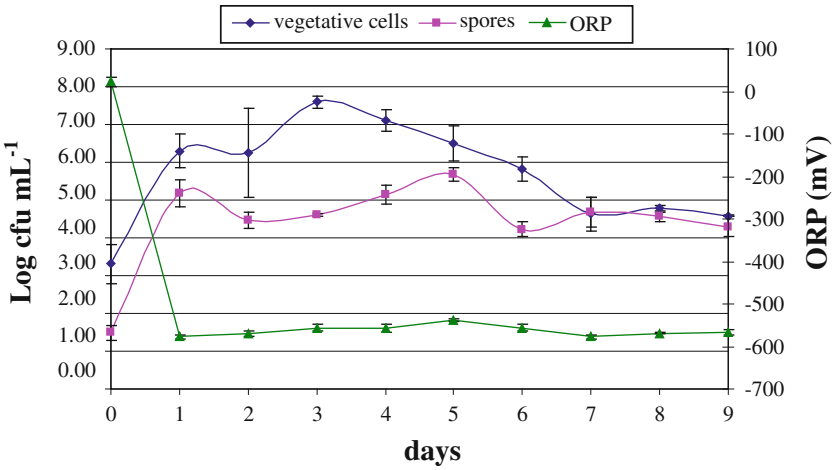

Fig. 2 Viable counts of vegetative cells (*diamonds*), thermo-resistant spores (*squares*) and oxidation/reduction potential (ORP) values (versus Ag/AgCl) (*triangles*) monitored during batch fermentation under microaerophilic conditions in corn steep liquor (CSL) broth added with 140 g L^{-1} of woad powder sterilised at 121 °C for 15 min

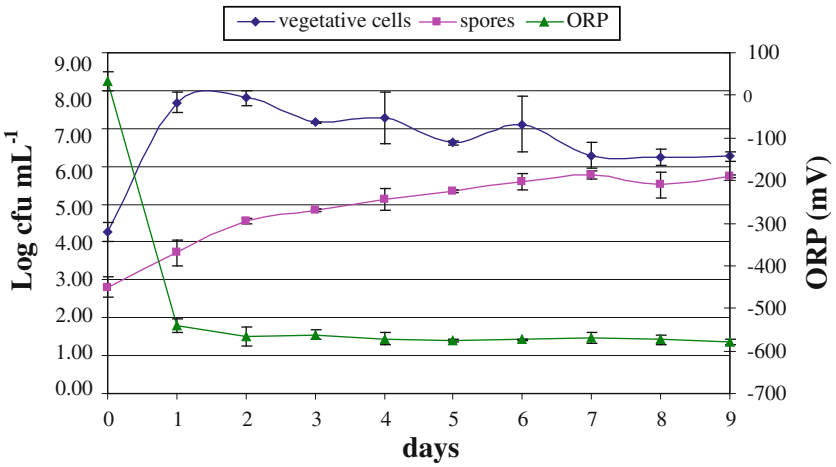

Fig. 3 Viable counts of vegetative cells (*diamonds*), thermo-resistant spores (*squares*) and oxidation/reduction potential (ORP) values (*triangles*) monitored during batch fermentation under strict anaerobic conditions in corn steep liquor (CSL) broth added with 140 g L^{-1} of woad powder sterilised at 121 °C for 15 min

A different trend was observed when viable counts of thermo-resistant spores produced under microaerophilic and strict anaerobic conditions were comparatively evaluated. In the first case, an up-and-down pattern was found from day 0 to day 7, when the spores reached a value of 4.68 ± 0.39 Log cfu mL^{-1}, which remained almost stable until day 9 (4.30 ± 0.27 Log cfu mL^{-1}). On the contrary, a continuous increase from 2.81 ± 0.27 Log cfu mL^{-1} at day 0 to 5.74 ± 0.02 Log cfu mL^{-1} at day 9 was recorded under strict anaerobic conditions.

Laboratory dyeing trials

The daily monitoring of indigo reduction during the fermentation of CSL broth added with woad powder allowed the different behaviour of the broth cultures fermented under microaerophilic or strict anaerobic conditions to be observed. In the former case, a pronounced change in the reduction intensity, revealed by the broth becoming a green-yellowish colour, was seen from day 14 to day 15, whereas in the broth continuously flushed with sterile oxygen-free N_2, this change occurred much earlier, namely between day 2 and day 3. In both cases, indigo reduction was associated with the formation of the typical coppery surface scum of oxidised indigo. These preliminary evaluations were confirmed by the results of laboratory dyeing trials. In fact, the cotton fabrics and wool yarns immersed in the broth cultures fermented under strict anaerobiosis began to be clearly stained light blue on day 3 (data not shown), whereas those dyed in the broth cultures under microaerophilic conditions became a barely visible light-blue colour only on day 15. As expected, the fabrics took on progressively more intense shades of blue when subjected to further dips in the broth cultures flushed with N_2 from day 4 to day 15.

