一株嗜热子囊菌产生的碱性耐热过氧化氢酶及其应用潜力

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摘 要 研究了一株嗜热子囊菌产过氧化氢酶的摇瓶发酵条件,并对其在纺织工业中的应用潜力进行了评价。以 20 g/L 糊精和 1%(*V*/*V*)乙醇为混合碳源时,过氧化氢酶酶活达到 1594 u/mL,比以糊精和乙醇单独为碳源时过氧化氢酶的活力之和还高 23%。改变培养基的初始 pH、提高发酵液中的溶氧水平及添加外源过氧化氢,过氧化氢酶的产量进一步提高到 2762 u/mL,比优化前提高了 5.8 倍。将嗜热子囊菌的过氧化氢酶同来源于牛肝、黑曲霉的过氧化氢酶进行了热(70℃, 80℃, 90℃)、碱(pH 9.0, pH 10.0, pH 11.0)稳定性的比较。结果显示,产自嗜热子囊菌的过氧化氢酶对高温和强碱性的耐受性能明显优于其它来源的酶,在纺织染整工艺中具有良好的应用潜力。

关键词 过氧化氢酶, 嗜热子囊菌, 混合碳源, 过氧化氢, 热、碱稳定性

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过氧化氢酶(Catalase,简称 CAT)是一种能够高效催化分解过氧化氢的酶,广泛用于食品消毒、临床分析、医学诊断以及纺织、造纸、制浆等工业。近年来,随着绿色环保意识与要求的不断加强,在染整工艺中利用酶替代传统的强碱高温工艺对棉织物进行前处理已成为大势所趋。CAT 在棉织物前处理过程中的主要作用是去除织物漂白废液中残余的 H_2O_2,以免给后续染色工序带来问题[1]。应用 CAT 实现漂染同浴,不仅能节省大量的水、电、气的消耗,提高生产效率,还能减少废水的排放,有利于环保。但是,现有商品 CAT 在高温和强碱性环境下易失活,限制了 CAT 在染整工艺中的应用[2]。

CAT 来源丰富,几乎存在于所有好氧生物中。但来自动物脏器以及一些微生物(如溶壁微球菌、球形红假单孢菌、大肠杆菌、粗糙脉孢霉、微紫青霉、黑曲霉)的 CAT,均不能忍受接近沸点的高温和 pH 大于 10 的强碱性[3]。近 20 年来,关于嗜热菌[4,5]、嗜碱菌[6]或嗜热嗜碱菌[7]产生 CAT 的报道陆续出现,特别是嗜热子囊菌所产的 CAT 是迄今为止已报道过的热、碱稳定性最好的 CAT[8],为开发耐热耐碱的 CAT 提供了可能。但是,大多数微生物体内 CAT 的合成因受到糖分解代谢物阻遏的影响[9]而无法实现高产,制约了发酵法生产 CAT 的工业化进程。本文研究了不同碳源对一株金黄色嗜热子囊菌(*Thermoascus aurantiacus*)生产 CAT 的影响,通过设计混合碳源添加策略,显著提高了 CAT 产量。作者进而考察了 *T. aurantiacus* 合成的 CAT 对高温和强碱性环境的耐受能力,以评价该酶在纺织染整工艺中的应用潜力。

1 材料与方法

1.1 材料

1.1.1 菌种:金黄色嗜热子囊菌(*Thermoascus aurantiacus*)WSH 03-01。

1.1.2 过氧化氢酶:牛肝 CAT,购自 Sigma 公司,黑曲霉 CAT,购自 Novozymes。

1.1.3 培养基:

斜面培养基 PDA(g/L):马铃薯 200,葡萄糖 20,琼脂 16,pH 6.0。

种子培养基(g/L):酵母膏 4,玉米淀粉 15,K_2HPO_4 1,Na_2HPO_4 1,$MgSO_4 \cdot 7H_2O$ 0.5,pH 6.8。

基础发酵培养基(g/L):葡萄糖 20,酵母膏 4,$(NH_4)_2SO_4$ 2,K_2HPO_4 5, $KH_2PO_4 \cdot 3H_2O$ 3,$MgSO_4 \cdot 7H_2O$ 2,微量元素液 2 mL,pH 7.0。

微量元素液(g/L):$FeC_6H_5O_7 \cdot nH_2O$ 6,$CaCl_2 \cdot$

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$2H_2O$ 4，$ZnSO_4 \cdot 7H_2O$ 2，$MnCl_2 \cdot 4H_2O$ 0.1，KI 0.1，$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0.1，$CoCl_2 \cdot 6H_2O$ 0.1，H_3BO_3 0.1，NaCl 5。

1.2 方法

1.2.1 培养方法：将成熟的孢子接种到 PDA 斜面上，45 ℃下培养 6～8 d。待孢子成熟后，刮取斜面上的孢子，用无菌水制成孢子悬液接种于种子培养基中（培养基中孢子浓度为 10^4 个/mL），45 ℃摇床培养 52 h。吸取种子培养液，按 10% 的接种量接种于基础发酵培养基中（装液量为 500 mL 摇瓶中装 100 mL 培养基），45℃摇床培养 84 h。每项实验均设 3 个平行样，取平均值。

1.2.2 测定方法：

发酵液挥发量的校正：由于发酵过程在高温下进行且周期较长，培养基中水分的挥发对实验结果的准确性会有较大影响，因此在发酵结束后采用称重补水法进行校正。将接种后的摇瓶用电子天平（±0.01 g）记录初始质量，发酵结束后，分别将去离子水补入相应摇瓶至初始值。恢复质量后的发酵液再用于发酵液各项参数的分析测定。

