原 创 性 声 明

本人郑重声明：所呈交的学位论文，是本人在导师的指导下，独立进行研究所取得的成果。除文中已经注明引用的内容外，本论文不包含任何其他个人或集体已经发表或撰写过的科研成果。对本文的研究作出重要贡献的个人和集体，均已在文中以明确方式标明。本声明的法律责任由本人承担。

论文作者签名：邵凌健　　日　期：2010.11.29

关于学位论文使用授权的声明

本人完全了解山东大学有关保留、使用学位论文的规定，同意学校保留或向国家有关部门或机构送交论文的复印件和电子版，允许论文被查阅和借阅；本人授权山东大学可以将本学位论文的全部或部分内容编入有关数据库进行检索，可以采用影印、缩印或其他复制手段保存论文和汇编本学位论文。

(保密论文在解密后应遵守此规定)

论文作者签名：邵凌健　导师签名：[illegible]　日　期：2010.11.29

目 录

摘 要 1
ABSTRACT 5
符号说明及缩略词 10
第一章 绪 论 11
1.1 生物表面活性剂概述 11
1.2 槐糖脂的结构 14
1.3 产槐糖脂的菌株 14
1.4 槐糖脂发酵条件的优化 15
1.4.1 补料发酵提高产量 15
1.4.2 以工农业废弃物为底物发酵槐糖脂 16
1.4.3 培养基组成对于槐糖脂结构的影响 16
1.4.4 下游工艺的优化 18
1.5 槐糖脂的结构修饰 18
1.5.1 脂肪酸部分的修饰 19
1.5.2 槐糖部分的修饰 22
1.6 槐糖脂产生菌在基因工程方面的研究进展 24
1.6.1 槐糖脂代谢过程中相关基因的克隆 25
1.6.2 转化及筛选系统的建立 25
1.6.3 基因敲除 26
1.7 槐糖脂在医药领域的应用 26
1.7.1 槐糖脂的抗菌活性 26
1.7.2 槐糖脂的抗肿瘤活性 27
1.7.3 槐糖脂的抗炎症反应 28
1.7.4 槐糖脂的抗病毒活性和杀精子活性 29
1.8 槐糖脂在环境修复中的应用 30
1.8.1 疏水性有机污染物的生物修复 30
1.8.2 土壤中重金属离子的去除 30

1.8.3 减轻有害藻华的危害.....30
1.9 本论文的研究目的和主要研究内容.....31
第二章 补料发酵生产槐糖脂.....33
引言.....33
2.1 材料和方法.....33
2.1.1 菌株.....33
2.1.2 仪器和试剂.....34
2.1.3 培养基.....34
2.1.4 培养方法.....34
2.1.5 检测方法.....35
2.2 结果与讨论.....38
2.2.1 摇瓶分批发酵生产槐糖脂.....38
2.2.2 摇瓶补料发酵生产槐糖脂.....39
2.2.3 发酵罐补料发酵生产槐糖脂.....40
2.2.4 提高发酵罐转速对补料发酵生产槐糖脂的影响.....41
2.3 本章小结.....42
第三章 槐糖脂的抑菌作用.....44
引言.....44
3.1 材料和方法.....44
3.1.1 仪器和试剂.....44
3.1.2 培养基.....45
3.1.3 样品.....45
3.1.4 抑菌试验菌株.....45
3.1.5 槐糖脂对细菌的抑菌曲线的绘制.....45
3.1.6 槐糖脂对皮肤癣菌的抑制作用.....46
3.1.7 槐糖脂对皮肤癣菌的菌丝延伸抑制率.....46
3.1.8 最小抑制浓度 (MIC) 和最小杀灭浓度 (MFC).....46
3.1.9 透射电镜观察药物作用后皮肤癣菌菌丝的变化.....47
3.2 结果与讨论.....47

3.2.1 槐糖脂对细菌的抑制作用.....47
3.2.2 槐糖脂对皮肤癣菌的抑制作用.....50
3.3 本章小结.....61
第四章 槐糖脂的分离纯化、结构鉴定及抗肿瘤活性.....63
引言.....63
4.1 材料和方法.....64
4.1.1 仪器和试剂.....64
4.1.2 槐糖脂生产菌株.....65
4.1.3 抗肿瘤研究所用的肿瘤细胞株.....66
4.1.4 培养基.....66
4.1.5 槐糖脂的提取.....67
4.1.6 粗提槐糖脂样品的分离纯化与制备.....67
4.1.7 MS 分析.....68
4.1.8 细胞传代培养方法.....68
4.1.9 槐糖脂样品的稀释.....68
4.1.10 MTT 法分析细胞存活率.....69
4.1.11 数据分析.....69
4.2 结果.....69
4.2.1 HPLC 分离纯化槐糖脂.....69
4.2.2 各槐糖脂组分结构鉴定.....70
4.2.3 乙酰基数量对槐糖脂抑制食管癌细胞作用的影响.....72
4.2.4 脂肪酸不饱和程度对槐糖脂抑制食管癌细胞作用的影响.....72
4.2.5 酸型槐糖脂对食管癌细胞的抑制作用.....73
4.3 讨论.....74
4.4 本章小结.....75
第五章 不同结构的槐糖脂诱导食管癌细胞系 KYSE450 细胞凋亡的研究.....77
引言.....77
5.1 材料和方法.....78
5.1.1 仪器和试剂.....78

5.1.2 样品的稀释.....79
5.1.3 细胞株.....79
5.1.4 细胞传代培养方法.....79
5.1.5 细胞的爬片.....79
5.1.6 阴性对照组和空白对照组的设立.....79
5.1.7 倒置相差显微镜观察样品作用后的细胞形态.....80
5.1.8 Giemsa 染色观察样品作用后的细胞形态.....80
5.1.9 吖啶橙染色观察样品作用后的细胞形态.....80
5.1.10 透射电镜观察样品作用后的细胞形态.....80
5.1.11 TUNEL (TdT-mediated dUTP nick end labeling) 检测细胞凋亡.....80
5.1.12 流式细胞技术检测细胞凋亡率及周期分布.....81
5.2 结果.....81
5.2.1 倒置相差显微镜观察槐糖脂作用后食管癌细胞形态的变化.....81
5.2.2 Giemsa 染色观察槐糖脂作用后食管癌细胞形态的变化.....82
5.2.3 吖啶橙染色观察槐糖脂作用后食管癌细胞形态的变化.....83
5.2.4 透射电镜观察槐糖脂作用后食管癌细胞细胞核形态的变化.....83
5.2.5 TUNEL 检测食管癌细胞的凋亡.....84
5.2.6 流式细胞技术检测槐糖脂作用后食管癌细胞的凋亡率及周期分布.....85
5.3 讨论.....86
5.4 本章小结.....87
全文总结与展望.....88
参 考 文 献.....90
攻读学位期间已发表和录用的论文.....99
致 谢.....100

摘　要

生物表面活性剂是微生物在一定条件下培养时，在其代谢过程中分泌出的具有一定表面活性的代谢产物。与化学合成的表面活性剂相比，生物表面活性剂除具有降低表面张力、稳定乳化液和增加泡沫等相同作用外，还具有一般化学合成的表面活性剂所不具备的无毒、生物可降解性、无污染、良好的选择性、专一性和生物相容性等优点。随着人类社会的进步与发展和人们环保意识的增强，生物表面活性剂部分取代化学合成表面活性剂，在人类生产、生活中发挥重要作用将成为必然趋势。

槐糖脂是一种由酵母产生的糖酯类生物表面活性剂，由于其生物可降解性，低毒性，良好的环境兼容性，以及产量高等很多优点，在石油工业、环境保护、化妆品、洗涤剂及药学领域都得到了广泛的应用。近年来，槐糖脂在医药领域的应用尤其得到了研究者的关注，很多报道证明了槐糖脂具有很好的抗微生物、抗肿瘤甚至抗艾滋病毒的活性。

本文的主要研究内容及结果如下：

1. 补料发酵提高槐糖脂的产量

在本实验室前期筛选到一株高产槐糖脂拟威克酵母菌株，并开展了部分发酵条件优化研究的基础上，本文通过补料发酵的方法，在发酵过程中向培养基中补加菜籽油提高了槐糖脂的产量。300 mL 摇瓶补料发酵，在发酵过程中补加三次5%菜籽油后，槐糖脂产量为 43.1 g/L，比分批发酵的槐糖脂产量提高了 32%。在 5 L 发酵罐中扩大生产槐糖脂，补料发酵 288 h 后，槐糖脂产量为 68.2 g/L，比摇瓶分批发酵提高了 109.2%，比摇瓶补料发酵产量提高了 58.2%。将 5 L 发酵罐的转速从 400 rpm 提高到 500 rpm，发酵 196 h 后，槐糖脂产量达到 71.1 g/L，与提高转速之前相比，槐糖脂的产量略高一点，但是发酵周期大大缩短，由 288 h 缩短到 196 h， 提前了 96 h，槐糖脂的容积生产率由 0.24 g/L/h 提高到 0.37 g/L/h。

2. 槐糖脂对细菌抑制作用的研究

研究了槐糖脂对几种常见细菌大肠杆菌（*Escherichia. coli*）、蜡状芽孢杆菌（*Bacillus. cereus*）、金黄色葡萄球菌（*Staphylococcus. aureus*）以及能引起龋齿的变形链球菌（*Streptococcus mutans*）的抑制作用。结果发现槐糖脂对革兰氏阳性菌有明显的抑制作用，当槐糖脂浓度达到 15 mg/L 时就能够完全抑制蜡状芽孢杆菌的生长。与对金黄色葡萄球菌的最低抑制浓度 40 mg/L 相比，槐糖脂对蜡状芽孢杆菌的最低抑制浓度更小。槐糖脂对大肠杆菌几乎没有抑制作用。首次报道了槐糖脂对引起龋齿的变形链球菌具有明显的抑制作用。当槐糖脂浓度达到 50 mg/L 时就能够完全抑制变形链球菌的生长，将其添加到牙膏或漱口水中可以有效的防治龋齿的发生。

3. 槐糖脂对皮肤癣菌抑制作用的研究

研究了槐糖脂对红色毛癣菌、石膏样毛癣菌和犬小孢子菌这三种常见的皮肤病致病菌的抑制作用，结果证明槐糖脂对这三种皮肤癣菌都具有抑制作用。培养 5 天时，内酯型槐糖脂完全抑制红色毛癣菌、石膏样毛癣菌和犬小孢子菌的浓度分别为 0.5 g/L、0.1 g/L 和 0.1 g/L；酸型槐糖脂完全抑制红色毛癣菌、石膏样毛癣菌和犬小孢子菌的浓度分别为 0.5 g/L、0.25 g/L 和 0.5 g/L。比较了培养 7 天后内酯型槐糖脂和酸型槐糖脂对这三种皮肤癣菌的作用效果的不同，发现对红色毛癣菌来说，内酯型槐糖脂的抑制效果比酸型槐糖脂的抑制效果好一点；对石膏样毛癣菌来说，在低浓度的条件下，内酯型槐糖脂的抑制作用要好于酸型槐糖脂，在高浓度的条件下，酸型槐糖脂的抑制作用要好于内酯型槐糖脂；对犬小孢子菌来说，内酯型槐糖脂的抑制效果比酸型槐糖脂的抑制效果好很多。

内酯型槐糖脂比酸型槐糖脂能更好的抑制三种皮肤癣菌的菌丝延伸。当内酯型槐糖脂浓度为 0.5 g/L 时，红色毛癣菌、石膏样毛癣菌和犬小孢子菌的菌丝延伸抑制率分别为 53.8%，62.5% 和 68.2%。内酯型槐糖脂对红色毛癣菌、石膏样毛癣菌和犬小孢子菌的 MIC_{50} 值分别是 0.0625, 0.125, 0.0625 mg/mL，MIC_{90} 值分别是 0.125, 0.25, 0.125 mg/mL，MFC 值分别是 0.5, 0.5, 0.25 mg/mL。

通过透射电镜观察，发现经过内酯型槐糖脂处理后，红色毛癣菌、石膏样毛癣菌和犬小孢子菌的细胞的显微结构都发生了明显改变。细胞壁变厚而且松散，

细胞质凝集，细胞器的膜消失，细胞质中没有完整的细胞器和核区。

4. 对槐糖脂粗品进行分离纯化，并鉴定槐糖脂纯品的结构

将拟威克酵母利用菜籽油为第二碳源发酵得到的槐糖脂粗品经 HPLC 分析，发现槐糖脂粗品中含有多达十几种以上的组分。其中含量较高的 10 种组分用制备型 HPLC 进行了制备，并用质谱分析这 10 种槐糖脂组分的结构。结果发现我们分离得到的槐糖脂组分的脂肪酸部分都是 18 碳脂肪酸，而它们的结构的区别在于脂肪酸部分的不饱和程度不同（无双键、一个双键或两个双键），槐糖脂部分的乙酰化程度不同（单乙酰基、双乙酰基、非乙酰化），以及是否存在内酯化（内酯型、酸型）。

5. 不同结构的槐糖脂组分对食管癌细胞抑制作用的研究

研究了不同结构的天然槐糖脂组分对食管癌细胞 KYSE 109 和 KYSE 450 的抗肿瘤活性，发现槐糖脂分子的乙酰化程度、脂肪酸部分饱和程度和是否内酯化都对其抗肿瘤活性有重要的影响。

（1）内酯型槐糖脂的抗肿瘤作用与槐糖部分的乙酰基的数量有关，双乙酰基内酯型槐糖脂对食管癌细胞的抑制效果（30 μg/mL 的浓度就可以完全杀灭细胞）好于单乙酰基内酯型槐糖脂（60 μg/mL 的浓度能够完全杀死细胞）。

（2）首次报道了内酯型槐糖脂的抗肿瘤作用与脂肪酸部分的不饱和程度有关。脂肪酸部分含有 1 个双键的内酯型槐糖脂分子对食管癌细胞具有最强的细胞毒性（30 μg/mL 的浓度就可以完全杀死细胞），脂肪酸部分含有 2 个双键的内酯型槐糖脂分子对食管癌细胞的抑制作用稍弱（60 μg/mL 的浓度能够完全杀死细胞），脂肪酸部分没有双键的内酯型槐糖脂分子对食管癌细胞的细胞毒性最弱（60 μg/mL 的浓度只能够抑制 20%的细胞生长）。

