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Recombinant *Candida rugosa LIP2* expression in *Pichia pastoris* under the control of the *AOX1* promoter

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ABSTRACT

The *LIP2* isoenzyme gene from *Candida rugosa* has been completely synthesised and functionally expressed under the *AOX1* promoter control in *Pichia pastoris*. The on-line monitoring and control of methanol, the key inducer carbon source in fed-batch cultures, has enhanced the yield product/biomass 7.8-fold and the productivity 12.8-fold compared to the best batch cultivation with the *Pichia* system and, 10-fold compared to the fed-batch cultivation process using the native *C. rugosa* strain.

Nevertheless, the high ionic strength of culture broth favoured aggregation of Lip2, leading to total loss of lipolytic activity. After cultivation, a diaultrafiltration process was implemented to diminish ionic strength, allowing for the recovery of lipolytic activity in the diaultrafiltrate. The developed bioprocess resulted into a reproducible product in terms of quality and productivity.

1. Introduction

Lipases (triacylglycerol acylhydrolases EC 3.1.1.3), in addition to the hydrolysis of their natural substrates, catalyse the enantio- and regioselective hydrolysis and synthesis of a broad range of natural and synthetic esters, constituting an alternative method to chemical asymmetric synthesis with a high increase in pharmaceutical applications [1–3].

Practically all microorganisms are potential lipase producers [4]. Between them, fungi lipases from *Candida antarctica*, *Humicula lanuginosa*, *Rhizomucor miehei*, *Rhizopus arrhizus*, *Geotrichum candidum* and *Candida rugosa* are the most widely used in fungal biotechnology [5]. Among them, *C. rugosa* is one of the most frequently used organism for lipase production [6] and it is commercialised by several companies [7].

Unfortunately, *C. rugosa* lipase (CRL) from commercial preparations is composed by a mixture of isoenzymes with different stereobias towards several substrates [8,9]. At least seven lipase genes, namely *LIP1*–*LIP7*, have been described in *C. rugosa*, and five of them (*LIP1*–*LIP5*) have been fully characterised [10]. However, only three of their products (Lip1, Lip2 and Lip3) have been identified in commercial crude enzyme preparations [11,12]. Using competitive reverse transcription-PCR (RT-PCR) it has been demonstrated that the levels of gene expression of the isoenzymes in native *C. rugosa* usually follow the order (from highest to lowest): *LIP1*, *LIP3*, *LIP2*, *LIP5* and *LIP4*. Furthermore, it was observed that the expression of *LIP1* and *LIP3* was always high and constitutive, according to the two main isoenzymes detected in commercial powders, while the expression of the other genes was affected by the culture conditions [13].

Besides, it has been suggested that the heterogeneity of lipase isoforms may be partially due to changes in gene expression, heteroglycosylation [14], non-covalent association of glycosylated impurities to the lipase component [11,15], partial proteolysis, or other post-translational modifications [7].

Consequently, in order to characterise the biocatalysis performance of CRLs, it is necessary to use pure isoenzymes. Otherwise, it is difficult to gain a firm mechanistic understanding of the enzyme performance [16].

Two different strategies can be applied: the purification of CRL isoenzymes and, the recombinant overproduction of pure CRL isoenzymes.

There are slight differences among CRLs isoenzymes in terms of isoelectric point and glycosylation degree; also the deduced amino acid sequences for Lip1–Lip5 share about 70% identity, with molecular weights of about 60 kDa [7]. Hence, the purification process

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requires several chromatographic steps to obtain the pure isoenzyme resulting in a low final yield. Thus, this methodology is not recommended from an industrial point of view [15,17].

On the other hand, the recombinant production of CRL isoenzymes is the most promising strategy for future industrial applications of pure/defined CRL isoenzymes preparations [7]. However, *C. rugosa* has an unusual codon usage in which the triplet CUG, a universal codon for leucine, is read as serine [18]. In *C. rugosa*, the CUG triplet accounts for about 40% of the total serine codons [19]. For instance, in the *LIP1* gene, 20 of its 47 serine residues, including the Ser209 present in the catalytic enzyme active centre, are encoded by CUG triplets. As a consequence, the direct heterologous expression of *LIP1* in *Saccharomyces cerevisiae* resulted in an inactive lipase [20]. Thus, substitution of most of the CUG codons by universal serine triplets (UCN, AGY) is required for the expression of a functional Lip1 protein in heterologous hosts [21].