生物量测定：取 50 mL 发酵液进行真空抽滤，收集湿菌体，用蒸馏水洗涤 2 次后，置 105 ℃下恒温干燥至恒重，用称重法测定生物量。

菌球粒径测定：将一滴均匀的菌体培养液滴在载玻片上，用目旋测微仪测显微镜 3 个视野中 3 个菌球的粒径，计算算术平均值。重复 3 次，取 3 次测量结果的平均值为整个培养液中菌球的平均粒径。

过氧化氢酶活性测定：采用分光光度法在 25℃下测定[10]。反应总体积为 3 mL，含 0.1 mL 酶液和 2.9 mL 含有 10 mmol/L H_2O_2 的 50 mmol/L 的磷酸二氢钾、磷酸氢二钠缓冲液（pH 7.0）。过氧化氢的分解速率用 752 型紫外-可见光分光光度计在 240 nm 下测定。酶活定义为：在 25℃下，每分钟分解 1 μmol H_2O_2 所需的酶量为一个酶活单位。

还原糖测定：3,5-二硝基水杨酸比色法[11]。

总糖的测定：蒽酮比色法[12]。

1.2.3 用于热、碱稳定性考察的粗酶制备方法：发酵液经真空抽滤后，向清液中加入等体积乙醇，离心，将沉淀溶于 20 mmol/L Tris-HCl 即得粗酶液。

2 结果

2.1 *T. aurantiacus* WSH 03-01 种子培养条件优化

对 *T. aurantiacus* WSH 03-01 种子培养条件进行优化是为了获得大小适宜的菌球和较多的菌体量。在接种孢子浓度不变的情况下，作者对影响菌球大小和菌体生长的两个主要因素——摇床转速和培养基装液量进行了研究（详细数据未给出）。结果表明，随着摇床转速的提高，菌球粒径逐渐变小，菌体量不断增加，而菌体生长达到对数生长期所需的时间也依次缩短。在 200 r/min 的转速下，500 mL 的摇瓶中种子培养基的装液量为 210 mL 时，菌体生长较好且菌球的平均粒径小于 2.0 mm。将此条件下培养 52 h 的种子接种进行发酵，发酵结束后发酵液中的 CAT 活性和菌体量均可达到最大。

2.2 培养基碳、氮源的研究

培养基碳、氮源优化以基础发酵培养基为依据，考察碳源时用其它碳源替换葡萄糖，其余成分不变。

2.2.1 碳源的选择：*T. aurantiacus* WSH 03-01 以单糖或二糖为碳源时（图 1A），当碳浓度超过 4 g/L 后 CAT 的活性便迅速降低；当碳浓度达到 20 g/L 时，发酵终了时发酵液的 pH 均降至 3 以下（数据未显示），且 CAT 酶活极低（< 10 u/mL）。以乙醇为单一碳源时（图 1A），其浓度变化对 CAT 活性的影响趋势虽与以单糖或二糖为碳源的情况类似，但 CAT 活性整体高于后者。以糊精或淀粉为碳源时（图 1B），其浓度升高对 CAT 活性的影响相对较小：当碳浓度超过 12 g/L 时 CAT 活性才出现明显下降。此外，以甘油为碳源时（图 1B），CAT 活性受其浓度变化的影响虽不如糖类碳源显著，但整体活性较低，因此甘油并不是适宜碳源。

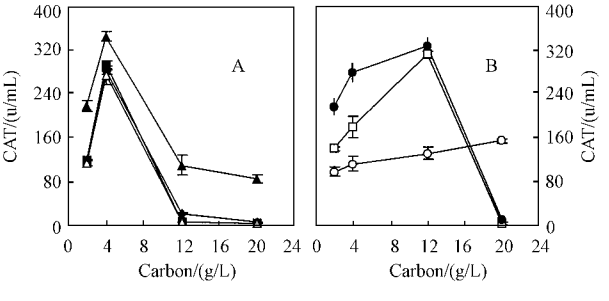

图 1 不同种类的碳源对 CAT 合成的影响

Fig. 1 Effect of different carbon sources on CAT biosynthesis

A：◆ Glucose ■ Sucrose △ Maltose ▲ Ethanol

B：● Dextrin □ Starch ○ Glycol

由于在相同碳浓度下，糊精和乙醇对 CAT 的得率高于其它碳源（图 1），故作者进一步研究了这两种碳源的最适浓度。如图 2 所示，以糊精和乙醇为单一碳源合成 CAT 时，其最适浓度分别为 20 g/L 和 1%（*V*/*V*），对应的碳浓度分别为 8.9 和 4.1 g/L。

2.2.2 氮源的优化：以 20 g/L 的糊精为碳源，考察

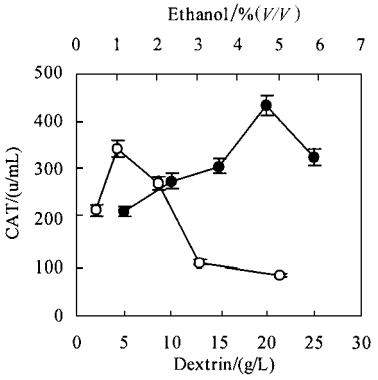

图 2　乙醇和糊精浓度对 CAT 合成的影响

Fig.2　Effect of the concentrations of ethanol or dextrin on CAT biosynthesis

● Dextrin ;○ Ethanol

了相同氮摩尔浓度下不同氮源对 CAT 合成的影响。其中有机氮源单独添加,无机氮源则与 0.4 g/L 的酵母膏一同添加。由图 3 看出,以蛋白胨为氮源合成 CAT 具有明显的优势。进一步研究表明(图 4),蛋白胨浓度为 10 g/L 时,CAT 产量最高,达 888 u/mL。