（3）酸型槐糖脂无论槐糖部分是双乙酰基还是单乙酰基，脂肪酸部分是一个双键还是两个双键，其对食管癌细胞都基本没有抑制作用。

6. 不同结构槐糖脂对于食管癌细胞 KYSE 450 抗肿瘤作用机制的研究

实验室之前的研究证明，脂肪酸部分为 C18:1 的双乙酰内酯型槐糖脂抑制人

肝癌细胞 H7402 的机制是引起细胞的凋亡。本文选取了 C18:1 MLSL 和 C18:1 DLSL 两种槐糖脂，以食管癌细胞 KYSE 450 作为研究对象，采用多种检测方法，细胞形态学观察（如光学显微镜观察、电子显微镜观察、荧光显微镜观察），流式细胞技术检测细胞周期分布及凋亡率以及 DNA 裂解的原位检测对不同结构槐糖脂对于食管癌细胞 KYSE 450 的抗肿瘤作用和机制进行了研究。

通过形态学观察，槐糖脂作用后， 细胞形态发生了变化，细胞收缩、变圆、体积变小、膜泡突出并且出现一些小体结构，有些细胞从培养板壁脱离下来。细胞核染色质凝集，凝聚于核膜内侧，发生边缘化，有的细胞核碎裂成多个块状，DNA 片断化，并出现膜包绕的凋亡小体。通过流式细胞仪分析说明细胞周期的分布发生了变化，并且出现了非常明显的亚二倍体峰。以上都表明，这两种结构的槐糖脂都能够在一定浓度范围内引起食管癌细胞的凋亡，说明槐糖脂抑制肿瘤细胞的机制对于不同的肿瘤细胞具有一致性，都是引起细胞凋亡。

另外，不同结构的槐糖脂在相同浓度下引起肿瘤细胞凋亡的水平也不相同，无论从细胞形态观察还是从细胞周期的变化以及凋亡率的大小，都可以说明，相同浓度的 C18:1 DLSL 比 C18:1 MLSL 能诱导食管癌细胞 KYSE 450 更大程度的发生凋亡。这一点也与双乙酰基的内酯型槐糖脂对食管癌细胞的抑制效果优于单乙酰基的内酯型槐糖脂的结果相符合。

关键词：生物表面活性剂，槐糖脂，拟威克酵母，补料发酵，抑菌作用，抗肿瘤作用，细胞凋亡

Abstract

Biosurfactants are the metabolic products of some microorganisms and have many advantages including low toxicity, good biodegradability and biocompatibility compared with synthetic surfactants by chemical methods. In order to meet the demands for environment protection, biosurfactants will play more and more role than chemically synthetic surfactants in our daily life.

Sophorolipids are glycolipid biosurfactants produced by several selected variety of yeast strains. Due to their properties of low toxicity, high biodegradability biocompatibility and high yields, sophorolipids have great application prospects in petroleum industry, environmental industry, cosmetics, food, detergent industries and pharmaceutical sector. Recently, sophorolipids have been proved to have good antimicrobial, anticancer activities and even the anti-HIV activity, which will broaden the applications of sophorolipids in pharmaceutical sector.

The main research aspects and results are as following:

1. Sophorolipid production by fed-batch fermentation

After feeding 5% rapeseed oil three times, the yield of sophorolipids in fed-batch fermentation was increased by 32% than batch fermentation in 300 mL flask. In 5 L fermentor, after 288 h of fed-batch fermentation, the yield of sophorolipids was 68.2 g/L and was increased by 109.2 % more than batch fermentation in 300 mL flask and 58.2% than fed-batch fermentation in 300 mL flask. After the rotatory speed was improved from 400 rpm to 500 rpm, the yield of sophorolipids 5 L fermentor reached 71.1 g/L at 196 h, the fermentation period shortened 96h, the productivity of sophorolipids was increased from 0.24 g/L/h to 0.37 g/L/h.

2. Inhibition of sophorolipids to bacterium

The inhibition effects of sophorolipids to some bacterium including *Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus*, and *Streptococcus mutans* were studied. It was found that sophorolipids showed strong antibacterial activities against

gram-positive bacteria. 15 mg/L sophrolipids could fully inhibit the growth of *B. cereus*, and 40 mg/L sophrolipids could fully inhibit the growth of *S. aureus*. Sophorolipids showed no antibacterial activities against gram-negative bacteria *E. coli*. This is the first report that sophorolipid had strong inhibition effect to *Streptococcus mutans* which could cause decayed tooth. 50 mg/L sophrolipids could fully inhibit the growth of *Streptococcus mutans*.

3. Inhibition of sophorolipids to Dermatophytes

The inhibition of sophorolipids to three common clinical dermatophytes, *Trichophyton rubrum*, *Trichophyton gypseum*, and *Microsporum canis* were investigated. We compared the inhibition of acidic sophorolipids with that of lactonic ones to the three dermatophytes. Lactonic or acidic sophorolipids showed inhibition on the growth of all the three dermatophytes and showed different inhibition towards each of them. To *Trichophyton rubrum*, the inhibition of lactonic sophorolipids was a little better than acidic ones. Toward *Trichophyton gypseum*, when at low concentrations, the inhibition of lactonic sophorolipids was much better than that of acidic ones. However, when at high concentrations, the inhibition of acidic sophorolipids was much better than lactonic ones. Toward *Microsporum canis*, the inhibition of lactonic sophorolipids was much better than acidic ones.

For the inhibition of hypha extension, lactonic sophorolipids could inhibit the extension of hypha much better than the acidic ones. When lactonic sophorolipids concentration was 0.5 mg/mL, the inhibition ratio on hypha extension of *Trichophyton rubrum*, *Trichophyton gypseum* and *Microsporum canis* was 53.8%, 62.5% and 68.2%, respectively. The MIC_{50} of lactonic sophorolipids to the three dermatophytes was 0.0625, 0.125, 0.0625 mg/mL, respectively. MIC_{90} of lactonic sophorolipids to *Trichophyton rubrum*, *Trichophyton gypseum* and *Microsporum canis* was 0.125, 0.25, 0.125 mg/mL respectively. MFC of lactonic sophorolipids to *Trichophyton rubrum*, *Trichophyton gypseum* and *Microsporum canis* was 0.5, 0.5, 0.25 mg/mL respectively.

The TEM observation results indicated that, after being treated by lactonic

sophorolipids, three dermatophytes have some obvious changes in their microstructures. The cell wall became thicker and loose, the cytoplasm agglomerated, the membranes of organelles were disappearing, and no integrated organelles and clear nuclear zone were found in the cytoplasm.

4. Purification and Structure elucidation of sophorolipid

After being analysed by HPLC, it was found that the crude sophorolipids produced by *Wickerhamiella domercqiae var. sophorolipid* are a mixture composed of more than ten molecules. Ten sophorolipid molecules were separately collected by preparative HPLC. The structures of the ten sophorolipid molecules were elucidated by MS analysis. It was found that all the sophorolipid molecules are sophorolipids with C18 fatty acid. Their structures differ in acetylation degree of sophorose, unsaturation degree of hydroxyl fatty acid and lactonization or ring opening.

5. The anticancer effects of sophorolipids with different structures on human esophageal cancer cell

The anticancer effects of sophorolipids with different structures on human esophageal cancer cell KYSE 109 and KYSE 450 were investigated. It was found that the differences of sophorolipid sructure in acetylation degree of sophorose, unsaturation degree of hydroxyl fatty acid, and lactonization or not can affect the anticancer activity of sophorolipid.

(1) The results indicated that the inhibition of diacetylated lactonic sophorolipid to esophageal cancer cells (totally inhibition at 30 μg/mL concentration) was stronger than momoacetylated lactonic sophorolipid (totally inhibition at 60 μg/mL concentration), which confirmed that anticancer activity of SLs was affected by their acetylation degree in sophorose moiety.

(2) Our results showed that sophorolipid with different unsaturation degree of hydroxyl fatty acid also had different cytotoxic effects on esophageal cancer cells. Sophorolipid having one double bond in fatty acid part had the strongest cytotoxic effect (totally inhibition at 30 μg/mL concentration) on esophageal cancer cells,

sophorolipid with two double bonds had a little weaker anticancer effect (totally inhibition at 60 μg/mL concentration), while sophorolipid with no double bond had the weakest cytotoxic effect (only 20% of cells were inhibited at 60 μg/mL concentration) among the three sophorolipid molecules. This was the first study to reveal the relationship of bioactivities of natural sophorolipid molecules with different unsaturation degree in hydroxyl fatty acid and their structures.

(3) No matter acidic SL with one or two double bond in fatty acid part, with momoacetylated group or diacetylated groups in sophorose part, they have little anticancer effect against esophageal cancer cells.

6. The inhibition mechanism of sophorolipids with different structures on human esophageal cancer KYSE450

In our previous studies, the inhibition mechanism of diacetylated lactonic sophorolipid with a C18 momounsaturated fatty acid on the human liver cancer cells H7402 has been proved to induce cell apoptosis. The inhibition mechanism of two sophorolipid molecules C18:1 MLSL and C18:1 DLSL on human esophageal cancer cell KYSE 450 was investigated by reverse phase contrast microscopy, cell staining, fluorescence microscopy, flow cytometer, and TUNEL assay in this study.

It was found that, after being treated with C18:1 MLSL and C18:1 DLSL, cell gradually shrank, turned round, membrane blebbing stood out. Chromatin condensation and margination, nuclear fragmentation and apoptotic bodies were observed. Cell cycle distribution change was observed and the sub-G1 population appeared. These changes can demonstrate the apoptosis of KYSE 450 induced by sophrolipids and the inhibition mechanism of sophrolipids to different cancer cells was all apoptosis.

The apoptosis level of KYSE 450 induced by sophrolipid with different structure of the same concentration was different. It was found that C18:1 DLSL could induce apoptosis of KYSE 450 at a greater extent than C18:1 MLSL by morphological changes, cell cycle distribution changes and apoptosis rate of KYSE 450, which agree

with the results that the inhibition of diacetylated lactonic sophorolipid to esophageal cancer cells was stronger than momoacetylated lactonic sophorolipid.

Key words: Biosurfactant, sophorolipid, fed-batch fermentation, *Wickerhamiella domercqiae*, antibacterial activity, anticancer activity, apoptosis

符号说明及缩略词

AO	Acridine orange	吖啶橙
bp	Base pair	碱基对
CMC	Critical micell concentration	临界胶束浓度
DAB	Diaminobenzidine	二氨基联苯胺
DNS	3, 5-dinitro salicylic acid	3,5-二硝基水杨酸
DSMO	Dimethyl sulfoxide	二甲基亚砜
HPLC	High performance liquid chromatography	高效液相色谱
IL	Interleukin	白细胞介素
LB(medium)	Luria-Bertani(medium)	细菌用培养基
MFC	Minimal Fungicidal Consistency	最小杀灭浓度
MIC	Minimum Inhibitive Consistency	最小抑制浓度
MS	Mass spectroscopy	质谱
MTT	3-(4,5 -dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide	四甲基噻唑蓝
OD	Optical density	光密度
PI	Propidium iodide	碘化丙啶
SDS	Sodium dodecyl sulfate	十二烷基磺酸钠
TGF	Transformation growth factor	转化生长因子
UV	Ultraviolet spectrum	紫外光谱

第一章 绪 论

1.1 生物表面活性剂概述

生物表面活性剂是微生物在一定条件下培养时，在其代谢过程中分泌出的具有一定表面活性的代谢产物。与化学合成的表面活性剂相比，生物表面活性剂除具有降低表面张力、稳定乳化液和增加泡沫等相同作用外，还具有一般化学合成的表面活性剂所不具备的无毒、生物可降解性、无污染、良好的选择性、专一性和生物相容性等优点。随着人类社会的进步与发展和人们环保意识的增强，生物表面活性剂取代化学合成表面活性剂将成为必然趋势。

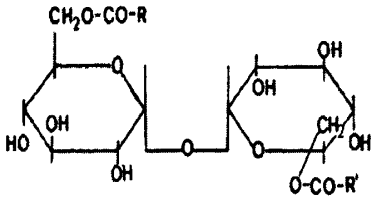
生物表面活性剂的特性决定其具有广泛的应用。在石油工业中，可以用于石油的生物降粘、脱沥青、提高原油采收率、重油污染土壤的生物修复、海洋石油污染修复等。在采矿工业中，可以用做金属螯合剂、矿石浮选的起泡剂、脱水剂、煤炭运输添加剂等。在环境保护方面，可以清除土壤中的有机物质、金属离子，促使污染物分散、乳化、增溶、生物修复等。在农业方面，可以用于土壤改良、肥料添加剂、饲料添加剂，病虫害防治，植物生长促进剂等。另外，生物表面活性剂作为稳定剂、润滑剂、分散剂、发泡剂等，在食品工业、化妆品、纺织、涂料等精细工业和医药等方面也越来越受到人们的青睐(张天胜等，2005)。

微生物产生的生物表面活性剂包括许多不同的种类。依据他们的化学结构可以分为以下几类：糖脂、磷脂、脂肽、脂蛋白、脂肪酸中性脂，及一些大分子脂蛋白和脂多糖聚合物。生物表面活性剂大多来源于微生物，其结构和微生物来源都呈现出多样性 (Healy & Devine, 1996; Rosenberg & Ron, 1999; Kim *et al.*, 1999; Bento *et al.*, 2005) (表 1-1，图 1-1)。

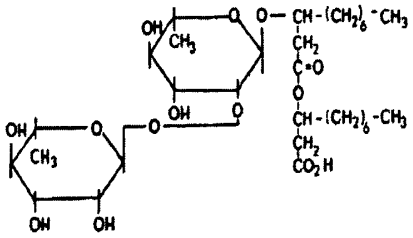
表 1-1 微生物产生的表面活性剂

Table 1-1 Biosurfactants from Microorganisms

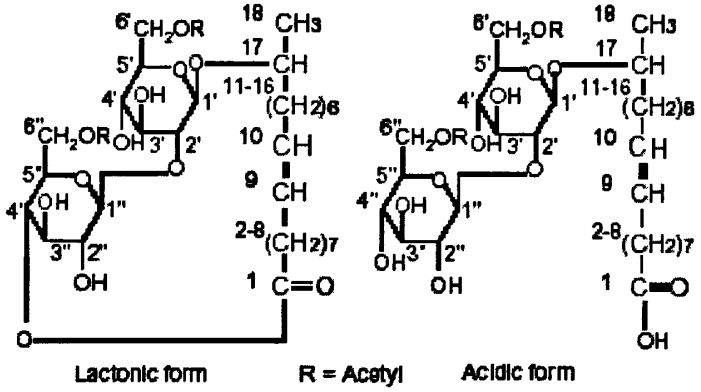
生物表面活性剂/ Biosurfactant	微生物/ Microorganisms
未知糖脂/Unidentified glycolipid	*Nocardioides* sp.(Tonkova & Gesheva, 2005)
鼠李糖脂/ Rhamnolipids	*Pseudomonas* species
葡萄糖脂/ glucose lipid	Marine bacterial strain MM1(Passeri *et al.*, 1992)
海藻糖脂/ Trehalose lipids	*Arthrobacter* species/ *Rhodococcus erythropolis*/ *Mycobacterium* sp.
槐糖脂/ Sophorolipids	*Candida* species
蔗糖脂/ Sucrose lipids	*Rhodococcus erythropolis*
果糖脂/ Fructose lipids	*Rhodococcus erythropolis*
甘露糖基赤藓糖醇脂 Mannosyl erythritol lipids	*Candida* species
磷脂/ Phospholipids	*Acinetobacter* specie/ *C. thiooxidans*
脂肽/ Lipopeptide	*Bucillus* species/ *C. petrophilium*
脂蛋白/ Protein complex	*C. tropicalis*
脂肪酸中性脂/ Fatty acid, glycerides	*Arthrobacter* species
脂蛋白和脂多糖聚合物 Proteo-lipid-carbohydrate complex	*Corynebacterium hydrocarboclastus*
戊糖脂/ Pentasaccharide	*Nocardia corynebacteroides* (Powalla et al., 1989)



