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Eco-efficiency analysis as a reaction-engineering tool—Case study of a laccase-initiated oxidative C–N coupling

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ABSTRACT

The laccase-mediated heterocoupling of 3-methylcatechol and *n*-hexylamine was investigated at the milligram to gram scale, and an early eco-efficiency analysis and evaluation revealed bottlenecks in enzymatic synthesis. Eco-efficiency analysis was used as an optimisation tool to create more sustainable solutions and improve subsequent reactions, enabling the scaled-up biocatalytic process to become more economical and competitive compared with the chemocatalysed procedure using sodium iodate. When working with higher substrate concentrations (up to 100 mM), the following key aspects must also be considered: (1) the low solubility of *n*-hexylamine and the reaction products in aqueous solution, and (2) the poor stability of enzymes in unconventional solvents. The present study includes activity and stability experiments using laccases in methanol, a suitable solvent for the hydrophobic compound *n*-hexylamine. Contrary to previously published reports, excellent activities were obtained using Novozym 51003 at high methanol contents and substrate concentrations up to 100 mM. In addition, the use of this volatile organic solvent drastically simplified downstream processing.

1. Introduction

Interest in laccases (E.C. 1.10.3.2) has increased in recent years because of the potential of these enzymes to provide unique green chemistry solutions for dye oxidation, lignin derivative polymerisation, and bleaching [1,2]. In the presence of oxygen, laccases oxidise a broad range of substrates, such as phenolic aromatic compounds, *via* one-electron oxidation to generate products ranging from dimers to macromolecules [3,4]. Depending on the enzyme source and substrates, native laccases typically remain active in aqueous solvents at low temperatures at a specific pH. These reactions have mainly been observed at the analytical scale at low concentrations and yields because the reaction compounds are often hydrophobic and poorly soluble in aqueous buffer solutions. Thus, we sought to address the following question: "What are the important characteristics of reaction media for larger-scale laccase-catalysed oxidations?" [5]. Various studies have investigated the partial or complete replacement of water as a solvent. Table 1 is not intended as an exhaustive list of studies on the influence of organic solvents, but it provides an overview of several laccase investigations at different scales. Many researchers observed that 10–30% v/v water-miscible organic solvents did not substantially improve biotransformations, mostly at the analytical scale. However, scaling up is often limited by poor substrate solubility. Notably, Rodakiewicz-Nowak summarised the effects of different organic solvents on several phenol-oxidising enzymes. Confirming the importance of solvent exchange, he reported that in aqueous solutions, radicals initiate undesirable, non-enzymatic polymerisation of substrates [6]. Organic solvent content is often limited by the resulting decreased activity of the native laccase enzyme.

Here, we describe a laccase-initiated oxidative C–N coupling. On the preparative scale, the water-miscible organic solvent methanol has a decisive influence on catalyst activity as well as reaction rate and selectivity. Consistent with the results of Wellington et al. [23], the robust, stable, commercially available Novozym 51003 represents a native laccase that can operate at exceptionally high methanol concentrations; such high methanol concentrations have not successfully used previously.

In addition, the use of eco-efficiency analysis in the early stages of reaction and process development enabled the identification of relevant process parameters to upgrade the biocatalytic process *via* reaction engineering. For the production of (new) chemicals, environmental issues and conventional pathways must be critically considered. New insights in biological research and development will enable novel applications of biocatalysis. The improved

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Table 1
Studies of the influence of organic solvents on laccase-catalysed biotransformations.