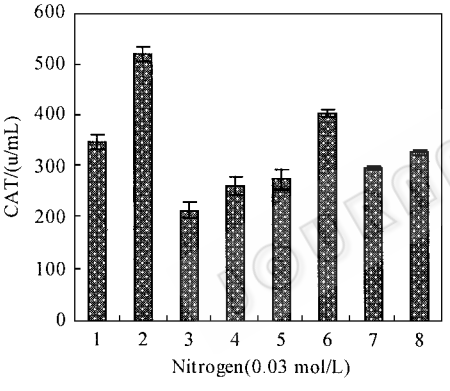

图 3　不同氮源合成 CAT 能力的比较

Fig.3　Comparison of CAT biosynthesis with different nitrogen sources

1 :yeast extract ;2 :peptone ;3 :corn steep liquid ;4 :$(NH_4)_2SO_4$; 5 :NH_4Cl ;6 :$(NH_4)_2HPO_4$;7 :$NaNO_3$;8 :urea

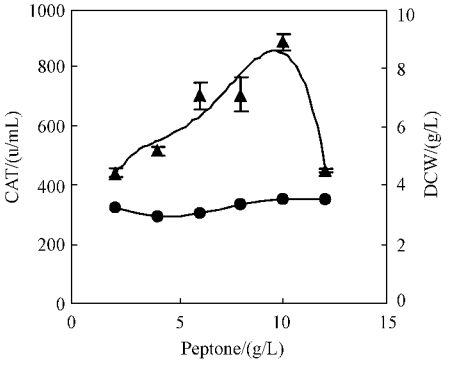

图 4　蛋白胨浓度对 CAT 合成的影响

Fig.4　Effect of the concentration of peptone on CAT fermentation

▲ CAT ;● DCW

2.2.3　混合碳源添加策略:为进一步提高 CAT 产量,作者研究了由 20 g/L 糊精和 1%(*V*/*V*)乙醇组成的混合碳源对 CAT 合成的影响。以 10 g/L 蛋白胨为氮源时,添加混合碳源后,CAT 的水平和菌体量均有较大幅度的提高。其中 CAT 产量与以乙醇和糊精分别为单一碳源时相比分别提高了 2.9 倍和 0.8 倍,即从 408 u/mL、882 u/mL 提高到 1594 u/mL,这比分别以乙醇和糊精单独为碳源时合成的 CAT 产量之和还高 23%,同时单位细胞产 CAT 的能力也分别提高了 62%和 31%(图 5)。

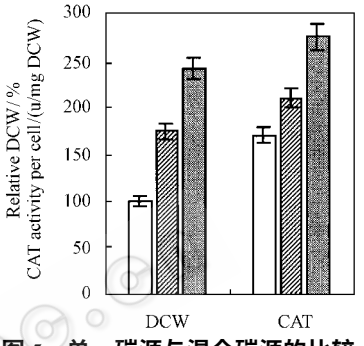

图 5　单一碳源与混合碳源的比较

Fig.5　Comparison of DCW and CAT activity per cell with single and co-carbon sources

□ Ethanol ; ▨ Dextrin ; ▩ Dextrin + Ethanol

2.3　通风量及外源过氧化氢对 CAT 合成的影响

2.3.1　培养基装液量对 CAT 合成的影响:由于本实验培养条件较为特殊,发酵过程中培养基的挥发量较大,在 500 mL 摇瓶中,培养基装量少于 70 mL 时无法进行测定,因此考察了装液量为 80 mL 以上的情况。图 6 显示,*T. aurantiacus* WSH 03-01 的生长对氧的需求不高,相对较低的溶氧水平有利于菌体生长,但装液量的变化对 CAT 的合成有较大影响,当装液量为 80 mL 时,CAT 的产量最高,达到 2325 u/mL,表明相对较高的溶氧水平对产 CAT 是有利的。

2.3.2　外源 H_2O_2 对 CAT 合成的诱导作用:H_2O_2 是 CAT 合成的直接诱导剂,它对微生物的作用是双重的。一方面,外源 H_2O_2 能直接进入细胞,有可能通过诱导作用促进微生物合成 CAT;另一方面,H_2O_2 具有强氧化性,毫摩尔/升级的 H_2O_2 就会抑制微生物的生长,因此超过一定浓度时它就成为微生物的生长抑制剂。实验发现,*T. aurantiacus* WSH 03-01 对 H_2O_2 的耐受能力远远高于一般微生物:在发酵初始加入 0.4%(约 118 mmol/L)的外源 H_2O_2,菌体依然生长良好,适宜浓度的 H_2O_2 甚至能促进菌体