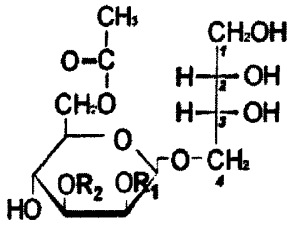
海藻糖脂



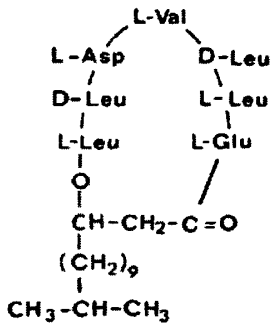
鼠李糖脂



槐糖脂



甘露糖基赤藓糖醇脂



脂肽

图 1-1 几种主要的生物表面活性剂分子结构

Fig. 1-1 The molecular formula of the main microbial surfactants

槐糖脂是一种由酵母产生的糖酯类生物表面活性剂，由于其具有生物可降解性，生物相容性、低毒性，良好的环境兼容性以及良好的抗菌抗肿瘤活性，并且可以利用可再生的原料进行生产，在所有的生物表面活性剂中，产量最高，因而引起了广泛的关注，在石油工业（Banat *et al.*，1995）、环境保护、化妆品、洗涤

剂及医药等领域都具有良好的应用前景。

1.2 槐糖脂的结构

天然合成的槐糖脂是由很多槐糖脂分子所组成的混合物。这些槐糖脂分子有一个共同的特征，分子由亲水性和疏水性两部分组成。亲水性部分是槐糖（两个葡萄糖分子以β-1, 2 糖苷键结合），疏水性部分是饱和或不饱和的长链ω-（或ω-1）羟基脂肪酸，这两部分以糖苷键相连。而它们的区别在于它们所含脂肪酸碳链的长度不同、饱和或不饱和的程度不同、槐糖部分乙酰化的程度和乙酰化位置不同，以及是否存在内酯化作用（图 1-2）（Asmer, 1988）。

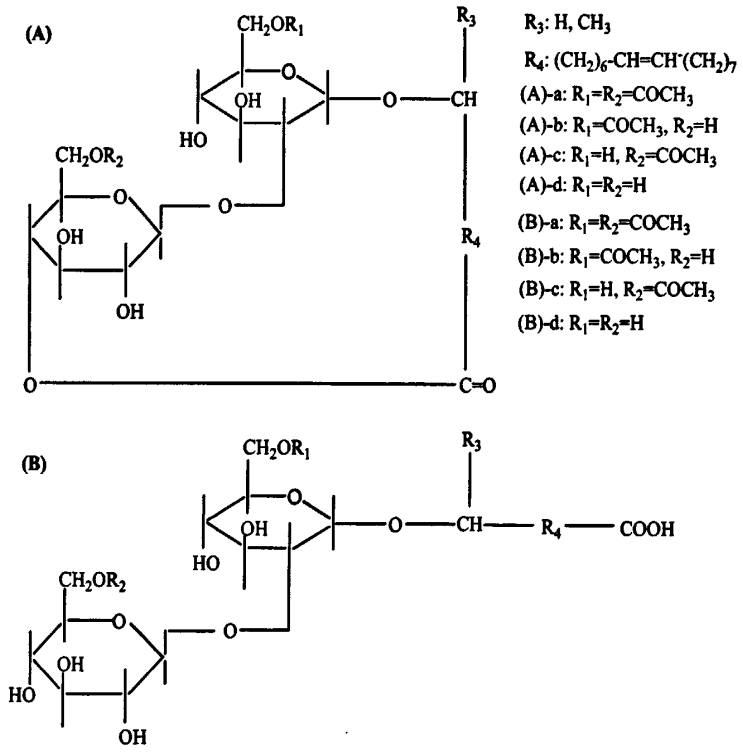


图 1-2 槐糖脂的结构。（A）内酯型槐糖脂，（B）酸型槐糖脂

Fig. 1-2 Structure of sophorolipids: (A) lactonic type; (B) acidic type.

1.3 产槐糖脂的菌株

根据文献报道，目前能够产生槐糖脂的微生物主要是球拟酵母属（*Torulopsis*）和假丝酵母属（*Candida*）的一些酵母菌，后来酵母菌鉴定系统将

球拟酵母属归类到假丝酵母属（Kreger, 1984; Hommel *et al.*, 1987）。包括*C. bombicola, C. apicola, C. bogoriensis, C. magnoliae, C lipolytica, C. gropengiesseri, C. petrophilum, Rhodotorula bogoriensis* 等菌株（Gorin PAJ *et al.*, 1961; Esders & Lights, 1972; Hommel *et al.*, 1993, 1994a; Otto *et al.*, 1999; Davila *et al.*, 1993; Hu & Ju, 2001a; Zhou *et al.*, 1992; Mulligan, 2005; Nunez *et al.*, 2004）。用于槐糖脂生产和研究最常用的菌株是*C. bombicola*。

我们实验室筛选得到了一株产槐糖脂的菌株，经过鉴定确定其为拟威克酵母，命名其为 *Wickerhamiella domercqiae var. sophorolipid*。这是国内外首次报道的能产槐糖脂的拟威克酵母菌株。

1.4 槐糖脂发酵条件的优化

槐糖脂的生产成本较高是制约其应用的一个重要因素（Rosenberg & Ron, 1999）。因此，降低槐糖脂的生产成本，提高槐糖脂的产量是进一步开发槐糖脂的新特性，并在各领域中广泛应用的先决条件。近年来，很多研究者开展了这方面的工作并取得了可喜成果。

1.4.1 补料发酵提高产量

Davila 等在槐糖脂产生阶段补加疏水性碳源，使槐糖脂产量高达 300 g/L（Davila *et al.*, 1997）。

Daniel 等采用了两步法的发酵过程，首先 *Cryptococcus curvatus* 以乳清中的乳糖为底物生长，来生产单细胞油。细胞裂解后，将细胞提取物中的甘油三酯直接用来作为第二步发酵的底物。当 *Candida bombicola* 把单细胞油利用完全后，再不断补加菜籽油作为疏水性碳源，发酵 550 h 后，最终槐糖脂产量达到 422 g/L（Daniel *et al.*, 1998）。

Rau 等用菜籽油与葡萄糖作为底物通过补料发酵以及两步连续发酵过程生产槐糖脂。优化培养条件后，产量达到了300 g/L以上，补料发酵和连续发酵的容积生产率分别为57 g/L/d和76 g/L/d（ Rau *et al.*, 2001）。作者总结了提高槐糖脂产量的几个重要因素：

1．接种的菌体一定要达到稳定期。

2．在菌种的生长期：要用铵离子作为氮源，要添加菜籽油，葡萄糖与菜籽油的质量比为50∶1。

3．在槐糖脂生产阶段：要限制氮源，葡萄糖要过量，菜籽油只能少量的过量（1 g/L），葡萄糖与菜籽油的质量比为3∶1。

2009 年，Kim 等用控制补料速率的补料发酵培养提高槐糖脂产量。作者首先研究了各种疏水性底物对槐糖脂产量的影响，确定效果最好的为菜籽油，并且确定了槐糖脂生产过程中葡萄糖的最优浓度为 30 g/L。研究发现，在控制 pH 为 3.5 的过程中，菜籽油的消耗量与 NaOH 的加入量成正比。在发酵过程中，采用菜籽油作为第二碳源，葡萄糖浓度控制在 30-40 g/L，并且通过控制 pH，计算菜籽油的补加速率。发酵 8 天后，槐糖脂产量达到 365 g/L（Kim *et al.*, 2009）。

1.4.2 以工农业废弃物为底物发酵槐糖脂

利用工农业废弃物来生产槐糖脂是降低生产成本的有效手段之一。动物脂肪、去蛋白乳清、废糖蜜都可以作为底物产生槐糖脂（Deslipande & Daniels, 1995; Daniel *et al.*, 1999; Solaiman *et al.*，2004）。2006年，Fleurackers 和Belgium 用煎炸废油为底物生产槐糖脂（Fleurackers & Belgium，2006）。2007年，Felse等用工业脂肪酸废料作为底物生产槐糖脂，并研究了脂肪酸链长和不饱和程度以及脂肪酸废料的来源对槐糖脂产量的影响（Felse *et al.*，2007），而Shah等也利用餐馆废油为底物来生产槐糖脂（Shah *et al.*, 2007a）。2008年，Daverey 和Pakshirajan 用低成本的甘蔗糖浆作为底物生产槐糖脂（Daverey & Pakshirajan，2008）。

1.4.3 培养基组成对于槐糖脂结构的影响

1994 年，Hommel 等人研究了培养基中起始铵离子浓度对槐糖脂产量以及结构的影响。随着起始铵离子浓度的增加，槐糖脂的产量不断增加，当起始铵离子浓度达到 73.6 mM 时，槐糖脂的产量大幅度下降。并且随着起始铵离子浓度的增加，含 ω-1 羟基脂肪酸的槐糖脂比例不断下降（Hommel *et al.*, 1994b）。

Cavalero 和 Cooper 以不同碳原子数的烷烃以及几种脂肪酸为第二碳源，发

酵得到的槐糖脂在产量、结构以及物理性质上都有所不同。随着第二碳源碳原子数的变化，也就是当烷烃的碳原子数由12增加到16时，槐糖脂的产量不断增加，而当烷烃的碳原子数由17增加到20时，槐糖脂的产量却不断下降。产生的槐糖脂的脂肪酸部分与其第二碳源的碳原子数相同的组分占总产物比例的情况是：十七烷最高，十六烷次之，从十二烷到十七烷不断增大，之后又不断下降。其中十六烷和十七烷所产生的槐糖脂大部分为双乙酰的内酯型槐糖脂，产物是一种晶体的形态。而其他底物产生的槐糖脂则是内酯型与酸型的混合物，产物是一种油脂的形态。在槐糖脂合成的过程中，脂肪酸主要由脂类的底物直接添加到槐糖脂分子中，而不是由细胞从头合成脂肪酸（Cavalero & Cooper，2003）。

Ashby等利用脂肪酸酯为底物生产槐糖脂来增加酸型槐糖脂的合成。首先通过大豆油和甲醇、乙醇和丙醇的转酯化作用分别合成 Me-Soy、Et-Soy、Pro-Soy 等脂肪酸酯，然后用甘油和脂肪酸酯作为发酵底物来生产槐糖脂。*Candida bombicola* 以 Me-Soy、Et-Soy、Pro-Soy 为底物通过补料分批发酵分别得到槐糖脂的产量为46 ± 4 g/L, 42 ± 7 g/L 和 18 ± 6 g/L。以Me-Soy为底物生成的槐糖脂中有71%为酸型槐糖脂，其中59%的分子仍然在脂肪酸的羧基末端带有酯基。以 Et-Soy 和 Pro-Soy 为底物分别得到的槐糖脂中分别包含43%和80%的游离酸型槐糖脂（脂肪酸部分为亚油酸和油酸，在ω-1位上通过羟基与槐糖分子相连），并且没有明显的残留的酯化作用（Ashby *et al.*, 2005）。

Shah 等以*Candida bombicola* 利用葡萄糖和花生四烯酸为底物生产出了脂肪酸部分为花生四烯酸的槐糖脂，产量为1.44 g/L。得到的槐糖脂为混合物，槐糖脂分子的结构区别在槐糖分子与花生四烯酸ω或ω-1碳原子上的羟基结合，乙酰化程度也不相同。酸水解槐糖脂得到的脂肪酸由气质联用分析其结构，为20-HETE(20-hydroxyeicosatetraenoic acid)和19-HETE(20-hydroxyeicosatetraenoic acid)，这两种物质在医药方面也有重要应用（Shah *et al.*, 2007b）。

1.4.4 下游工艺的优化

在实验室中，将槐糖脂从发酵液中分离出来的方法通常是有机溶剂萃取法，常用的有机溶剂为乙酸乙酯，萃取出槐糖脂后蒸发除去乙酸乙酯，用正己烷洗涤以除去残留油脂，再蒸干完全除去正己烷。也可以用其他溶剂代替正己烷，例如戊烷（Cavalero & Cooper，2003）和甲基叔丁基醚（Rau *et al.*，2001）。这种方法有很多缺点，包括有机溶剂成本较高，对环境有污染，操作复杂等，不适用于大规模发酵。

因为槐糖脂的密度比水大，发酵结束后会沉降到培养基的底部，在大规模发酵产生大量的槐糖脂时可以采用离心法将槐糖脂从发酵液中分离出来。离心得到的槐糖脂中残留的水分和杂质可以用多羟基醇萃取除去（Inoue *et al.*，1980）。