Figure 4 shows wool yarns (panels a, c) and cotton fabrics (panels b, d) subjected to single dips in CSL broth after 15 days of fermentation under microaerophilic (panels a, b) or strict anaerobic (panels c, d) conditions. The amount of indigo trapped in the matrix of the cotton fibres, quantified by spectrophotometric analysis and expressed as mg cm^{-2}, is reported next to each dyed fabric (panels b, d).

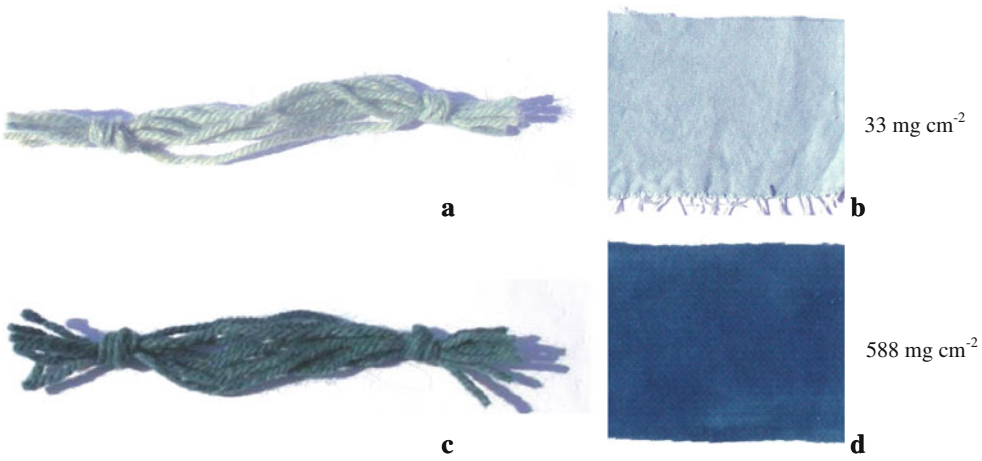

Fig. 4 Wool yarns (**a**, **c**) and cotton fabrics (**b**, **d**) subjected to single dips in corn steep liquor (CSL) broth added with 140 g L^{-1} of woad powder after 15 days of fermentation under microaerophilic (**a**, **b**) and strict anaerobic (**c**, **d**) conditions. The amount of indigo trapped within the cotton fibre structure, expressed as mg cm^{-2}, is reported next to each dyed fabric

Discussion

Over the last decades, there has been an increase in research efforts on clostridial fermentations and, concomitantly, renewed interest in the biochemistry, physiology and genetics of clostridia. Accordingly, several species, such as *Clostridium acetobutylicum*, *Clostridium propionicum*, *Clostridium butyricum*, *Clostridium tyrobutyricum* and *Clostridium perfringens*, have been exploited in either laboratory- or industrial-scale fermentation processes for production of organic solvents and acids [22]. In this study, the strain *C. isatidis* DSM 15098^T, first isolated by [18] from a fermenting woad vat prepared and maintained according to the mediaeval practice, was exploited for the development of an environmentally friendly biotechnological process for vat dyeing with woad.

The preliminary evaluation of the growth conditions confirmed the suitability of Clostridial Nutrient Medium (CNM) for cultivation of indigo-reducing bacteria, as previously suggested by [1]. As regards the incubation temperature, our findings are similar to those of [19], who described *C. isatidis* DSM 15098^T as a moderate thermophilic strain, capable of growing at between 30 and 55 °C with an optimum at 49–52 °C in Reinforced Clostridium Agar (RCA), pH 7.8 ± 0.2. This is a synthetic medium recommended for cultivation and enumeration of clostridia and other anaerobes, which has been successfully used for isolation of indigo-reducing bacteria in previous studies [1, 16, 19, 20, 30, 31].

The results concerning the effect of pH on growth differ slightly from those reported by [19]. According to those authors, *C. isatidis* DSM 15098^T showed optimal growth at incubation temperature of 50 °C and pH 7.2 ± 0.2, although pH values in the range 5.9–9.9 were found to allow bacterial multiplication at 45 °C. However, it is worth noting that [19, 20] and other authors [1] adjusted the pH of synthetic media to values ≥9.0 to successfully isolate and subculture strains assigned to *C. isatidis* or other indigo-reducing taxa. In our study, as the pH of CNM was increased from 6.8 ± 0.2 to 9.0 ± 0.1, greater enhancement in growth was seen in broth cultures than on agar medium. Furthermore, the type of alkali used for pH adjustment appeared to have an influence on the bacterial growth on the agar medium, but not in the broth cultures. The possible effect of the cation, associated with the alkali used to adjust the pH, on either the growth or the metabolic activity of bacteria has previously been elucidated [15]. In more detail, the greater growth of *C. isatidis* when KOH was used instead of NaOH for the pH adjustment of CNM agar may indicate that, under these specific culturing conditions, the cells were stressed by sodium ions.