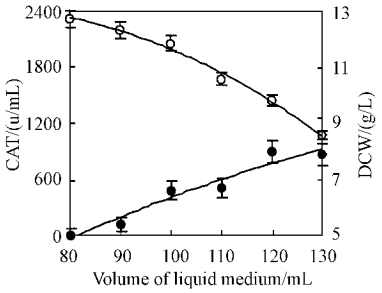

图 6 培养基装液量对 CAT 合成的影响

Fig.6 Effect of volume of medium on CAT fermentation

○ CAT；● DCW

生长(图 7)。对 CAT 合成的诱导效果则以在发酵初始加入 0.3%的 H_2O_2 为最好，发酵结束时发酵液中的 CAT 酶活可达 2762 u/mL。

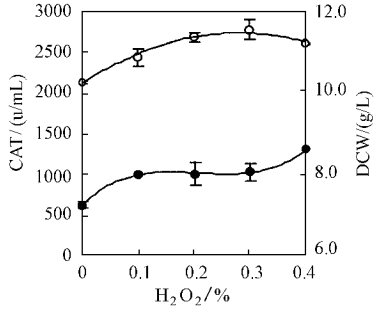

图 7 添加外源过氧化氢对 CAT 合成的影响

Fig.7 Effect of H_2O_2 addition on CAT fermentation

○ CAT；● DCW

2.4 培养基初始 pH 对 CAT 合成的影响

改变发酵培养基的初始 pH 对 *T. aurantiacus* WSH 03-01 菌体生长无较大影响(数据未显示)，但对于 CAT 的合成有一定影响。图 8 显示，适合 *T. aurantiacus* WSH 03-01 产 CAT 的培养基初始 pH 在一略偏碱性的范围内(7.0～7.6)。虽然真菌的生存环境一般偏酸性，但 *T. aurantiacus* WSH 03-01 属嗜热菌，其耐高温的细胞结构可能使得它对于环境 pH 的改变也会有一定的耐受性。

2.5 酶应用潜力的研究

根据 CAT 在纺织行业中的应用条件(表 1)，将 *T. aurantiacus* WSH 03-01 所产 CAT 加入到一定条件下(温度分别为 70℃、80℃、90℃，pH 为 9.0、10.0、11.0)的模拟漂白浴液中，维持不同时间后测定 CAT 的残余活性，并与其它来源的 CAT 进行比较，由此来评价 *T. aurantiacus* WSH 03-01 所产 CAT 的热和碱稳定性(图 9)。在 70℃、pH9.0(或 10.0)的浴液中处理 60 min，*T. aurantiacus* WSH 03-01 所产 CAT 活性基本无变化；pH 11.0 下处理 60 min 后 CAT 的残余活性仍有 90%。浴液温度升高到 80℃后，*T. aurantiacus* WSH 03-01 所产 CAT 的碱稳定性有所下降，但在 pH9.0 和 10.0 的条件下处理 60 min，仍保留 55%以上的酶活，仅在强碱性环境中(pH11.0)活性迅速下降。其余两种 CAT 则不具备耐受高温和强碱性环境的特性。其中来自牛肝的 CAT，70℃、pH 9.0 下处理 10min 后活性基本丧失，80℃时则完全失活。另一 CAT 来源于重组黑曲霉，能忍受一定程度的高温和碱性刺激(70℃，pH9.0 的条件下 60 min，酶活残余 27.5%)，但随着温度和浴液 pH 的升高，其活性也会完全丧失。90℃时，由于温度接近沸点，两种商品酶迅速失活，而 *T. aurantiacus* WSH 03-01 所产 CAT 在 pH 9.0、保温 10 min 酶活仍能检测出来(数据未显示)。由此显示，来源于 *T. aurantiacus* WSH 03-01 的 CAT 具有良好的热、碱稳定性，在纺织行业中具有很好的应用前景。

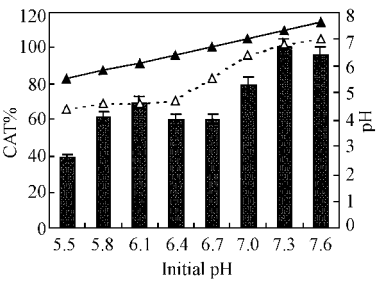

图 8 发酵培养基初始 pH 对 CAT 合成的影响

Fig.8 Effect of initial pH on CAT fermentation

■ CAT；△ Final pH；▲ Initial pH

表 1 纺织行业中应用 CAT 的条件[13]

Table 1 Bleaching effluents conditions when using CAT before dye process[13]

Process	Temperature	pH	Treatment time/min
A	60～100℃	9～11	10～30
B	60～70℃	9～10.5	10～60

3 讨论

丝状真菌在发酵液中的理想形态是菌球，菌球的大小对代谢有较大影响[14]。培养液中的营养物质(包括溶氧)是通过菌球表面向内部渗透，在 2.5 mm 深度的范围内，菌球内菌丝的生长和分枝不受限制，一旦超过 2.5 mm，氧的进入量不足将成为限制菌体增殖的因素。所以菌球的粒径应严格控制在低于 5 mm 的范围内，以利于营养成分的渗入和代谢产物向外扩散[15]。影响菌球大小的因素主要是摇床转速和接种孢子浓度，一般接种孢子浓度为 10^4