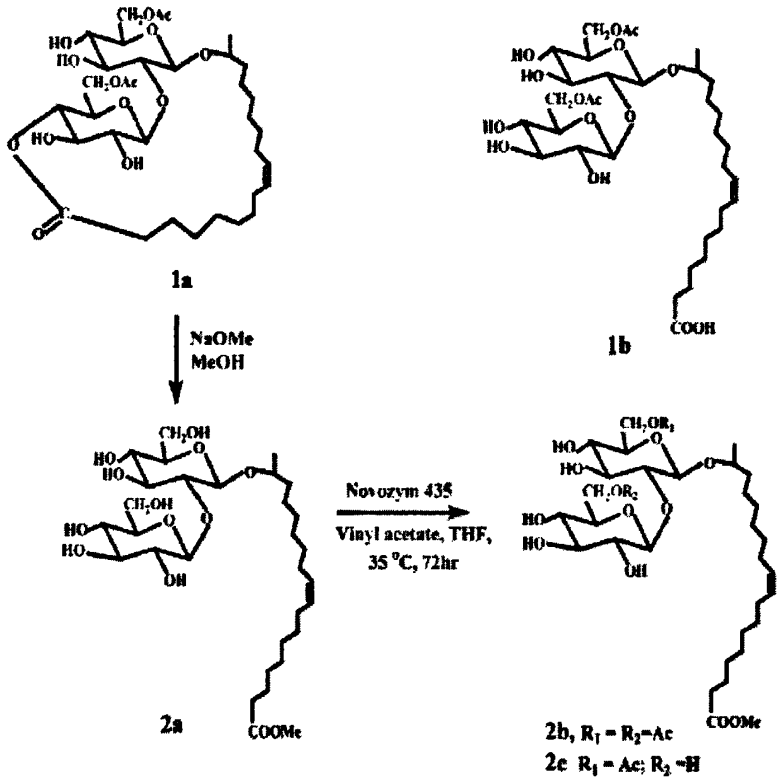
在特定发酵条件下，高浓度的内酯型槐糖脂会以晶体形式析出，更易于从发酵液中分离纯化。内酯型槐糖脂溶于乙醇且溶解度随温度的升高而增大，酸型槐糖脂微溶于乙醇，其溶解度不受温度影响，因此也可采用乙醇高温溶解内酯型槐糖脂，再降低温度使内酯型晶体析出的方法，但这种方法会导致一部分内酯型槐糖脂残存在乙醇中。Hu和Ju利用酸型槐糖脂溶于水而内酯型不溶于水特别是高pH水溶液的特性，用邻苯二甲酸盐和磷酸盐缓冲液代替乙醇作为溶剂，酸型槐糖脂溶于水中，内酯型槐糖脂结晶析出，可以很容易将内酯型槐糖脂从粗品槐糖脂中分离出来，该方法不用有机溶剂，收率较高（Hu & Ju，2001b）。

1.5 槐糖脂的结构修饰

通过酶催化的方法对槐糖脂进行结构改造也是对槐糖脂研究的一个重要方面，研究者希望通过对槐糖脂的结构改造得到新的具有更好表面活性或者生物活性的槐糖脂分子。这些结构改造包括对于槐糖脂分子上槐糖部分的修饰和对于脂肪酸分部分的修饰两方面。

1.5.1 脂肪酸部分的修饰

Nunez 等利用脂肪酶的作用，将槐糖脂的脂肪酸部分加上半乳糖分子，变为槐糖脂酸半乳糖酯。首先通过甲醇钠的作用将双乙酰化的内酯型槐糖脂脱乙酰化并且开链，变为槐糖脂甲酯。然后利用脂肪酶将槐糖脂甲酯重新乙酰化，再利用同一种脂肪酶将 1,2-3,4-di-*O*-isopropylidene-D-galactopyranose 转酯到槐糖脂的脂肪酸链上，最后水解掉 di-*O*-isopropylidene 得到槐糖脂肪酸半乳糖酯（图 1-3）（Nunez *et al.*，2003）。



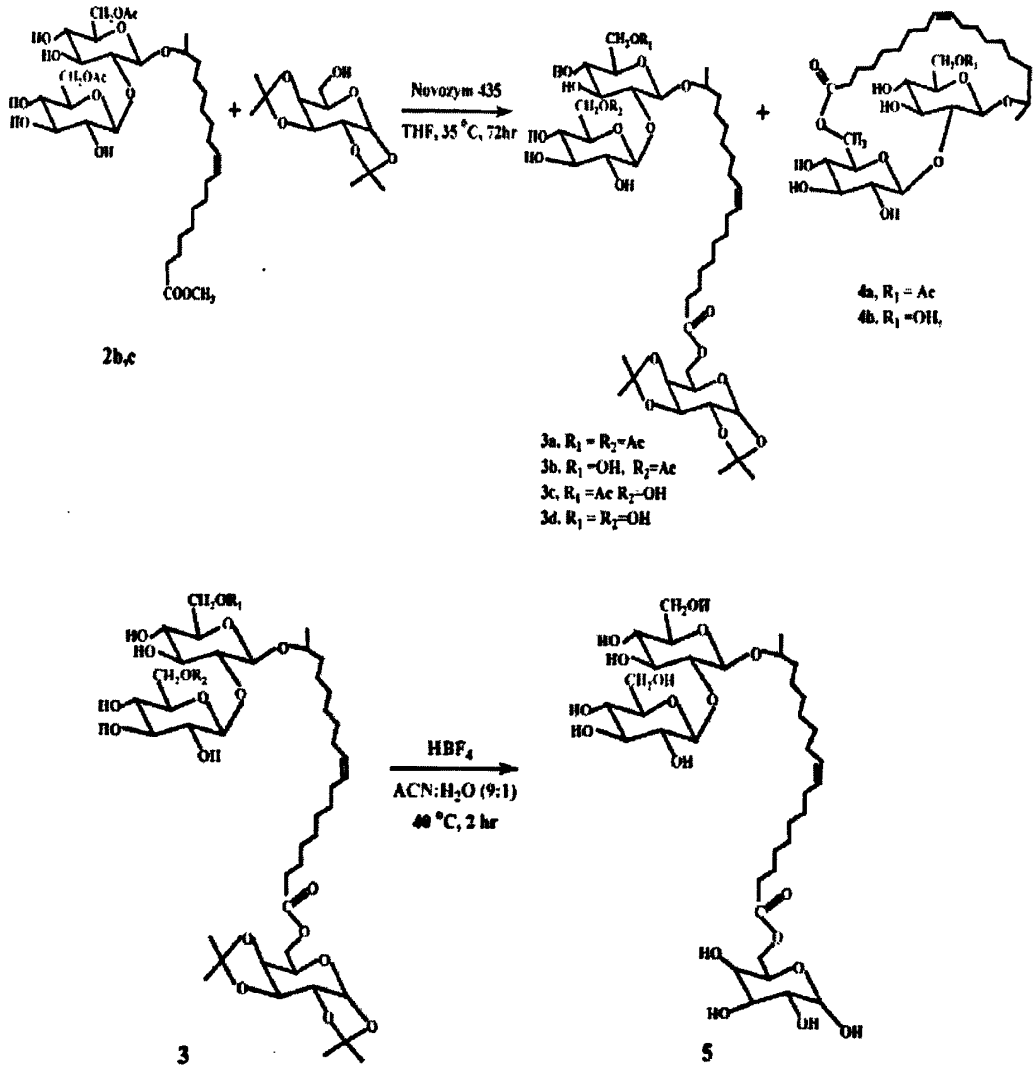


图 1-3 槐糖脂通过结构修饰变为槐糖脂半乳糖酯的过程

Fig. 1-3 Enzymatic formation of galactopyranose sophorolipid esters

Carr等利用脂肪酶的作用在槐糖脂的脂肪酸部分加上正丁基或异丁基，变为槐糖脂正丁酯或者槐糖脂异丁酯。首先通过甲醇钠的作用将双乙酰化的内酯型槐糖脂脱乙酰化并且开链，变为槐糖脂甲酯。然后将槐糖脂甲酯重新乙酰化或者丙酰化，最后在脂肪酶的作用下将槐糖脂甲酯转化为槐糖脂正丁酯或者槐糖脂异丁酯（图1-4）（Carr *et al.*，2003）。

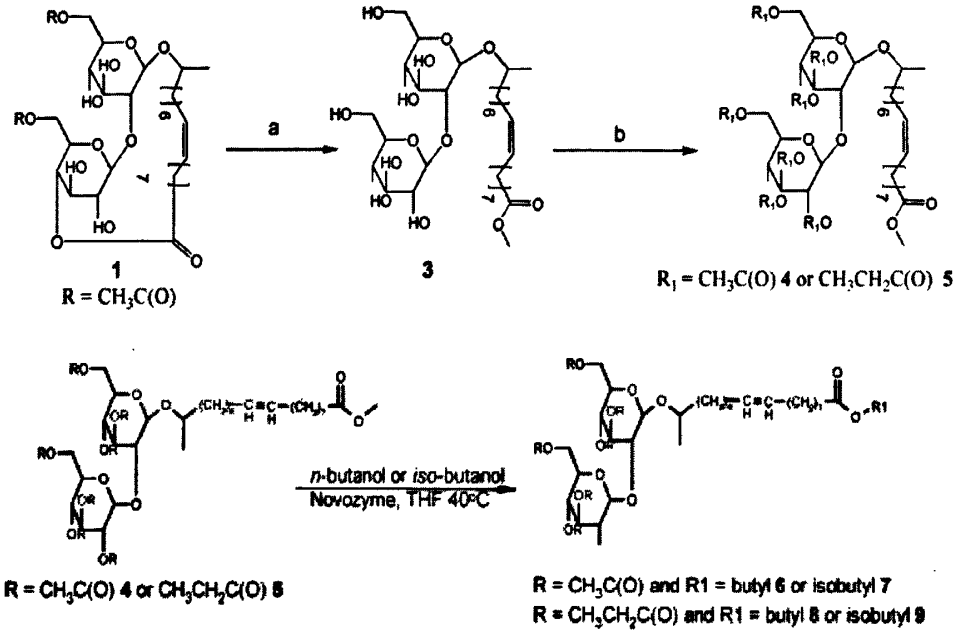


图 1-4 槐糖脂通过结构修饰变为槐糖脂正丁酯或者槐糖脂异丁酯

Fig. 1-4 Enzymatic formation of sophorolipid butyl esters and sophorolipid isobutyl esters. (a) 0.022N sodium methoxide, reflux, 30 min; (b) acetic anhydride or propionic anhydride, DMAP, dry THF.

Azim等用化学法体外合成槐糖脂的衍生物，在酸型槐糖脂的脂肪酸部分的羧基上结合各种氨基酸分子，得到了各种不同的槐糖脂的氨基酸衍生物。并且测定了不同的氨基酸-槐糖脂衍生物的生物活性，包括抑菌活性、抗病毒活性和杀精子活性，来研究槐糖脂衍生物结构与生物活性之间的关系。

制备氨基酸-槐糖脂衍生物包括以下几个步骤：（1） 将发酵得到的天然槐糖脂通过碱水解的方法变为酸型的槐糖脂。（2） 将氨基酸的羧基部分通过酯化形式保护起来。（3） 氨基酸的α-氨基与槐糖脂脂肪酸部分的羧基相连（图1-5）。（4）去掉氨基酸羧基端的保护。氨基酸的种类包括天冬氨酸、丝氨酸、亮氨酸、苯丙氨酸、谷氨酸、甘氨酸以及它们的乙酯形式（Azim *et al.*，2006）。

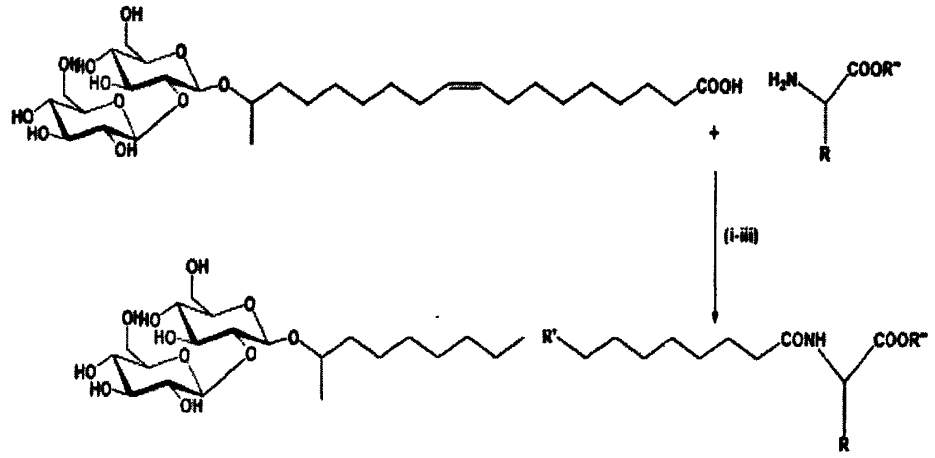


图1-5 氨基酸的α-氨基与槐糖脂脂肪酸部分的羧基相连形成氨基酸-槐糖脂衍生物

Fig. 1-5 Formation of amino acid-sophorolipid derivatives. i) DCC, DCM/DMF (4:1), RT, 24 hr; (ii) 13 f 14, TFA/DCM, 0 °C, 30 min; (iii) Pd/C ethanol, H_2

1.5.2 槐糖部分的修饰

Rau 等通过酶催化的方法将槐糖脂槐糖分子上的一个葡萄糖分子释放出来变为葡萄糖脂。首先通过碱水解将双乙酰化的内酯型槐糖脂变为脱乙酰化的酸型槐糖脂，随后利用几种糖苷酶对其进行催化作用。其中一种桔皮苷酶能够最有效的使槐糖脂的一个葡萄糖分子释放出来，将槐糖脂变为葡萄糖脂（图 1-6）。新合成的葡萄糖脂与内酯型以及酸型的槐糖脂在表面活性方面相比较，内酯型的槐糖脂的表面活性最好，葡萄糖脂和酸型槐糖脂的表面活性相似（Rau *et al.*，1999）。