Both NaOH and KOH can be interchangeably used to control pH in a variety of fermentation processes, including those which involve use of corn steep liquor [26]. At an industrial level, NaOH is generally preferred, owing to its low cost, whereas KOH is more suitable for agricultural applications, such as production of fertilisers, due to both its complete dissociation in water to hydroxyl and potassium ions and its relatively low toxicity for aquatic organisms, as documented by the International Programme on Chemical Safety (IPCS) (http://www.inchem.org/). Considering these advantages, and the positive results obtained with KOH in broth cultures, this alkali was selected for the control of pH during batch fermentations in laboratory bioreactors. This choice was also dictated by the need to develop a biotechnological process fully respectful of the environment.

During inoculum preparation, intense acidifying activity was shown by *C. isatidis* DSM 15098^T when fermented in Erlenmeyer flasks. This finding is again similar to the results reported by [19] regarding the ability of this strain to lower the pH of glucose-based media during growth. Unfortunately, neither of the two buffers used in this study for the maintenance of pH within an optimal range allowed

growth of the strain under study, although in a very recent investigation [10] both of these buffers were found not to inhibit multiplication of a protease-producing strain of *Bacillus subtilis*, which is a related endospore-forming species. In a previous study, [1] successfully used NaOH–Na_2CO_3 buffer (pH 10) for cultivation of indigo-reducing bacteria isolated from a dye vat prepared and maintained according to the Japanese tradition; this buffer might represent a valid alternative for the maintenance of alkaline pH during fermentation associated with the acid production carried out by *C. isatidis* strains.

In this study, two simple media were assayed in batch fermentations for their ability to sustain either the growth or the reducing activity of *C. isatidis* DSM 15098^T. These media were formulated including molasses as a carbon and energy source and either freshly prepared yeast extract (YE) or corn steep liquor (CSL) as a source of nitrogen compounds, amino acids, peptides, salts and vitamins, especially those belonging to the B group, as previously reported by other authors [12, 28].

The comparative evaluation of viable counts obtained in the two media revealed the greater suitability of CSL broth for cultivation of the strain under study, although an abrupt and rapid drop in the oxidation/reduction potential (ORP) to values lower than −470 mV versus Ag/AgCl was seen in both YE and CSL broth. For decades, either YE or CSL has been used in formulation of microbiological media [12, 24]. However, these components are both characterised by great (and sometimes lot-to-lot) variability in their chemical composition, which largely depends upon a multitude of factors involved in the processing of yeast cells and starch, respectively [12, 28, 32]. This means that the principle constituents in CSL that best support *C. isatis* growth cannot be identified without more extensive fermentation studies and chemical analyses.

On the one hand, these preliminary findings clearly deny the assertion made by [1] regarding the lack of or low culturability of clostridia; on the other hand, they justify the choice of CSL broth for fermentation assays with *C. isatidis*. As a by-product of the sugar beet refining industry, CSL has the advantage of being a cheap alternative to much more expensive ingredients for the fermentation industry, such as yeast extract or peptone [12].

In our dyeing trials, carried out in broth cultures containing 140 g L^{-1} of woad powder, providing an amount of indigo dye (2.4 g L^{-1}) which corresponds to that currently used in industrial processes, ranging from 1 to 3 g L^{-1} [23], ORP values from −540 to −579 mV and from −538 to −577.5 mV versus Ag/AgCl were measured under microaerophilic and strict anaerobic conditions, respectively. These values are fully comparable to those generated by the same strain in RCM added with 0.01 % (w/v) of indigo [17]. An oxidation/reduction potential of at least −600 mV has previously been proposed by [3] as a minimum for successful indigo reduction in industrial dyeing with synthetic indigo, whereas other authors [17, 18] have considered values ≤−470 mV versus Ag/AgCl generated by bacterial fermentation as sufficiently negative for successful reduction of natural indigo to its leuco form. An indicative ORP value of −474 mV was measured by [27] in water maintained at 50 °C in the presence of indigo powder and 0.5 mM leucoindigo. By contrast, more negative ORP values, ranging from −440 to −670 mV, were recorded by [1] during fermentation of broth cultures added with *Sukumo*, which is a dye product obtained from the Japanese indigo-bearing plant *P. tinctorium*.