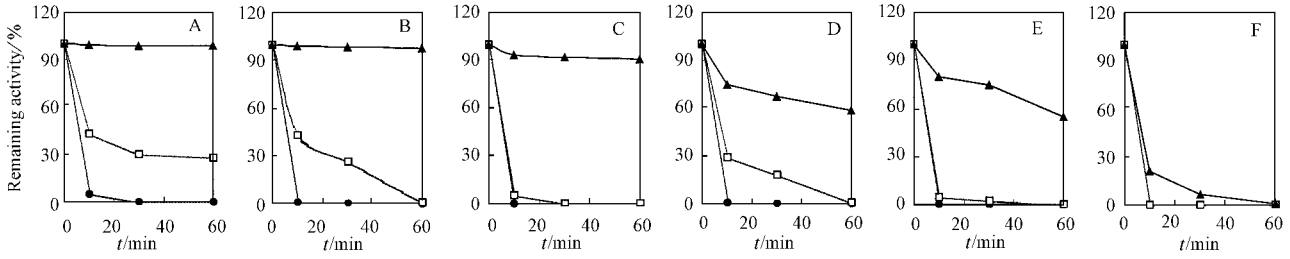

图 9　不同来源 CAT 热、碱稳定性的比较

Fig.9　Comparison of CAT activities at high temperature and alkaline condition

▲ CAT from *T. aurantiacus*；□ CAT from Aspergillus niger；● CAT from bovine liver

Panel A-C：70 ℃；pH 9.0，pH 10.0，pH 11.0；Panel D-F：80 ℃；pH 9.0，pH 10.0，pH 11.0

个/mL 较为适宜。在孢子浓度适宜的情况下，提高摇床转速可使孢子聚集体的尺寸下降，最终形成较小的菌球。

已经证实，许多微生物体内 CAT 的合成受葡萄糖分解代谢物阻遏调节[9]。以葡萄糖为碳源时，当碳浓度超过 4 g/L 时 CAT 活性便迅速下降（图 1A），表明 *T. aurantiacus* WSH 03-01 产 CAT 的过程可能也存在类似调节机制。微生物以糊精为碳源时也会产生分解代谢物阻遏作用，但这种阻遏作用较以葡萄糖为碳源时为弱。对于 *T. aurantiacus* WSH 03-01，图 1B 中糊精的碳浓度要达到 12 g/L 才会对 CAT 合成产生明显负作用也支持这一观点。因此有可能存在一个既适合细胞生长又满足 CAT 合成的适宜糊精浓度，实验确定为 20 g/L（碳浓度 8.9 g/L）。

乙醇虽然可以作为 *T. aurantiacus* WSH 03-01 生产 CAT 的唯一碳源，但以 20 g/L 糊精和 1% 乙醇为混合碳源时，CAT 酶活比以乙醇和糊精单独为碳源时的产量之和还高 23%（图 5），表明乙醇除了作为碳源外，还可能具有刺激 CAT 产生的作用。该作用可能包括两个方面：(1) 直接诱导。根据表 2 的途径 1 和 2，乙醇有可能直接诱导 CAT 的产生。(2) 间接诱导。根据表 2 的途径 3，乙醇在氧化为乙醛的同时会产生 NADH，后者在好氧条件下有可能会产生一部分 H_2O_2（正常生理条件下，约有 1% ~ 4% 的氧变成 H_2O_2[16]），其诱导效率低于直接诱导。实验发现，在含有 20 g/L 糊精的培养基中添加 1% 的乙醇或 0.3 % 的 H_2O_2，CAT 活性分别提高 1.4 倍和 1.2 倍（详细数据未给出），而 1% 的乙醇（170 mmol/L）几乎是 0.3 % 的 H_2O_2 摩尔浓度的两倍（88 mmol/L），表明乙醇对 CAT 产生的刺激作用可能是直接诱导和间接诱导双重作用的结果。在动物体内，肝脏是乙醇解毒的主要器官，其 CAT 含量往往也较高，这预示着乙醇代谢和 CAT 活性之间存在某种联系。本文发现 *T. aurantiacus* WSH 03-01 中乙醇代谢与 CAT 合成之间存在相关性，如能对这种相关性深入研究，不仅有助于进一步理解微生物产生 CAT 的生理学本质，也有助于优化 CAT 的生产过程。

水的消耗和工业生产废水的处理正在变得越来越昂贵，因此大多数染色和整理工厂正密切注视着减少水的消耗和废水排放的节约潜力。用耐高温、碱性的 CAT 在染色前处理漂白浴液，可实现水和能源相当大的节省。对 *T. aurantiacus* WSH 03-01 所产 CAT 粗酶液的研究表明，此酶的最适反应温度为 80℃（数据未显示），且具有耐受高温和强碱性环境的特性，完全可以适应苛刻的工业化应用条件，预期

表 2　生物体内的乙醇代谢途径[16]

Table 2　Metabolic pathway of ethanol in living organisms[16]

Metabolism pathway	Equations and key enzymes
1. NADPH oxidase—CAT	$NADPH + H^+ + O_2 \xrightarrow{NADPH\,oxidase} NADP^+ + H_2O_2$
	$H_2O_2 + CH_3CH_2OH \xrightarrow{CAT} 2H_2O + CH_3CHO$
2. Hypoxanthine oxidase—CAT	$hypoxanthine + H_2O + O_2 \xrightarrow{hypoxanthine\ oxidase} oxanthine + H_2O_2$
	$H_2O_2 + CH3CH_2OH \xrightarrow{CAT} 2H_2O + CH_3CHO$
3. Alcohol dehydrogenase	$CH_3CH_2OH + NAD^+ \xrightarrow{ADH} CH_3CHO + NADH + H^+$
	$CH_3CHO \xrightarrow{ADH} CH_3COOH \rightarrow acetyl\text{-}CoA \rightarrow Krebs\ cycle$

在纺织行业中将有广阔的应用前景。

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Thermo-alkali-stable Catalase from *Thermoascus aurantiacus* and Its Potential Use in Textile Bleaching Process