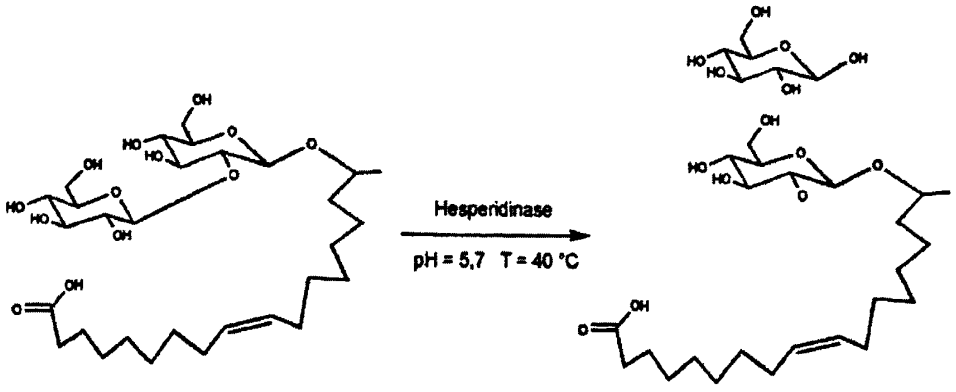
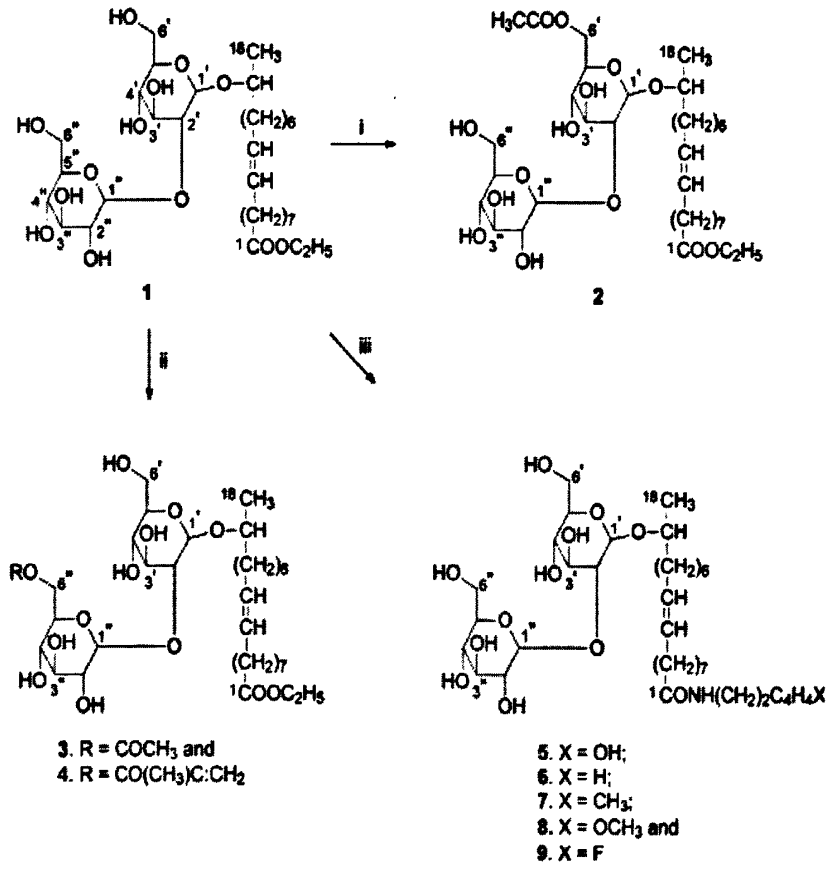


图 1-6 槐糖脂在桔皮苷酶的作用下生成葡萄糖脂

Fig. 1-6 Enzymatic formation of a glucolipid

不同的脂肪酶在不同条件下来催化槐糖脂的乙酰化反应，可以得到不同结构的槐糖脂衍生物。利用Novozym 435和Lipase PS-C这两种酶可以精确地选择在槐糖分子的6′或 6″的位置进行单乙酰化，或者双乙酰化。Novozym 435可以催化槐糖脂乙酯在槐糖的6′碳的位置进行单乙酰化，Lipase PS-C可以催化槐糖脂乙酯在槐糖的6″碳的位置进行单乙酰化，Novozym 435在不同的条件下还可以催化槐糖脂乙酯进行双乙酰化。Novozym 435除了催化乙酰化反应外，还可以催化槐糖脂乙酯转化为槐糖脂氨基化合物。槐糖脂氨基化合物在Novozym 435或Lipase PS-C的催化下也可以精确地选择在槐糖分子的6′或 6″的位置进行单乙酰化，或者双乙酰化。另外Novozym 435还能够将槐糖脂乙酯转化为槐糖脂氨基化合物和双乙酰化同时进行，直接合成双乙酰化的槐糖脂氨基化合物（图1-7）（Singh *et al.*，2003）。



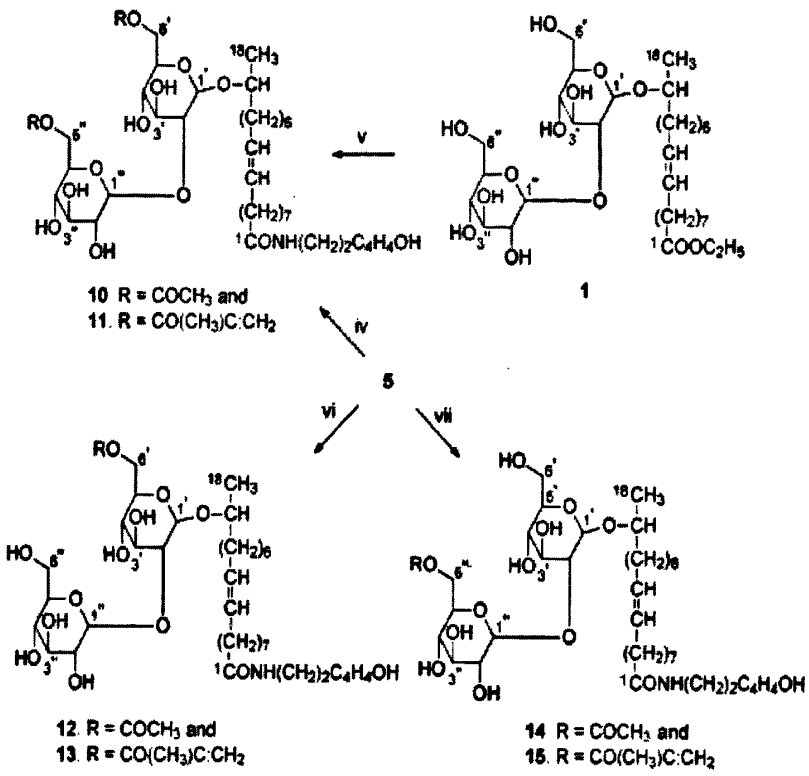


图 1-7 脂肪酶区域选择性催化槐糖脂乙酰化反应

Fig. 1-7 Regioselective enzyme-catalyzed synthesis of acetylated sophorolipid esters, amides. (i) vinyl acetate, Novozym 435, dry THF, 40°C, 2.5 h; (ii) vinyl ester (vinyl acetate for 3 and vinyl methacrylate for 4), Lipase PS-C, dry THF, 40°C, 72 h; (iii) primary amine (tyramine for 5, phenethylamine for 6, (*p*-tolyl) amine for 7, *p*-methoxyphenethylamine for 8, *p*-fluorophenethylamine for 9), Novozym 435, dry THF, 50°C, 24 h. (iv) vinyl acetate for 10 and vinyl methacrylate for 11, Novozym 435, dry THF, 50°C, 80 h; (v) tyramine, Novozym 435, dry THF, 50°C, 24 h; vinyl ester (vinyl acetate for 10 and vinyl methacrylate for 11), 50°C, 80 h (one-pot reaction); (vi) vinyl ester (vinyl acetate for12 and vinyl methacrylate for 13), Novozym 435, dry THF, 40°C, 20 h; (vii) vinyl ester (vinyl acetate for 14 and vinyl methacrylate for 15), Lipase PS-C, dry THF, 40°C, 72 h.

1.6 槐糖脂产生菌在基因工程方面的研究进展

近年来，关于槐糖脂产生菌在基因工程方面的研究越来越多。研究者希望

通过基因工程的方法，调节槐糖脂代谢途径来提高槐糖脂的产量或者生产新的槐糖脂衍生物。

1.6.1 槐糖脂代谢过程中相关基因的克隆

细胞色素 P450 是槐糖脂合成过程中重要的酶，能将脂肪酸羟基化成为 ω 或 ω-1 羟基脂肪酸，在此基础上才能将槐糖分子与脂肪酸分子通过糖苷键连接起来形成槐糖脂分子。

1996 年，Lottermoser 等研究者从槐糖脂产生菌 *Candida apicola* 中克隆得到了 2 种细胞色素 P450 基因，并将其分别命名为 *CYPS2E1* 和 *CYPS2E2*。这 2 个基因编码的蛋白分子都含有 519 个氨基酸，分子量分别为 58.7 kDa 和 58.6 kDa。（Lottermoser *et al.*，1996）

1.6.2 转化及筛选系统的建立

要对槐糖脂产生菌进行基因工程方面的研究，建立一个有效的转化和筛选系统是非常重要的。

为了建立这样一个系统，Van Bogaert 等研究者从槐糖脂产生菌 *Candida bombicola* 中克隆得到了乳清酸核苷-5-磷酸脱羧酶基因（*URA3*），这个基因编码的蛋白分子含有 216 个氨基酸，与其他酵母的乳清酸核苷-5-磷酸脱羧酶具有很高的相似度（Van Bogaert *et al.*，2007）。

后来，他们又筛选得到了 *Candida bombicola* 的尿嘧啶营养缺陷型菌株，并将从野生型菌株中克隆得到的 URA3 基因转化到了营养缺陷型菌株中，使其重新变为野生型菌株，验证了此尿嘧啶营养缺陷型菌株可以作为一个对 *Candida bombicola* 进行基因工程研究的有效的转化和筛选系统（Van Bogaert *et al.*，2008a）。

随后 Van Bogaert 等人又从 *Candida bombicola* 中克隆得到了甘油醛-3-磷酸脱氢酶（glyceraldehyde-3-phosphate dehydrogenase，GPD）基因。GPD 基因的启动子可用于同源或者异源基因的高效表达，Van Bogaert 等用不同长度的 GPD 基因启动子片段构建潮霉素抗性表达盒，并将其转化到 *Candida bombicola* 中。含

有 190 bp 启动子片段的表达盒能使 *Candida bombicola* 得到潮霉素抗性。短的启动子序列也有利于构建表达盒或表达载体，为同源或者异源基因在 *Candida bombicola* 中的高效表达提供了条件（Van Bogaert *et al.*，2008b）。

1.6.3 基因敲除

为了扩大槐糖脂的应用范围，得到脂肪酸部分为中等链长的新型槐糖脂，Van Bogaert 等将多功能酶 2 (MFE-2) 基因从 *Candida bombicola* 基因组中敲除（Van Bogaert *et al.*，2009）。要得到中等链长的槐糖脂就要利用中等链长的脂肪酸或烷烃，但是这种中等链长的底物通常对细胞有毒性，并且在代谢过程中被β氧化，不会被直接利用产生中等链长的槐糖脂。MFE-2 基因是β氧化过程中的重要酶，将 *Candida bombicola* 基因组中的 MFE-2 基因敲除后就可以阻断β氧化过程，使细胞在发酵过程中能直接利用中等链长的底物合成中等链长的槐糖脂。

1.7 槐糖脂在医药领域的应用

槐糖脂作为表面活性剂的一种，由于其生物可降解性，低毒性，良好的环境兼容性，以及产量高等很多优点，在石油工业、环境保护、化妆品、洗涤剂及药学领域都得到了广泛的应用。近年来，槐糖脂在医药领域的应用尤其得到了研究者的关注，很多报道证明了槐糖脂具有很好的抗微生物、抗肿瘤甚至抗艾滋病毒的活性。

1.7.1 槐糖脂的抗菌活性

2002 年 Kim 等报道了槐糖脂具有抗微生物的特性（Kim *et al.*, 2002）。

2005 年 Yoo 等又报道了槐糖脂能够抑制植物病原真菌 *Phytophthora* sp. 和 *Pythium* sp. 的生长。500 mg/L 的槐糖脂能够抑制 8%菌丝体的生长，并且 100 mg/L 的槐糖脂使 *Phytophthora* sp. 游动孢子的游动性降低 90%；同时还发现致使游动孢子裂解的浓度比抑制其游动高两倍，孢子的裂解率与槐糖脂的类型有关，100 mg/L 内酯型的槐糖脂处理后，最大孢子裂解率达 80%。

由于槐糖脂的分子结构与细胞膜结构相似，导致细胞膜受到破坏，是槐糖脂

抗菌机理之一。槐糖脂能够强烈抑制革兰氏阳性菌如 *Bacillus*, *Staphylococcus*, 和 *Streptococcus* sp 的生长（Yoo *et al.*, 2005）。

2007 年，Shah 等用葡萄糖、果糖、木糖、核糖、乳糖、甘露糖、树胶醛糖和半乳糖等多种糖类为第一碳源生产槐糖脂，并将这些不同来源的槐糖脂用于抑菌实验。结果显示槐糖脂对革兰氏阳性菌的作用明显大于对革兰氏阴性菌的作用。不同来源的槐糖脂的抑菌效果也不同。以树胶醛糖为底物得到的槐糖脂对于几种革兰氏阳性菌的作用与以葡萄糖为底物得到的槐糖脂的作用相比更好，但是对大肠杆菌完全没有抑制作用。以乳糖为底物得到的槐糖脂对枯草芽孢杆菌的抑制效果更好（Shah *et al.*，2007b）。

1.7.2 槐糖脂的抗肿瘤活性

1997年，Isoda等发现粗品槐糖脂能够诱导人早幼粒白血病细胞HL60分化，10 μg/mL 的粗品槐糖脂能将 HL60 细胞诱导成为单核细胞。但是该研究没有弄清楚到底是那种槐糖脂发挥的作用（Isoda *et al.*，1997）。

1998 年，Scholz 等纯化得到内酯型槐糖脂，然后通过醇解和脂肪酶催化的方法制备了一些槐糖脂的衍生物。用内酯型槐糖脂及其衍生物作用于两种肿瘤细胞，悬浮细胞 Leukemic Jurkat 和贴壁细胞 Head and neck cancer Tu 138。结果发现，对 Leukemic Jurkat 细胞的生长抑制，12.5 μg/mL 的粗品槐糖脂达到 89%，25 μg/mL 的乙酰化槐糖脂乙酯为 90%，25 μg/mL 的乙酰化槐糖脂丁酯为 99.7%，而脱乙酰化的槐糖脂乙酯和丁酯对细胞生长基本没有抑制作用。对 Head and neck cancer Tu 138 细胞来说粗品槐糖脂和乙酰化槐糖酯对细胞生长有很强的抑制作用。因此作者得出槐糖脂的抗肿瘤活性与槐糖脂分子上的乙酰基团有关（Scholz *et al.*, 1998; Gross *et al.*, 1999）。

2006 年，我们实验室报道了脂肪酸部分为油酸的双乙酰基内酯型槐糖脂对四种肿瘤细胞系，H7402（人肝癌细胞），A549 （人肺腺癌细胞），HL60（人急性早幼粒白血病细胞）和 K562（人慢性粒细胞白血病细胞），具有很强的抑制作用（Chen *et al.*, 2006a）。随后又进一步研究了脂肪酸部分为油酸的双乙酰基内酯型槐糖脂对人肝癌细胞 H7402 的作用机制，采用多种检测方法，细胞形态

学观察（如光学显微镜观察、电子显微镜观察、荧光显微镜观察、激光扫描共聚焦显微镜观察），流式细胞技术检测细胞周期分布及凋亡率，DNA 裂解的原位检测，凋亡蛋白质酶 Caspase-3 活性的检测及细胞内钙离子浓度的检测对双乙酰内酯型槐糖诱导肿瘤细胞的凋亡进行了多方面系统的研究，发现槐糖脂的抗肿瘤机制是引起细胞的凋亡（Chen *et al.*, 2006b）。