Different genetic strategies, namely total gene synthesis with codon adaptation to the host's usage or, substitution of all native serine-encoding codons by universal codons using site-directed mutagenesis strategies, have been applied to obtain functional heterologous *C. rugosa* isoenzymes in different hosts systems. For instance, *LIP1* has been expressed in *S. cerevisiae* [22] and *Pichia pastoris* [22,23], as well as fused to green fluorescent protein [24]. Also, this gene was expressed in *Candida maltosa* [25], which has the same codon usage for serine as *C. rugosa*. In addition, *LIP2* and *LIP3* have been expressed in *P. pastoris* [26,27], and *LIP4* in *Escherichia coli* [28] and *P. pastoris* [29].

Overall, the methylotrophic yeast *P. pastoris* has been shown to be a suitable expression system for *C. rugosa* isoenzymes. A key advantage of *P. pastoris* as a host system is that combines the unique capacity of growing in minimal medium at high cell densities with low levels of endogenous protein secretion and the ability to efficiently secrete heterologous proteins, i.e. simplifying their recovery. Also, it performs many of the higher eukaryotic post-traductional modifications as protein folding, proteolytic processing, disulphide bond formation and glycosylation [30]. Two promoters have been used for the heterologous expression of *C. rugosa* isoenzymes in *P. pastoris*: the alcohol oxidase 1 promoter (P*AOX1*) for *LIP1* [22] and, the glyceraldehyde phosphate promoter (P*GAP*) for *LIP1* [23], *LIP2* [26], *LIP3* [27] and *LIP4* [29] expression. However, production studies of heterologous CRL isoenzymes in *P. pastoris* reported in the literature involved cultivations operated in batch mode or non-controlled methanol fed-batch addition strategies.