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Abstract The effect of growth and fermentation conditions on the production of catalase by *T. aurantiacus* WSH 03-01 was investigated in shaking flasks. Catalase activity reached 1594 u/mL when the culture was grown on a complex carbon source containing 20 g/L dextrin and 1% (*V*/*V*) ethanol, which was 23% higher than the sum produced on 20 g/L dextrin and 1% (*V*/*V*) ethanol, respectively. It was concluded that dextrin might act as a major carbon source in the complex, while ethanol was rather a stimulator than a carbon source. The stimulation effect of ethanol on catalase production was postulated to be two aspects; catalase-dependent alcohol metabolism is activated by acute alcohol, thus more catalase need to be synthesized for that use, named direct induction. As for indirect induction, which may result from little amount of H_2O_2 generation in process of NADH regeneration in respiratory chain. Peptone was shown to be a favorable nitrogen source for catalase production and its optimum concentration was found to be 10 g/L. Catalase production by *T. aurantiacus* WSH 03-01 was further improved by optimizing the initial pH, volume of medium in flasks as well as the concentration of external H_2O_2. Under the optimum culture conditions, the activity of catalase reached 2762 u/mL, which was nearly 6.8 times higher than that of the initiate conditions. Furthermore, the potential application of this novel catalase in the treatment of textile bleaching effluents was evaluated. Thermo-and alkaline stability of this catalase was compared with the commercial available catalases produced from bovine and *Aspergillus niger*. The crude enzyme from *T. aurantiacus* WSH 03-01 showed stronger stabilities at (70 ℃, 80 ℃, 90 ℃) and (pH 9.0, pH 10.0, pH 11.0) than the other two types of catalases, indicating a great application potential in the clean production process of textile industry.

Key words catalase, *Thermoascus aurantiacus*, complex carbon source, hydrogen peroxide, thermo-and alkaline stability

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Thiocyanate Elution Measurement of Relative Affinity of Phage Antibodies

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Abstract Human antibodies generated by phage antibody technology have been widely used in the immunotherapy of various diseases. Among the characteristics of these therapeutic antibodies, affinity is one of the most important determinants of their biological efficacy. The binding of an antibody and its corresponding antigen could be disrupted by thiocyanate solution of different concentrations, depend upon the affinity of the antibody. This mechanism has been adopted to determine the relative affinity of monoclonal or polyclonal antibodies in routine immunological practice. Correlation between the elution method and other techniques that measure the affinity such as equilibrium dialysis and biospecific interaction analysis (BIA) has been established. Here we describe the applications of the thiocyanate elution method in the determination of the relative affinity index (RAI) of phage antibodies (Phabs). Five clone antibodies, including 3 clones of anti-keratin antibodies (AK1, AK2 and AK3) and 2 clones of anti-HBsAg antibodies (HB1 and HB2) were selected to express Phabs and Fabs, and the RAI were determined by ELISA after thiocyanate elution. A HRP-conjugated anti-M13 was used as secondary antibody for Phabs and HRP-goat-anti-human Fab was used for Fabs. The affinity ranks of the Phabs were compared with that of the Fab fragments. The results showed that all the Phabs tested were tolerant to thiocyanate treatment. The relative affinity rank of 5 Phabs coincided well with that of their corresponding Fabs. We conclude that the thiocyanate elution can be used as an easy and rapid method to measure and compare the relative affinity of Phabs.

Key words thiocyanate elution, phage antibody, antibody affinity

The display of antibody molecules on the surface of filamentous phage that encode the antibody genes, and the selection of phage with antigen binding activities by panning, offer a powerful way of antibody production. It is one of the main techniques for human antibody preparation. Other strategies include humanization of mouse monoclonal antibodies and transgenic mice[1-6]. Human antibodies generated by these methods have been widely used in the immunotherapy of various diseases. Among the characteristics of therapeutic antibodies, affinity has been shown in many instances to be an important determinant of the biological efficacy of the antibodies. It is well-known that many of the antibodies in a combinatorial library are likely to have a lower affinity, though higher affinity antibodies can be obtained when very large libraries are used[2]. It is therefore desirable to improve the affinity *in vitro* by mimicking somatic hypermutation *in vivo* through antigen driven affinity maturation[7-10]. There is a need for methods and procedures to evaluate the antibody affinity more easily and quickly so as to select high affinity phage antibodies (Phabs) more conveniently. This provides a series of technical and experimental challenges.

Thiocyanate elution is a traditional method using elution ELISA for the determination of relative affinities of antibodies[11,12]. Correlation between the elution method and other measuring techniques such as equilibrium dialysis and biospecific interaction analysis (BIA) has been achieved[13]. The mechanism of the elution method is that the binding of an antibody with its corresponding antigen could be disrupted by thiocyanate solution of different concentrations, depend upon the affinity of the antibody. To our knowledge, there has been no report of using thiocyanate elution method in phage antibody technology. Here we describe the applications of thiocyanate elution to measure the relative affinity index (RAI) of Phabs.

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Abbreviations: AMP, Ampicillin; Fab, fragment of antigen binding; HRP, horseradish peroxidase; pfu, plaque-forming unit; Phab, phage displaying antibody; PBS, Phosphate-buffered saline; RAI, Relative affinity index

1 Materials and Methods

1.1 Antibodies, antigens and other reagents

Five Phab clones, including 3 clones of anti-keratin antibodies (AK1, AK2 and AK3)[14] and 2 clones of anti-HBsAg antibodies (HB1 and HB2)[15] were developed previously by our group. Phagemid vector p3MH was derived from pCOMB3H by adding 9E10/c-myc epitope and histidine tail to the 3′ end of Fd. Human epidermal keratin (5 mg/mL) was extracted and purified from normal human epidermis as previously described[16]. Recombinant HBsAg was purchased from National Vaccine and Serum Institute (Beijing). Ammonium thiocyanate (NH_4SCN) is a product of Sigma. HRP-goat-anti-human Fab was purchased from Pierce. HRP-anti-M13 was purchased from Pharmacia Biotech. The *E. coli* strain XL1-Blue and helper phage VCSM13 were purchased from Stratagene.