2008年，Fu等报道了天然槐糖脂混合物以及槐糖脂衍生物（包括槐糖脂甲酯、槐糖脂乙酯、单乙酰基槐糖脂乙酯、双乙酰基槐糖脂乙酯、酸型槐糖脂、双乙酰基内酯型槐糖脂）对胰腺癌细胞的抑制作用（Fu *et al.*，2008）。结果显示，天然槐糖脂混合物在测验的所有剂量都对胰腺癌细胞有相似的细胞毒性（大约20%），与其他槐糖脂衍生物相比，槐糖脂甲酯对胰腺癌细胞的细胞毒性最高（大约63%），双乙酰基槐糖脂乙酯对胰腺癌细胞的细胞毒性为36%，单乙酰基槐糖脂乙酯对胰腺癌细胞的细胞毒性为18%。双乙酰基内酯型槐糖脂和酸型槐糖脂对胰腺癌细胞的细胞毒性与槐糖脂浓度成反比，双乙酰基内酯型槐糖脂在浓度为0.5 mg/mL的浓度时细胞毒性为40.3%，在2.0 mg/mL的浓度时细胞毒性为3.4%，酸型槐糖脂在浓度为0.5 mg/mL的浓度时细胞毒性为49%，在2.0 mg/mL的浓度时无细胞毒性。

1.7.3 槐糖脂的抗炎症反应

研究者 Napolitano 报道了槐糖脂对患腹部脓血症小鼠的作用。分析了小鼠的生存率和炎症反应细胞因子的基因表达，结果发现静脉注射和腹腔注射槐糖脂的小鼠生存率分别提高了 34%和 14%；白细胞介素 1（IL-1）和转化生长因子（TGF）的 mRNA 表达分别下调 42.5%和上调 11.7%。通过实验数据分析，作者认为脓血症小鼠生存率的升高是由于巨噬细胞的减少抑制了炎症反应（Napolitano, 2006）。

2007年，Hardin等研究了不同剂量（一次剂量和连续多次剂量）槐糖脂以及天然槐糖脂混合物和单一的槐糖脂衍生物对脓血症相关的存活率的影响。结果发现5 mg/kg剂量的槐糖脂处理腹膜炎脓血症小鼠后，24 h和72 h小鼠的存活率比只注射溶剂的对照组分别提高了28%和42%。使用连续剂量（每24 h 5 mg/kg剂量，共3次）的槐糖脂后，24 h和72 h小鼠的存活率比只注射溶剂的对照组分别提高了39%和26%。5 mg/kg剂量的天然槐糖脂和槐糖脂乙酯处理72 h后脓血症小鼠的存

活率分别提高了32%和20%。而内酯型槐糖脂处理后脓血症小鼠的存活率反而降低了10%。同样的，在体外实验中发现，槐糖脂乙酯能够减少炎症细胞的产生，而内酯型槐糖脂却不能。作者认为可能是需要不同结构的槐糖脂协同作用才能提高脓血症的存活率，而单一的成分却不一定有效（Hardin *et al.*，2007）。

由于引起脓血症的因素也很有可能引起菌血症，而槐糖脂具有抗菌活性，所以槐糖脂能提高脓血症的存活率的部分原因也有可能是由于槐糖脂具有抗菌活性。

Hagler等报道了槐糖脂作用于骨髓瘤细胞系（U266），可以降低IgE的生成，并推测这种作用是通过影响血浆细胞活性产生的。随后的机制研究证实槐糖脂可以减少IgE协同因子BSAP（Pax5），TLR-2，STAT 3和IL-6的mRNA表达，而不影响B肌动蛋白、细胞结构、增生和凋亡、IgA的产生以及FceRI和IL-6R mRNA的表达（Hagler *et al.*，2007）。最近，Bluth等用槐糖脂对小鼠哮喘模型喷雾给药，证实槐糖脂能抑制哮喘病和卵白蛋白特异性IgE产生（Bluth *et al.*，2008）。

以上研究为槐糖脂作为抗炎、IgE失调疾病以及哮喘病的治疗药物的应用提供了依据。

1.7.4 槐糖脂的抗病毒活性和杀精子活性

Shah 等研究了槐糖脂及其衍生物的抗艾滋病毒以及杀精子活性。文章所研究的槐糖脂及其衍生物包括天然的槐糖脂混合物、内酯型槐糖脂、酸型槐糖脂、槐糖脂脂甲酯、乙酯、己酯以及单乙酰化的槐糖脂乙酯和双乙酰化的槐糖脂乙酯。（Shah *et al.*，2005）

结果表明，双乙酰化的槐糖脂乙酯的杀精活性最好，其最低有效浓度（EMC）为 0.18 mg/mL，低于商业常用的杀精剂 Nonoxynol-9（EMC 为 0.25 mg/mL）。双乙酰化的槐糖脂乙酯在 20 μg/mL 的浓度下在 2 分钟内就能抑制大部分的精子的运动性，而内酯型和槐糖脂己酯在 2 分钟内能抑制 50%的精子的运动性。双乙酰化的槐糖脂乙酯的抗艾滋病毒活性也是最高的，仅次于 Nonoxynol-9，内酯型槐糖脂和酸型槐糖脂相比，酸型槐糖脂的抗艾滋病毒活性更高。

作者根据结果分析得出，槐糖脂碳链的长度会影响其生物活性，碳链越长生

物活性越高，乙酰化也会使槐糖脂的生物活性增加。

1.8 槐糖脂在环境修复中的应用

1.8.1 疏水性有机污染物的生物修复

槐糖脂用于生物修复的原理之一是将其添加在污染处，使不溶或难溶性的污染物与水形成乳化液，有利于微生物的吸收和分解；之二是添加槐糖脂后疏水性污染物与水形成乳化液，通过多次冲洗达到除去目的。

添加槐糖脂到含有10%的土壤和1.35%的烃类混合物中，90%的烃类物质在79 h 内降解掉；而不加槐糖脂在114 h 内仅降解81%（Oberbremer *et al.*，1990）。Schippers研究了槐糖脂在菲的生物降解中的作用，实验结果表明：使用 *Sphingomonas yanoikuyae* 菌株降解菲，当加入500 mg/L的槐糖脂，在36 h内，菲的浓度从80 mg/L降为0.5 mg/L，而不添加时则降为2.3 mg/L；菲的降解速率由不加槐糖脂时的0.8 mg/L/h升高为1.3 mg/L/h（Schippers *et al.*，2000）；且添加槐糖脂后土壤悬浮介质的临界微团浓度CMC从45 mg/L降到4 mg/L。荧光测定显示，这种效果并不是由于增加的生物数量，而是通过增加明显的溶解颗粒来增加菲的生物利用度，说明槐糖脂对菲污染的土壤具有一定的修复作用。

1.8.2 土壤中重金属离子的去除

Mulligan等用鼠李糖脂、槐糖脂和表面活性素（surfatin）去除沉积物中的重金属。其中，4%的槐糖脂可以去除25%的Cu和60%的Zn。这主要是由于金属离子先与表面活性剂的亲水端结合，随后胶束形成而从土壤上解吸下来，多次淋洗去除金属离子（Mulligan *et al.*，1999；Mulligan *et al.*，2001）。

1.8.3 减轻有害藻华的危害

与抗菌活性类似，槐糖脂还有抑制藻类细胞生长的作用。Sun等通过研究槐糖脂对有害藻类塔玛亚历山大藻（*Alexandrium larnarense*），赤潮异弯藻（*Heterosigma akashiwo*）和多环旋沟藻（*Cochlodinium polykrikoides*）细胞的不可逆抑制作用，揭示了槐糖脂通过对细胞膜不可逆的破坏，使胞内物质核苷酸流

出而对细胞产生不可逆的伤害，10~20 mg/L的槐糖脂即可杀死90%的藻类细胞。通过藻类沉降实验证实了槐糖脂和黄土抑制有害藻类的协同作用，5 mg/L槐糖脂和1 g/L黄土联合作用时除去多环旋沟藻细胞效果最好。同样效果下，联合使用槐糖脂和黄土的用量分别降低25%和10%，总成本降低60%（Sun *et al.*，2004a, 2004b）。Lee等对某海域*Cochlodinium*藻类进行了抑制实验，同时也研究了槐糖脂和黄黏土混合物对海里细菌、异养原生生物和浮游动物的影响，结果在海面喷洒槐糖脂和黄黏土混合物30 min后藻类细胞杀死率为95%，黄黏土对照组为79%，对其他生物的抑制率较低，表明槐糖脂和黄黏土联合使用可以有效减轻有害藻类危害，对水域上层生态系统影响较小（Lee *et al.*，2008）。

1.9 本论文的研究目的和主要研究内容

槐糖脂作为一种生物表面活性剂，由于其生物可降解性，低毒性，良好的环境兼容性，以及高产量，在石油工业、环境保护、化妆品、洗涤剂及药学领域都具有良好的应用前景，目前对槐糖脂的主要应用研究集中在医药领域。在此背景下，本论文的研究内容主要包括以下几个方面：

1．通过补料发酵的方法，在发酵过程中往培养基中补加第二碳源，提高拟威克酵母以菜籽油为第二碳源为底物发酵生产槐糖脂的产量。

2．研究了拟威克酵母产生的槐糖脂对几种常见细菌以及能引起龋齿的变形链球菌的抑制作用。并且研究了槐糖脂对红色毛癣菌、石膏样毛癣菌和犬小孢子菌这三种临床上常见的皮肤病致病菌的抑制作用。

2．将拟威克酵母利用菜籽油为第二碳源发酵得到的天然槐糖脂混合物通过HPLC进行分离纯化，并通过质谱分析鉴定出10种槐糖脂组分的结构。

4．研究了不同结构的天然槐糖脂组分的抗肿瘤活性。将分离纯化得到的几种不同结构的槐糖脂组分用于对食管癌细胞KYSE 109和KYSE 450抗肿瘤实验中，分析了槐糖脂分子的乙酰化程度、脂肪酸部分饱和程度和是否内酯化对其抗肿瘤活性的影响。

5．采用多种检测方法，细胞形态学观察（如光学显微镜观察、电子显微镜观察、荧光显微镜观察），流式细胞技术检测细胞周期分布及凋亡率以及 DNA

裂解的原位检测对不同结构槐糖脂对于食管癌细胞 KYSE450 的抗肿瘤机制进行了研究。

第二章 补料发酵生产槐糖脂

引言

槐糖脂的生产成本较高是制约其应用的一个重要因素（Rosenberg & Ron, 1999）。因此，降低槐糖脂的生产成本，提高槐糖脂的产量是槐糖脂研究工作的一个重要方面。近年来，很多研究者开展了这方面的工作并取得了可喜成果。Davila 等在槐糖脂产生阶段补加疏水性碳源，使槐糖脂产量高达 300 g/L（Davila *et al.*, 1997）。Daniel 等采用了两步法的发酵过程，先以乳清中的乳糖为底物生产单细胞油，然后利用单细胞油为第二碳源，并不断补加菜籽油，发酵 550 h 后，最终槐糖脂产量达到 422 g/L（Daniel *et al.*, 1998）。Rau 等用菜籽油与葡萄糖作为底物通过补料发酵以及两步连续发酵过程生产槐糖脂。优化培养条件后，产量达到了 300 g/L 以上，补料发酵和连续发酵的产率分别为 57 g/L/d 和 76 g/L/d。Kim 等用控制补料速率的补料发酵培养提高槐糖脂产量。在发酵过程中，采用菜籽油作为第二碳源，葡萄糖浓度控制在 30~40 g/L，研究发现，在控制 pH 为 3.5 的过程中，菜籽油的消耗量与 NaOH 的加入量成正比，因此通过控制 pH，计算菜籽油的补加速率。发酵 8 天后，槐糖脂产量达到 365 g/L（Kim *et al.*, 2009）。

以上这些补料发酵生产槐糖脂所用菌株都是 *Candida bombicola*。我们实验室所分离得到的新的槐糖脂产生菌 *Wickerhamiella domercqiae* 经过前期的发酵条件优化，摇瓶发酵的产量在 30 g/L 左右。为了提高 *Wickerhamiella domercqiae* 发酵生产槐糖脂的产量，我们通过补料发酵的方法，在发酵过程中向培养基中补加菜籽油以增加槐糖脂的产量。

2.1 材料和方法

2.1.1 菌株

拟威克酵母，由本实验室自污水中分离。

2.1.2 仪器和试剂

本实验所用试剂为化学纯或分析纯，所用植物油为食用级。LD2X-40B1 型立式自动电热压力蒸汽灭菌器(上海申安医疗器械厂),恒温培养箱，Sigma 4K15 冷冻离心机，HZQ-Q 全温振荡器（哈尔滨东联电子技术开发有限公司），旋转蒸发器（上海申胜生物技术有限公司），722 光栅分光光度计（上海第三仪器厂），5 L 全自动磁力搅拌发酵罐（镇江东方生物工程设备技术有限公司）。

2.1.3 培养基

2.1.3.1 斜面及种子 YEPD 培养基

葡萄糖 2.0 %，蛋白胨 2.0 %，酵母粉 1.0 %，琼脂粉 2.0 %（w/v, 液体种子培养时不加）

2.1.3.2 发酵基本培养基

葡萄糖 8%，菜籽油 6%，KH_2PO_4 0.10 %，$Na_2HPO_4 \cdot 12\ H_2O$ 0.10 %，$MgSO_4 \cdot 7\ H_2O$ 0.05 %（w/v）。

2.1.4 培养方法

2.1.4.1 液体种子培养

于 300 mL 三角瓶中装 50 mL 种子培养基，接种一环已活化的斜面种子，160 r/min，30℃培养 24 h。

2.1.4.2 摇瓶培养

于 300 mL 三角瓶中装 50 mL 发酵培养基，按 2%（v/v）的接种量接入液体种子，160 r/min，30℃培养 168 h。测定发酵液中槐糖脂含量、菌体浓度以及葡萄糖残余量。

2.1.4.2 发酵罐培养

于 5 L 发酵罐中装 3.5 L 发酵培养基，按 2 %（v/v）的接种量接入液体种子，30℃培养，补料发酵，测定发酵液槐糖脂含量、菌体浓度以及葡萄糖残余量。

2.1.5 检测方法

2.1.5.1 葡萄糖浓度的测定

用 DNS 法测定还原糖。

(1) 配制 DNS 试剂

1. 准确称取 104 g 氢氧化钠溶于 1300 mL 蒸馏水中；
2. 准确称取 3,5-二硝基水杨酸 30 g 加入(1)中混匀；
3. 准确称取 910 g 酒石酸钾钠溶于 2500 mL 蒸馏水中；
4. 准确称取 25 g 重蒸苯酚，25 g 无水亚硫酸钠，加入（3）中混匀；