As regards the monitoring of biotic parameters, significantly higher counts of both vegetative cells and thermoresistant spores were seen in broth cultures maintained under anaerobiosis. This finding was in fact expected, considering the nature of *C. isatidis*, which is described as an obligately aerotolerant anaerobe [19]. It is worth noting that viable counts of vegetative cells were never higher than 10^7 cfu mL^{-1}, irrespective of the incubation conditions and the duration of the fermentation process. These results resemble those reported by [19] for a fermenting vat inoculated with couched woad, where anaerobic bacteria increased from 4×10^5 cfu mL^{-1} after 30 h of fermentation to 3×10^6 cfu mL^{-1} after 8 days. This evidence could be attributed to nutrient limitation or by-product inhibition, or even to the activation of a self-limiting mechanism of cell population density, based on a quorum sensing system. Indeed, the ability of clostridial cells to exchange metabolic signals for control of different cellular functions has recently been highlighted in *Clostridium difficile* [7], *Clostridium thermocellum* [6], *Clostridium perfringens* [29] and *Clostridium proteoclasticum* [13].

The oxidation/reduction potential results showed that a sufficiently negative value for reduction of indigo to its soluble form was reached at as early as 24 h of fermentation and maintained up to the end of the monitoring period, irrespective of the incubation conditions. In a recent investigation of the microbial dynamics occurring in a traditional woad vat, thermophilic aerobic bacilli were found to be responsible for the creation of a reducing environment and hence for the further growth of fermentative obligately anaerobic bacteria with indigo-reducing activity [4]. While the maintenance of the reducing environment generated by *C. isatidis* was expected in the broth cultures continuously flushed with sterile oxygen-free N_2, in those fermented without N_2 flushing it could be attributed to progressive self-generation of an anaerobiosis regime through production of CO_2 and H_2 by decarboxylation of pyruvic acid, as previously reported for this species when subcultured in glucose-based media [21]. As clearly elucidated by [4], maintenance of a reducing environment

is essential to ensure that indigo remains in the leucoindigo form.

A few considerations should be made concerning the laboratory dyeing trials. Although a similar trend in ORP was seen during fermentation under either microaerophilic or anaerobiotic conditions, a marked difference in the dyeing capacity of the two types of broth cultures was observed, and a positive impact of N_2 flushing on the maturation of the dye bath was seen. This finding could be either the result of the higher viable counts reached by *C. isatidis* in the broth cultures fermented under strict anaerobiosis, or due to a different ability of *C. isatidis* to act directly on the dye particles under the two particular conditions assayed. In fact, it has previously been hypothesised that, in the absence of a redox mediator, direct contact between *C. isatidis* and solid indigo particles is required for bacterial-driven indigo reduction [9]. This latter hypothesis is supported by previous experimental evidence, which revealed a significant reduction in the size of indigo dye particles incubated in *C. isatidis* cell-free supernatants, as a consequence of the activity of lytic enzymes, whose mode of action was, and remains, unknown [17]. The hypothesis that the generation and maintenance of a sufficiently negative oxidation/reduction potential is necessary but not sufficient for bacterial-driven indigo reduction has also been suggested by the results of further fermentation assays carried out in our laboratory with an isolate of *B. thermoamylovorans* from an indigo dye vat prepared and maintained in the traditional way; although this isolate was able to generate ORP lower than −470 mV during fermentation in CSL broth, no reduction of indigo to its leuco form was seen up to the end of the monitoring period (15 days), as revealed by both visual inspection of the dye bath and laboratory dyeing trials (data not shown).

Conclusions and future perspectives

After going into decline for more than a century, cultivation of indigo has progressively been revived in Europe. This is partly due to sponsored research projects aimed at developing new sustainable woad cultivation techniques and indigo production processes to satisfy the market demand for naturally sourced indigo. Notwithstanding the great effort made to replace the use of chemical indigo reduction agents with more attractive, eco-friendly alternatives, no reduction systems that use a biological approach are available to date. Our study provides a preliminary positive answer to this challenge, by laying some sound bases for the development of a biotechnological process. The novelty of our study lies in the exploitation of a well-characterised indigo-reducing strain in a standardised vatting process carried out in a simple broth medium under continuously controlled pH and T conditions. The main advantages of the novel vatting process proposed are: the revival of an ancient eco-textile tradition for exploitation of natural indigo dye; the absence of toxic by-products; the adoption of mild temperatures, leading to low energy consumption; the exploitation of inexpensive industrial by-products as fermentation ingredients; the possibility to treat wastewaters in simple waste disposal systems; the possible automation of fermentation and dyeing processes; the continuous on-line monitoring of process parameters; the notably faster maturation of the dye bath with respect to the traditional process, which takes approximately 1 month. The technical problems to be solved with a view to a future scale-up of this vatting process from laboratory to plant size are connected with the production of strong-smelling fermentation products, such as dimethyl sulphide, dimethyl disulphide and methanethiol, which could be overcome using suitable output air filtration systems, and the need for standardisation of the number of dyeing cycles in order to obtain the desired shade of colour.

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