Study/reaction	Laccase	Solvent system	Scale	Results	Ref.
Reaction of 1-aminobenzotriazole with dihydroxybenzoic acid esters	*Pc*L, *Mt*L	Buffer/MeOH	**A**	Negligible influence of 10% v/v MeOH; no product formation at MeOH content >75%	[7]
C–N coupling of 3-methylcatechol with primary amines	*Pc*L, *Tv*L	Buffer/MeOH	**A**	Increasing activity of *Tv*L and *Mt*L up to 30% v/v MeOH; completely inactivation of all tested laccases at 80% v/v MeOH	[8]
Characterisation of organic solvent-tolerant laccase	*Gf*L	Water/org. solvent	**A**	>80% residual activity in 50% v/v EtOH, MeOH, DMF, or DMSO	[9]
Oxidation of tetrahydro-2-naphthol derivatives and medium engineering	immobilised *Mt*L, *Tp*L	Aromatic solvent mixtures or biphasic system	**C**	Water-immiscible org. solvent (presaturated with buffer) influences enzyme selectivity; high conversions in toluene and benzene (93–95%); ≤14% isolated yield of dimeric derivatives; no reaction in pure org. solvent	[10]
Oxidation of phenolic compounds	*Rv*L	Biphasic system Water/non-polar solvents, *e.g.*, hexane	**A**	*Rv*L more efficient in hexane than in aqueous medium; highest activity in 6.5% v/v water in hexane; different oxidation products in org. solvents (no structure determination)	[11]
Oxidation von syringaldazine or 2,6-DMP	immobilised *Tv*L	Org. solvents (presaturated with water)	**A**	Rate of oxidation in several org. solvents with 7% v/v water was only 10–20% that in buffer; no oxidation in org. solvents with native laccase	[12]
Oxidation of totarol to C–O or C–C linked dimers	*Tp*L, *Mt*L	Buffer/acetone, CH_3CN, MeOH (50% v/v), or biphasic systems	**B**	Nature of the org. solvent affected the product ratio and spectrum; best results were obtained in buffer/acetone (1:1), which resulted in 96.3% conversion (two products, symmetrical linked C-C dimer was dominant)	[1]
Nuclear amination of *p*-hydroquinones with primary aromatic amines	*Mt*L, *Ts*L	Buffer or 100% MeOH	**A**	No products in 100% v/v MeOH; reaction using sodium iodate was comparable to laccase-catalysed reaction	[13]
Influence of water-miscible solvents on phenol oxidising enzymes	PO, Tyr, *Cu*L, *Pr*L	*e.g.*, MeOH, EtOH, DMSO, acetone	**A**	Overview; kinetic experimental series up to 3.5 M org. solvent; effects on V_{max} for syringaldazine oxidation (EtOH < acetone < DMSO)	[6,14–16]
Effect of organic solvents on laccase activity in oxidation of 2,6-DMOP	native and immobilised *Pr*L, *Cu*L	Buffer/org. solvent (0–90% v/v)	**A**	High native enzyme activities in ≤20% v/v MeOH/EtOH; protein denaturation begins at 20–50% v/v; highest stability in EtOH	[17,18]
Oxidative deprotection of *p*-methoxyphenyl-protected amines	*Tv*L	Buffer/solvent, *e.g.*, MeOH (10–40% v/v)	**B**	10% v/v MeOH resulted in 87% conversion (at 40% v/v, the conversion was only 75%); the best conversion (91%) was obtained in buffer with 10% v/v DMSO	[19]
Effects of water-immiscible and miscible organic solvents	native and immobilised *Rv*L	Water/several org. solvent	**B**	Highest *Rv*L activity in glycerol/water (1:1); immobilised *Rv*L displayed high activities in up to 80% v/v ethanol/acetone; non-polar solvents required a minimum water content (~2.5% v/v)	[20]
One-pot-synthesis of (di-)aminobenzoquinones	*Denilite*® *II Base*	Buffer/MeOH or DMF (≤20% v/v)	**C**	Best yield (58%) for one selected diaminated product in 20% v/v DMF	[21–23]
and 1,4-naphthoquinone-2,3-bis-sulfides	Novozym 51003	Buffer/DMF (5:1)		Yield up to 85% yield of monoaminated product in 20% v/v DMF	
Tandem synthesis of naphthoquinones	*Tvi*L	Buffer or buffer/MeOH	**B**	Lower isolated yield (18%) in buffer/MeOH (1:1 v/v) than in buffer (47%); higher reactivity and selectivity in aqueous medium	[2]

Abbreviations used: *Cu*L (*Cerrena unicolor*), *Gf*L (*Ganoderma fornicatum*), *Mt*L (*Myceliophthora thermophila*), *Pc*L (*Pycnoporus cinnabarinus*), PO (peroxidase), *Pr*L (*Phlebia radiate*), *Rv*L (*Rhus vernificera*), *Tp*L (*Trametes pubescens*), *Ts*L (*Trametes* sp.), *Tv*L (*Trametes versicolor*), *Tvi*L (*Trametes villosa*), Tyr (tyrosinase); **A** analytical scale (1–10 mM), **B** semi-preparative scale (10–50 mM), **C** preparative scale (>50 mM).

speed of development in protein engineering should create great opportunities for enzymatic pathways [24]. The ability of biocatalysis to substitute for chemocatalysis is an increasingly urgent question [25]. The aim of this study was to identify eco-efficient conditions for innovative laboratory applications in a manner similar to that used for industrial production processes. Therefore, various parameters must be evaluated, such as selectivity, ratios of reaction components, catalysts, solvents, product titre, product purification, energy input, and waste prevention. The key driving factors studied in this work are shown in Scheme 1.

Two alternative processes were examined and compared regarding their environmental impact and economy *via* the eco-efficiency analysis method developed by BASF [26]. This analysis is a comparative lifecycle assessment tool that considers raw material extraction, production and use of the product, and options for recycling and disposal [27–29]. The quantification of the sustainability of the products and processes enables recommendations for action in future scenarios, suggesting reserves for process intensification. Because it is time-consuming and complex, quantitative comparisons of biocatalytic and conventional chemical process alternatives in the same dimension are rarely performed [25]. However, achieving more sustainable production processes for fine chemicals *via* the optimisation of economic and environmental opportunities and risks must begin at an early stage when the process design is still flexible. In the present study, we demonstrate that early assessment can help identify the key parameters influencing biotechnological pathways in fine chemical synthesis.