将以上各步骤的溶液混合，加入 1200 mL 蒸馏水后，放在棕色试剂瓶中，于暗处放置一星期后，即可使用。

(2) 配制 1 mg/mL 的无水葡萄糖溶液

准确称取 0.1000 g 分析纯的无水葡萄糖（预先在 105℃干燥至恒重），用蒸馏水溶解后移至 100 mL 容量瓶中，并用蒸馏水洗称量瓶数次，以使全部葡萄糖移入容量瓶中，最后定容到刻度，摇匀，其浓度为 1 mg/mL。

(4) 葡萄糖标准曲线的绘制（DNS 法）

按表 2-1，依次用移液管准确吸取各试剂，加入各号具塞试管中。将试管置沸水浴中加热 5 min。取出试管，用冰水冷却。将试管中溶液定容至 12.5 mL，摇匀。550 nm 测 OD 值。以葡萄糖浓度（mg/mL）为纵坐标，以 OD 值为横坐标，绘制标准曲线（图 2-1）。

表 2-1 绘制葡萄糖标准曲线所需试剂及体积

Table 2-1 Different reagents and their volume of glucose standard curve

编号	0	1	2	3	4	5	6	7
无水葡萄糖溶液(mL)	0	0.2	0.4	0.6	0.8	1.0	1.2	1.5
蒸馏水（mL）	1.5	1.3	1.1	0.9	0.7	0.5	0.3	0
DNS 试剂	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

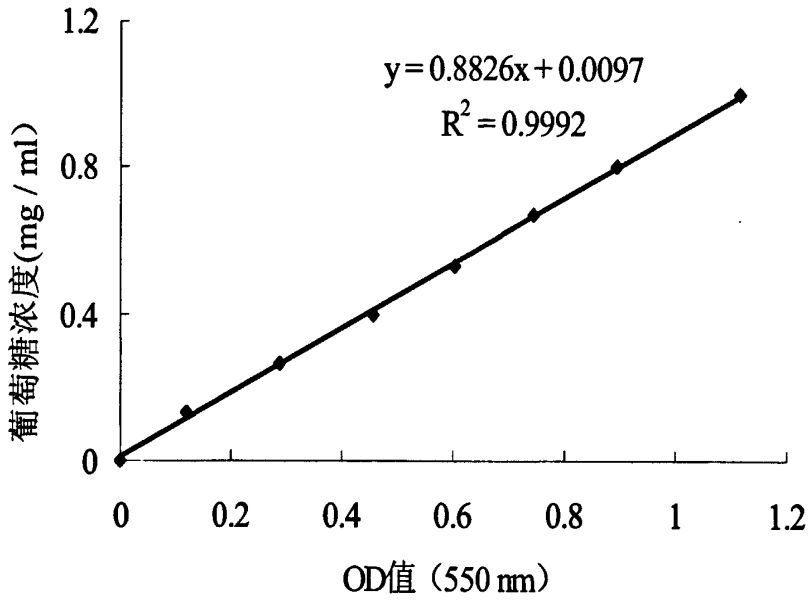


图 2-1 葡萄糖标准曲线（DNS 法）

Fig. 2-1 The standard curve of glucose by DNS method

(5) 葡萄糖测定步骤

1. 取 1 mL 发酵液，加入 1 mL 乙酸乙酯，经漩涡混合器混匀后静置 10 min， 然后 5000 rpm 离心 10 min；
2. 取 0.1 mL 水相，加入 0.9 mL 蒸馏水稀释 10 倍，混匀；
3. 取 0.75 mL 加入具塞试管中；
4. 加入 1 mL DNS 试剂，混匀后沸水浴加热 5 min；
5. 取出试管，冰水冷却后，定容至 12.5 mL，混匀；
6. 测量 550 nm 下的 OD 值。

2.1.5.2 槐糖脂含量的测定

用蒽酮法测定总糖 (Hu & Ju, 2001; 李建武, 2000)。

(1) 蒽酮试剂的配制

准确称取蒽酮 0.1 g，加入 100 mL 浓硫酸，混匀后即为蒽酮试剂。

(2) 蒽酮法测定总糖的原理

蒽酮法是一种测定总糖的方法，能用来测定己糖、戊醛糖、己糖醛酸等各种糖，不论是游离形式还是存在于多糖之中，各种糖在与蒽酮试剂反应后都呈现出蓝绿色。620 nm 下，有最大光吸收。

(3) 蒽酮法绘制葡萄糖标准曲线

分别吸取蒽酮试剂 4 mL 和标准葡萄糖溶液 1 mL 于具塞试管中，混匀后迅速于冰浴中冷却，然后在沸水浴中加热 10 min，冰浴冷却后，在 620 nm 下，用分光光度计测量 OD 值（1 mL 蒸馏水为空白对照）。以葡萄糖浓度（mmol/L）为横坐标，OD 值为纵坐标，绘制标准曲线（图 2-2）。

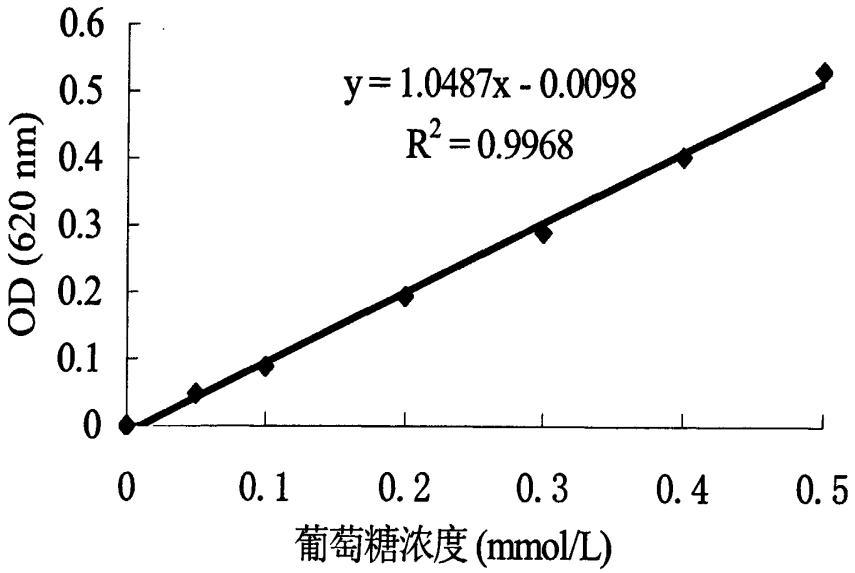


图 2-2 葡萄糖标准曲线（蒽酮法）

Fig. 2-2 The standard curve of glucose by anthrone method

(4) 槐糖脂测定原理

槐糖脂是由槐糖和羟基脂肪酸两部分所组成得，用蒽酮法测定槐糖脂样品在620 nm下的OD值，根据OD值从利用蒽酮法测定的葡萄糖标准曲线上求出葡萄糖的含量，利用葡萄糖分子量和槐糖脂之间的比值，即1 g葡萄糖相当于1.91 g槐糖脂，求出样品中槐糖脂的含量（Deslipande & Daniels, 1995）。

(6) 槐糖脂测定步骤

1. 取1 mL发酵液，加入1 mL乙酸乙酯，经过漩涡混合器混匀后静置10 min，然后5000 r/min离心10 min；
2. 取10 μL上清液加入具塞试管中，水浴5 min蒸发掉乙酸乙酯；
3. 加入1 mL水、4 mL蒽酮试剂，沸水浴10 min，然后用冰水冷却；
4. 620 nm下测定OD值。

2.1.5.3 菌体浓度的测定

取1 mL发酵液，加入2 mL $V_{正丁醇}$：$V_{乙醇}$：$V_{氯仿}$=10: 10: 1的溶液，经漩涡混合器混匀后，5000 r/min离心5 min，然后用蒸馏水洗涤2遍，干燥，称重。

2.2 结果与讨论

2.2.1 摇瓶分批发酵生产槐糖脂

在300 mL摇瓶中分批发酵生产槐糖脂，发酵曲线如图2-3所示。发酵24 h时，菌体生长达到稳定期，这时培养基中氮源耗尽，开始有槐糖脂产生。随着发酵时间的延长，培养基中的葡萄糖不断消耗，槐糖脂产量不断增加，发酵120 h时，葡萄糖基本消耗完全，槐糖脂产量继续增加，168 h后，槐糖脂产量达到32.6 g/L。

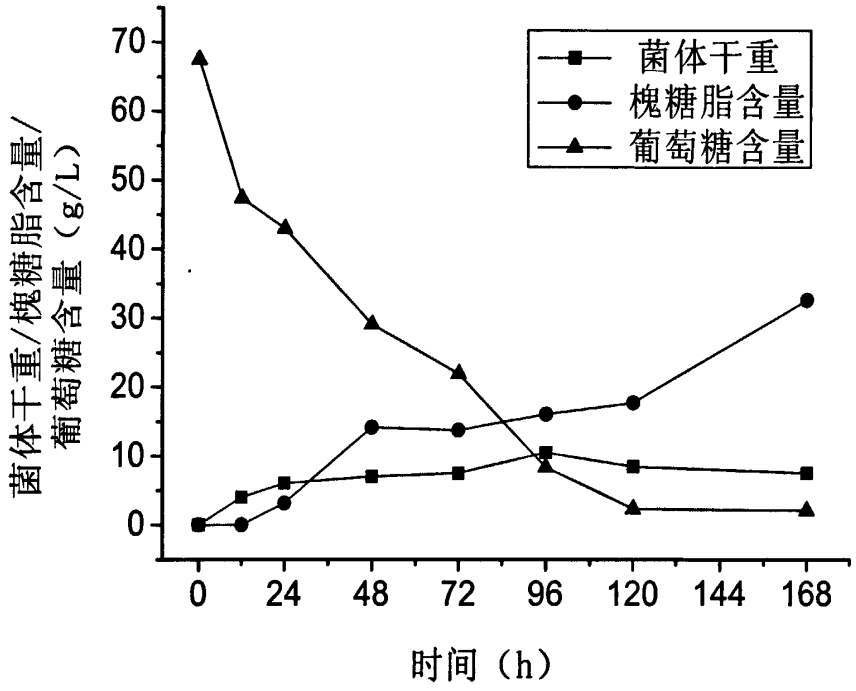


图 2-3 摇瓶分批发酵生产槐糖脂

Fig. 2-3 sophorolipid production with batch fermentation in 300 mL flask

2.2.2 摇瓶补料发酵生产槐糖脂

在摇瓶发酵生产槐糖脂的过程中通过补加菜籽油来提高槐糖脂的产量。如图2-4所示，发酵24 h时，菌体生长达到稳定期，培养基中氮源耗尽，开始有槐糖脂产生。葡萄糖同样在发酵120 h时消耗完全，72 h、144 h、168 h时分别补加5%的菜籽油，发酵168 h后，槐糖脂产量为37.8 g/L，比分批发酵提高了16%。168 h补油后，由于油的利用速度比较慢，继续取样会严重破坏发酵体系，所以只在288 h油基本利用完之后取样，测得槐糖脂产量为43.1 g/L，比分批发酵的产量提高了32%。

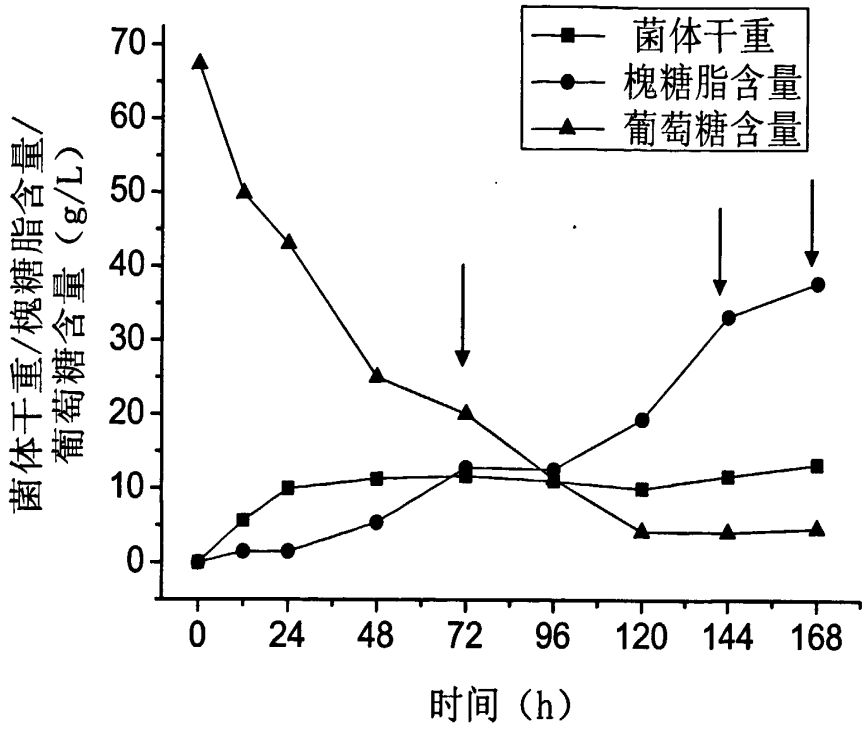


图 2-4 摇瓶补料发酵生产槐糖脂。箭头表示加入 5%的菜籽油。

Fig. 2-4 Sophorolipid production with fed-batch fermentation in 300 mL flask. The arrow means addition of 5% rapeseed oil.