1.2 Preparation of phage antibodies

The XL1-Blue stocks harboring Phab expression vectors were plated on standard LB agar plates supplemented with 100 μg/mL ampicillin and incubated overnight at 37℃. Single colonies were inoculated into 1.5 mL LB broth containing 0.1% glucose and 100 μg/mL ampicillin and grown with shaking at 37℃ until the A_{600} of 0.6 was reached. One hundred microliters of the culture was transferred into 10 mL SB-AMP (super broth medium containing 30g tryptone, 20g yeast extract and 10g Mops buffer per liter at pH 7.0 and 100 μg/mL ampicillin) and shaken at 37℃ for 1 hour. Then 30 μL of helper phage VCSM13 (2×10^{11} pfu/mL) was added and the culture was continued to shake at 30℃ overnight. Supernatants containing Phabs of the 5 clones were tested for antigen binding by ELISA. The titers of the Phabs were measured and adjusted to 2×10^{12} cfu/mL, stored at 4℃ for future use.

1.3 Preparation of soluble Fab fragments

Soluble Fab expression vectors were constructed by removing gene 3 fragment from Phab vectors by *Spe* Ⅰ/*Nhe* Ⅰ double digestion followed by self-ligation. XL1-Blue cells containing Fab expression vectors were cultured similarly to the preperation of phage antibodies, and induced to express Fabs by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mmol/L. The cultures were grown overnight at 30℃. Supernatants containing soluble Fabs were harvested and tested for antigen binding by ELISA. The concentrations of the Fabs were measured by ELISA using purified human IgG as reference to plot the standard curve.

1.4 Effects of NH_4SCN on phage ELISA

The 96-well microtiter plates (Costar, Cambridge, MA, USA) was coated at 4℃ with 50 μL of VCSM13 (1×10^{10}) overnight. After incubation with blocking buffer (3% BSA in PBS), 50 μL of 0 ~ 6 mol/L NH_4SCN was added and incubated at room temperature for 15 min. The plates were washed 3 times with TPBS (PBS containing 0.05% Tween20) and then incubated at 37 ℃ with HRP-goat-anti-M13 for 1 h. Following another 3 washes with TPBS, 100 μL OPD (*o*-phenylenediamine) substrate was added and color development was monitored at 490 nm.

1.5 Measurement of relative affinity

Relative affinity index (RAI) was measured by ELISA with the inclusion of an extra step of thiocyanate elution in standard ELISA. Keratin or HBsAg antigen was coated onto 96-well microtiter plates (Costar) by incubating 50 μL/well of 1 μg/mL dilutions in 50 mmol/L sodium hydrogen carbonate, pH 9.6, at 4℃ overnight. The plates were then washed 3 times with PBS, blocked with 300 μL/well of 3% (*W*/*V*) bovine serum albumin (BSA) in PBS at 37℃ for 1 hour. Anti-keratin or anti-HBsAg Phabs or soluble Fabs (50 μL /well) were dispensed in duplicate into the corresponding antigen coated wells. After an 2 hour incubation at 37℃ and the subsequent 3 washes with TPBS, ammonium thiocyanate solution was added to the appropriate wells (60 μL/well) in duplicate in concentrations ranging from 0 to 6 mol/L. The plates were allowed to stand for 15 min at room temperature, followed by 3 washes in TPBS. Fifty microliters of peroxidase-conjugated goat anti-M13 or anti-human Fab diluted 1∶2000 in 1% BSA-PBS was added to each well, and the plates were incubated at 37℃ for 1 hour. After the 3 washes, 100 μL OPD substrate was added to each well. The reaction was stopped by adding 80 μL of 20% H_2SO_4. The absorbance of the solution at 490 nm was measured using an automatic microtiter plate reader. The RAIs of the tested antibodies were calculated according to Macdonald *et al*[12].

2 Results

2.1 Effects of NH_4SCN on phage ELISA

In order to determine the relative affinity of phage antibodies by elution ELISA we first tested if the incubation of NH_4SCN with coated phage would influence the ELISA signal detected by anti-M13 antibody. VCSM13 was coated on ELISA plate and different concentrations of NH_4SCN were used to treat the phage coated wells during standard ELISA. The results showed that the treatment of NH_4SCN up to 6 mol/L had no effect on the ELISA signals (Fig.1), indicating NH_4SCN did not affect the integrity of phage.

2.2 Measurement of relative affinity

The relative affinities of both Fabs and Phabs of 3 anti-keratin clones (AK1, AK2 and AK3) and 2 anti-

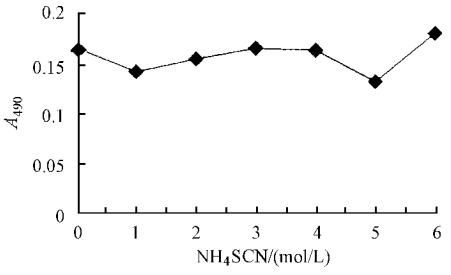

Fig.1 Effects of 0 ~ 6 mol/L NH_4SCN treatment on ELISA of phage

HBsAg clones (HB1 and HB2) were investigated using the thiocyanate elution ELISA. Fig.2 is the measurement of the relative affinities of anti-keratin Phabs. Upon adding increasing concentrations of NH_4SCN, the RAIs of anti-keratin Phabs dropped significantly. Fig.3 shows the curves produced for anti-keratin Fabs, and a good correlation with the results of the corresponding Phabs was demonstrated. Fig.4 and Fig.5 show the curves produced for anti-HBsAg Phabs and Fabs respectively. It could be educed that the relative affinities of anti-HBsAg Phabs coincided very well with that of their corresponding Fabs. The RAIs were calculated as the molar of NH_4SCN required to produce a 50% reduction of A_{490}. The results from the test are summarized in Table 1. RAIs of the Fabs and Phabs can be ranked in the same order for both anti-keratin (AK3 > AK2 > AK1) and anti-HBsAg (HB2 > HB1), although their RAI values were different. These results indicated that the relative affinity of phage antibodies are readily detected by thiocyanate elution ELISA.