The objective of this work is to develop an integrated process for recombinant production of a functional Lip2 isoenzyme (rLip2)

```
gaa ttc gca cca aca gct act tta gct aac gga gac act ata acc gga tta aac gcc atc gtt aat
 E   F   A   P   T   A   T   L   A   N   G   D   T   I   T   G   L   N   A   I   V   N
gag aaa ttt ttg ggc atc cca ttt gct gaa cca ccg gtg ggc acc tta aga ttc aaa cct cca gtt
 E   K   F   L   G   I   P   F   A   E   P   P   V   G   T   L   R   F   K   P   P   V
cct tat tcc gcc tcc tta aat ggt caa cag ttc act tcg tac gga cca tcc tgt atg cag atg aat
 P   Y   S   A   S   L   N   G   Q   Q   F   T   S   Y   G   P   S   C   M   Q   M   N
cca atg gga tcc ttc gaa gat act cta ccc aaa aat gca ctg gat ctg gtg ttg cag tca aag atc
 P   M   G   S   F   E   D   T   L   P   K   N   A   L   D   L   V   L   Q   S   K   I
ttt cag gtc gtg tta cca aat gat gaa gac tgc ctc aca att aac gta atc aga cct cca ggt acc
 F   Q   V   V   L   P   N   D   E   D   C   L   T   I   N   V   I   R   P   P   G   T
cgt gca agt gcg ggt ttg cct gta atg ctt tgg att ttc gga ggt ggt ttc gaa ctg ggt ggt agt
 R   A   S   A   G   L   P   V   M   L   W   I   F   G   G   G   F   E   L   G   G   S
tct cta ttc ccg ggt gac caa atg gtg gcc aaa agc gtg ctc atg ggc aaa ccg gtc atc cac gtt
 S   L   F   P   G   D   Q   M   V   A   K   S   V   L   M   G   K   P   V   I   H   V
tcg atg aac tac aga gta gct tcc tgg gga ttt ctg gca ggg ccc gat att caa aat gaa ggt tcc
 S   M   N   Y   R   V   A   S   W   G   F   L   A   G   P   D   I   Q   N   E   G   S
ggt aac gct ggt cta cat gac caa aga tta gct atg caa tgg gtg gca gac aat ata gcg gga ttt
 G   N   A   G   L   H   D   Q   R   L   A   M   Q   W   V   A   D   N   I   A   G   F
ggt ggg gat cct agt aaa gtg aca att tat gga gag tcg gcc ggt tct atg tcc act ttc gtt cat
 G   G   D   P   S   K   V   T   I   Y   G   E   S   A   G   S   M   S   T   F   V   H
ttg gtg tgg aac gat gga gat aac acc tat aac ggt aaa cca tta ttc aga gcc gct att atg cag
 L   V   W   N   D   G   D   N   T   Y   N   G   K   P   L   F   R   A   A   I   M   Q
agc gga tgt atg gtc cct tct gac ccc gta gat gga aca tat gga act gaa ata tac aac caa gtt
 S   G   C   M   V   P   S   D   P   V   D   G   T   Y   G   T   E   I   Y   N   Q   V
gtt gcc tcc gct ggt tgt ggt tct gca tct gat aag ttg gcc tgt cta cga ggt ttg tca caa gac
 V   A   S   A   G   C   G   S   A   S   D   K   L   A   C   L   R   G   L   S   Q   D
act ctt tac cag gca aca agc gat acc ccc ggg gtt ttg gcc tac ccc tcc ttg agg ctg tcc tat
 T   L   Y   Q   A   T   S   D   T   P   G   V   L   A   Y   P   S   L   R   L   S   Y
ctt cct aga ccc gat ggt acc ttt att acc gac gac atg tac gca cta gtg cga gat gga aag tac
 L   P   R   P   D   G   T   F   I   T   D   D   M   Y   A   L   V   R   D   G   K   Y
gca cat gtt cct gtt att atc ggt gat cag aat gat gaa ggc acc ctt ttc gga cta tca agt ttg
 A   H   V   P   V   I   I   G   D   Q   N   D   E   G   T   L   F   G   L   S   S   L
aac gtt acc acc gat gct caa gcc cgg gct tac ttc aag caa agt ttt att cat gcc tcc gat gcc
 N   V   T   T   D   A   Q   A   R   A   Y   F   K   Q   S   F   I   H   A   S   D   A
gag att gat acc cta atg gct gcc tac acg agt gat att act caa ggt tca cct ttc gac acc ggt
 E   I   D   T   L   M   A   A   Y   T   S   D   I   T   Q   G   S   P   F   D   T   G
att ttt aat gct att act ccc caa ttt aaa aga att agt gcc ctg ctt gga gat ctt gca ttc aca
 I   F   N   A   I   T   P   Q   F   K   R   I   S   A   L   L   G   D   L   A   F   T
ttg gca cgt agg tat ttc ttg aat tat tac cag gga ggt acc aag tac tct ttc ctg tca aaa cag
 L   A   R   R   Y   F   L   N   Y   Y   Q   G   G   T   K   Y   S   F   L   S   K   Q
tta tca ggc ttg ccg gtc ctg gga acc ttc cac gga aat gac atc ata tgg caa gat tat ttg gtt
 L   S   G   L   P   V   L   G   T   F   H   G   N   D   I   I   W   Q   D   Y   L   V
gga agc ggc tca gtt ata tac aat aat gca ttc att gct ttt gcg aat gat cta gac cca aat aaa
 G   S   G   S   V   I   Y   N   N   A   F   I   A   F   A   N   D   L   D   P   N   K
gct ggc ctc tgg acg aac tgg cca aca tac acc agt tct tcc cag tcc gga aac aac ttg atg caa
 A   G   L   W   T   N   W   P   T   Y   T   S   S   S   Q   S   G   N   N   L   M   Q
atc aat ggc cta ggt tta tat act ggc aag gac aac ttt aga cca gat gct tac agt gcc ctc ttt
 I   N   G   L   G   L   Y   T   G   K   D   N   F   R   P   D   A   Y   S   A   L   F
agt aat ccc cca tca ttt ttt gta tag gcg gcc gca agc ttc ag
 S   N   P   P   S   F   F   V   Stop
```

Fig. 1. *LIP2* gene optimised sequence for expression in *P. pastoris* and the corresponding deduced amino acid sequence. DNA regions in bold and underlined denote the restriction sites EcoRI and NotI, respectively, used for insertion of the *LIP2*-containing DNA fragment into the pPICZαA expression vector. The N-terminal residue of the mature native Lip2 enzyme is shown in bold and underlined.