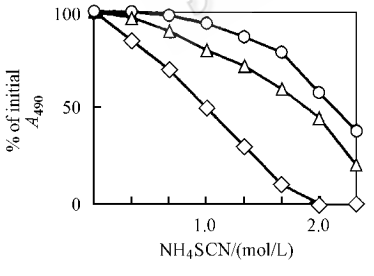

Fig.2 Relative affinities of anti-keratin Phabs
—□— AK1; —△— AK2; —○— AK3

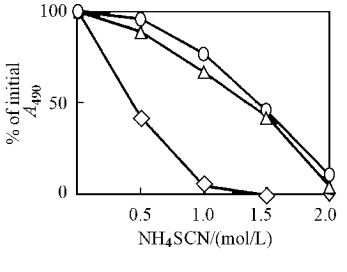

Fig.3 Relative affinities of anti-keratin Fabs
—□— AK1; —△— AK2; —○— AK3

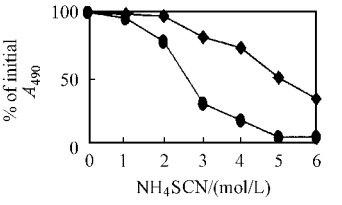

Fig.4 Relative affinities of anti-HBsAg Phabs
—●— HB1; —◆— HB2

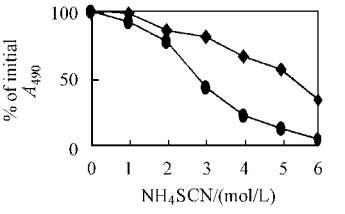

Fig.5 Relative affinities of anti-HBsAg Fabs
—●— HB1; —◆— HB2

Table 1 Relative affinity index of anti-keratin and anti-HBsAg antibodies measured by thiocyanate elution

Antibody clones	RAIs for Fabs/(mol/L)	RAIs for Phabs/(mol/L)
anti-keratin		
AK1	0.48 ± 0.001	0.90 ± 0.008
AK2	1.35 ± 0.002	1.98 ± 0.012
AK3	1.51 ± 0.010	2.32 ± 0.002
anti-HBsAg		
HB1	2.56 ± 0.014	2.77 ± 0.086
HB2	5.01 ± 0.032	5.48 ± 0.038

3 Discussion

Phage display technology offers a powerful way of making antibodies, especially the therapeutic human antibodies. The produced therapeutic antibodies are expected to have high affinity. If the affinity level of the therapeutic antibodies is not desirable, there are ways of improving it. Such strategies include improving the diversity and the content of the library[2,17-19], applying the mutant technique[8,10,20] or chain-shuffling[7] to achieve the *in vitro* affinity maturation or using various panning procedures to selectively isolate the high affinity antibodies[21,22]. In the experiments of antibody affinity maturation, a method that can evaluate the antibody affinity easily and quickly is needed to select the best ones from many isolated binders. Up to date the obtained phage antibodies from panning should be engineered into soluble Fabs to determine their affinities. However, that would be very time consuming. What is more, if the antibody molecule is scFv, generally it can not be measured by ELISA due to the lack of proper enzyme-conjugated bridge antibody. To deal with such problems, we tried to establish a simple method for the direct measurement and comparison of affinity levels of Phabs.

Pullen *et al*[11] reported an ELISA method using

thiocyanate elution to determine relative avidities of anti-rubella virus antibodies in sera from naturally infected and immunized persons, based on an index representing 50% effective antibody binding. In a later communication from the same laboratory, disruption of antigen-antibody binding by thiocyanate as a means of determining relative affinities of conventional McAbs was examined in more details. When compared with the determination by equilibrium dialysis method, a significant correlation was observed between the affinity ranking orders obtained using the two methods, thereby demonstrating the applicability of the elution technique[12]. The simple method has been widely used since to assess the affinity or avidity levels of both monoclonal antibodies and polyclonal antibodies or antiserum in numerous investigations[23-26]. McCloskey *et al*[13] utilized the thiocyanate elution method and compared with the most advanced biospecific interaction analysis (BIA) to analyze the affinity data of a panel of antibodies. A good agreement between the two methods was achieved by ranking the affinities in the same order, further suggesting that the simple elution ELISA is feasible and reliable. We have once employed the thiocyanate elution for the detection of relative affinities of genetically engineered Fabs and it proved to be a very useful technique[14], but we are still uncertain whether this method can be used for the evaluation of relative affinities of antibody fragments displayed on the phage surface. In the present study, we first proved that the ELISA reaction against phage was not affected by NH_4SCN treatment and the thiocyanate elution method was used to detect the RAIs of Phabs directly and compare their corresponding Fabs. The results ranked the affinity order of the tested Phabs as the same to the Fabs of both anti-keratin and anti-HBsAg antibodies. The relative simplicity of the technique and the ease with which the solid phase elution assay can be performed make it suitable for screening large numbers of Phabs isolated from phage antibody library, thus providing affinity data on a scale not previously possible.

To summarize, we have shown a good correlation between relative affinities of Phabs and Fabs measured by thiocyanate elution. It is the first report of a direct method to detect and compare the relative affinities of Phabs and will surely be useful in the phage antibody technology.

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