2.2.3 发酵罐补料发酵生产槐糖脂

在 5 L 发酵罐中扩大生产槐糖脂，发酵罐转速为 400 rpm，同样在发酵过程中补加菜籽油。如图 2-5 所示，发酵 48 h 时，菌体生长达到稳定期，开始有槐糖脂产生。葡萄糖同样在发酵 120 h 时消耗完全，72 h、120 h、168 h 时分别补加 5%的菜籽油，发酵 288 h 后，槐糖脂产量为 68.2 g/L，比摇瓶分批发酵提高了 109.2%，比摇瓶补料发酵产量提高了 58.2%。拟威克酵母为好氧菌，发酵罐的通氧量要大大高于摇瓶的通氧量，因此在 5 L 发酵罐中发酵得到的槐糖脂的产量要大大高于摇瓶发酵的产量。

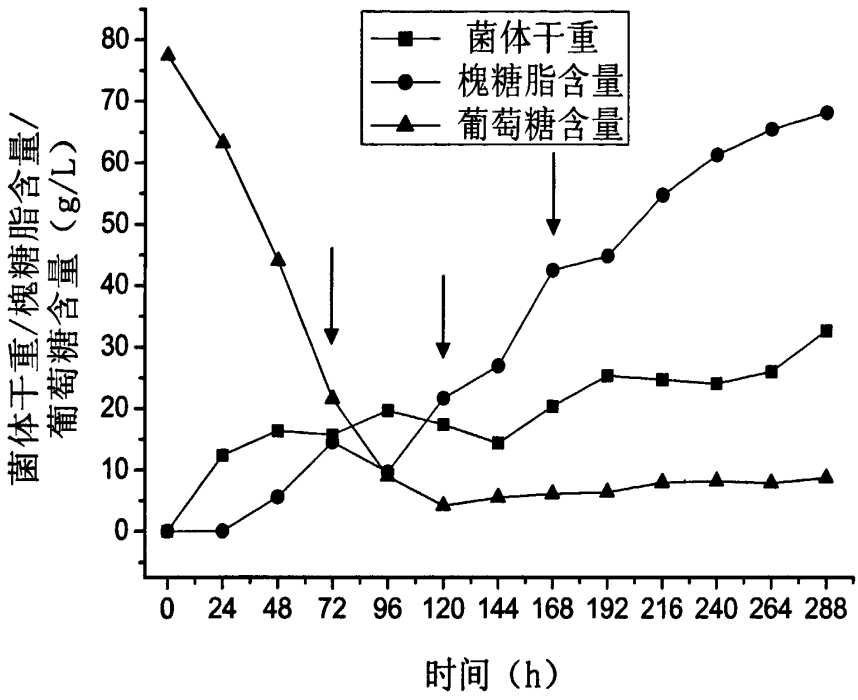


图 2-5　5 L 发酵罐补料发酵生产槐糖脂。箭头表示加入 5%的菜籽油。转速为 400 rpm

Fig. 2-5 Sophorolipid production by fed-batch fermentation in 5 L fermentor. The arrow means addition of 5% rapeseed oil.

2.2.4 提高发酵罐转速对补料发酵生产槐糖脂的影响

将 5 L 发酵罐的转速从 400 rpm 提高到 500 rpm，同样在发酵过程中补加菜籽油。如图 2-6 所示，发酵 48 h 时，菌体生长达到稳定期，开始有槐糖脂产生。葡萄糖同样在发酵 120 h 时消耗完全，96 h、120 h、168 h 时分别补加 5%的菜籽油，发酵 196 h 后，槐糖脂产量达到 71.1 g/L，与提高转速之前相比，槐糖脂的产量略高一点，但是发酵周期大大缩短，由 288 h 缩短到 196 h，提前了 96 h，槐糖脂的容积生产率由 0.24 g/L/h 提高到 0.37 g/L/h。

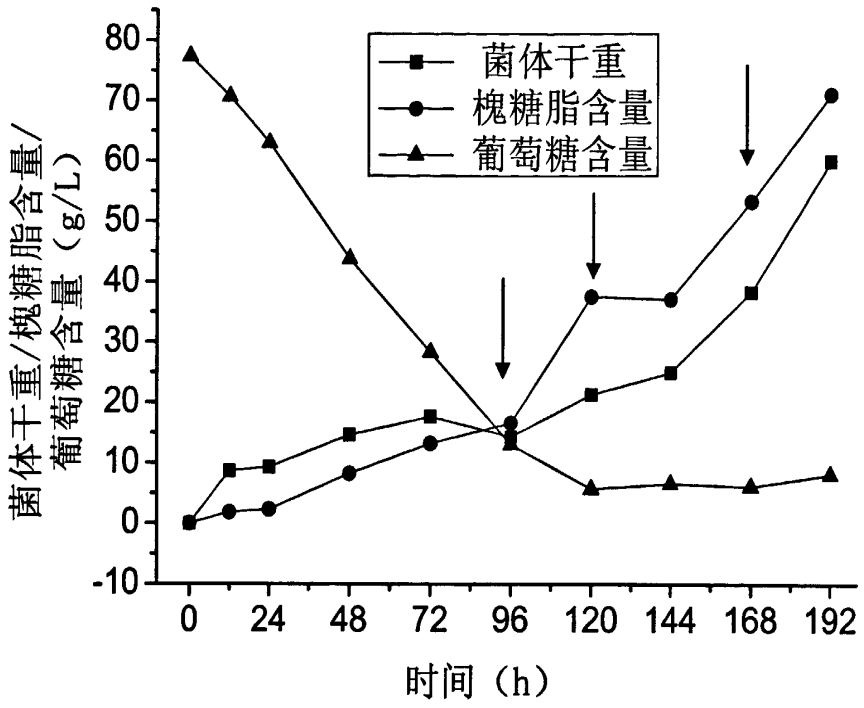


图 2-6　5 L 发酵罐补料发酵生产槐糖脂。箭头表示加入 5%的菜籽油。转速为 500 rpm。

Fig. 2-6 Sophorolipid production by fed-batch fermentation in 5 L fermentor. The arrow means addition of 5% rapeseed oil.

2.3 本章小结

（1）300 mL 摇瓶补料发酵中，补加三次 5%菜籽油之后，槐糖脂产量为 43.1 g/L，比分批发酵的槐糖脂产量提高了 32%。

（2）在 5 L 发酵罐中扩大生产槐糖脂，发酵过程中补加三次 5%菜籽油，发酵 288 h 后，槐糖脂产量为 68.2 g/L，比摇瓶分批发酵提高了 109.2%，比摇瓶补料发酵产量提高了 58.2%。

（3）将 5 L 发酵罐的转速从 400 rpm 提高到 500 rpm，同样在发酵过程中补加三次 5%菜籽油。发酵 196 h 后，槐糖脂产量达到 71.1 g/L，与提高转速之前相

比，槐糖脂的产量略高一点，但是发酵周期大大缩短，由 288 h 缩短到 196 h，提前了 96 h，槐糖脂的容积生产率由 0.24 g/L/h 提高到 0.37 g/L/h。

第三章 槐糖脂的抑菌作用

引言

近年来，槐糖脂在医药领域的应用引起了研究者的广泛关注，很多报道证明了槐糖脂具有很好的抗微生物、抗肿瘤、抗炎症反应以及抗艾滋病毒的活性。据报道，槐糖脂具有抑制细菌的活性，并且对革兰氏阳性菌的抑制作用要高于对革兰氏阴性菌的抑制作用（Shah *et al.*，2007）。除了能抑制细菌的生长外，槐糖脂还能作为真菌抑制剂，很好的抑制植物病原性真菌 *Phytophthora* sp. 和 *Pythium* sp. 的生长（Yoo *et al.*, 2005），以及抑制有害藻华的发生（Sun *et al.*，2004）。

红色毛癣菌(*Triehophyton rubrum*)、犬小孢子菌(*Microsporum canis*)和石膏样毛癣菌(*Trychophyton mentagrophyte*)是三种最常见的皮肤癣菌，常常是头癣、体癣和少数甲癣以及其他少见真菌病的病原菌。目前还未有槐糖脂对于皮肤癣菌抑制作用的报道。

本章首先研究了拟威克酵母产生的槐糖脂对几种常见细菌以及能引起龋齿的变形链球菌的作用效果。此外，我们选取了红色毛癣菌、犬小孢子菌和石膏样毛癣菌三种皮肤癣菌作为研究对象，研究了槐糖脂对于皮肤癣菌的抑制作用。

3.1 材料和方法

3.1.1 仪器和试剂

超净工作台（苏州净化设备厂），LD2X-40B1 型立式自动电热压力蒸汽灭菌器（上海申安医疗器械厂），HZQ-Q 全温振荡器（哈尔滨东联电子技术开发有限公司），恒温培养箱， Spectra Max 190 微板光谱仪（USA）。试剂均为化学纯或分析纯。

3.1.2 培养基

(1) 细菌培养基，即 LB 培养基

NaCl 10 g，蛋白胨 10 g，酵母粉 5 g，琼脂粉 20 g，蒸馏水 1000 mL，pH 7.0-7.2

(2) 真菌培养基，即沙氏培养基

葡萄糖 40 g，蛋白胨 10 g，琼脂粉 20 g，蒸馏水 1000 mL，自然 pH

3.1.3 样品

用二甲亚砜稀释成不同浓度的粗品内酯型槐糖脂和用水稀释成不同浓度的粗品酸型槐糖脂。

3.1.4 抑菌试验菌株

(1) 细菌：大肠杆菌 (*E. coli*)、蜡状芽孢杆菌（*B. cereus*）、金黄色葡萄球菌（*S. aureus*）、变形链球菌（*Streptococcus mutans*）。

(2) 真菌：红色毛癣菌（*Trichophyton rubrum*）、石膏样毛癣菌（*Trichophyton gypseum*）、犬小孢子菌（*Microsporum canis*）。

3.1.5 槐糖脂对细菌的抑菌曲线的绘制

1. 配制 0、5、10、15、20、25、30、40、50、100 mg/mL 槐糖脂粗品的二甲亚砜溶液；
2. 将不同浓度的样品加入 LB 液体培养基中，使其终浓度为 0、5、10、15、20、25、30、40、50、100 mg/L；
3. 接入菌液，37 ℃培养；
4. 定时取样，测定菌液的 OD_{600} 值；
5. 绘制菌体生长曲线。

3.1.6 槐糖脂对皮肤癣菌的抑制作用

1. 配制 0、8、20、40 mg/mL 样品溶液；
2. 各平皿分别加入 0.25 mL 一定浓度的样品液；
3. 每皿倒入 20 mL 培养基，充分混匀，样品终浓度为 0、0.1、0.25、0.5 mg/mL。以不加样品的培养基作阴性对照；
4. 凝固后加入 100 uL 孢子悬液（10^3 个/mL）涂布均匀，30 ℃培养 5 天；
5. 观察菌落生长情况。

3.1.7 槐糖脂对皮肤癣菌的菌丝延伸抑制率

1. 配制 0、5、10、20、40 mg/mL 样品溶液；
2. 各平皿分别加入 0.25mL 一定浓度的样品液；
3. 每皿倒入 20 mL 培养基，充分混匀。样品终浓度为 0、0.0625、0.125、0.25、0.5 mg/mL。以不加样品的培养基作阴性对照；
4. 将 6 mm 的菌丝饼正面朝下接于培养基上，30 ℃培养 5 天；
5. 测量菌落直径，计算菌丝延伸抑制率。

3.1.8 最小抑制浓度 (MIC) 和最小杀灭浓度 (MFC)

1. 50 μL 不同浓度的槐糖脂溶液加入到 5 mL 液体沙氏培养基中，使槐糖脂终浓度为 0, 0.0625, 0.125, 0.25, 0.5 mg/mL；
2. 加入 100 μL 孢子悬液（10^6 个/mL），30 ℃培养 3 天；
3. 没有菌丝生长的最小浓度即为 MIC；
4. 将没有菌丝生长的含有不同浓度槐糖脂的培养基接到沙氏固体培养基上，涂布均匀，30 ℃培养 5 天；
5. 没有菌丝生长的最小浓度即为 MFC。

3.1.9 透射电镜观察药物作用后皮肤癣菌菌丝的变化

终浓度为 0.5 mg/mL 的样品作用 24 h 后，收集菌丝，用 2.5%的戊二醛固定液固定后，4℃过夜；pH 7.2 的 PBS 洗涤 3 次；1%的锇酸 4℃下固定 2 h；梯度丙酮脱水（25%-100% 丙酮）；包埋剂包埋；LKBV 型超薄切片机切片，JEM-1200EX 透射电镜观察并拍照（Catelas *et al.*, 2005; 郭延奎, 2002）。

3.2 结果与讨论

3.2.1 槐糖脂对细菌的抑制作用

3.2.1.1 槐糖脂对大肠杆菌的抑制曲线

大肠杆菌是一种最常见的革兰氏阴性菌，在某些情况下是机会致病菌。由图 3-1 可以看出，随着槐糖脂在培养基中的浓度增大，从 5 mg/L 增加到 100 mg/L，大肠杆菌的生长曲线与对照相比都没有明显变化，菌体生长正常，说明槐糖脂对于大肠杆菌的生长没有抑制作用。这结果与之前文献报道的结果相符，说明槐糖脂对于革兰氏阴性菌没有明显的抑制作用。

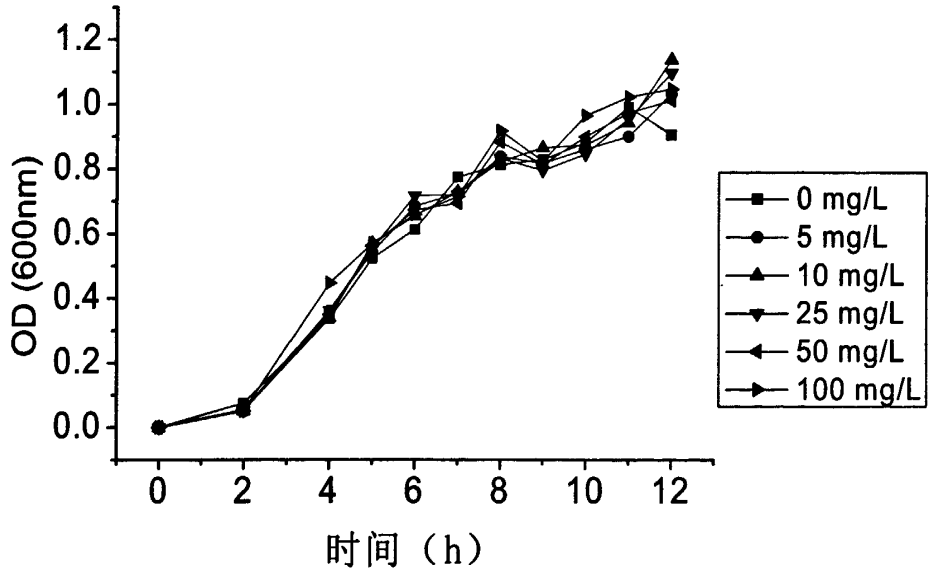


图 3-1 槐糖脂对大肠杆菌的抑制曲线

Fig. 3-1 Inhibition of sophorolipids to *E. coli*. Concentration of sophorolipids were 0、5, 10, 25, 30, 40, 50, 100 mg/L

3.2.1.2 槐糖脂对金黄色葡萄球菌的抑制曲线

金黄色葡萄球菌是一种革兰氏阳性菌，能引起多种感染，而且能对多种抗生素产生耐药性。槐糖脂对于金黄色葡萄球菌的抑制作用如图 3-2 所示。随着培养基中槐糖脂浓度从 10 mg/L 增加到 50 mg/L，菌体的浓度不断的减小，当槐糖脂浓度达到 40，50 mg/L 时，能够完全抑制住金黄色葡萄球菌的生长。说明槐糖脂对金黄色葡萄球菌有很好的抑制作用，在低浓度范围内就能完全抑制住菌体的生长。

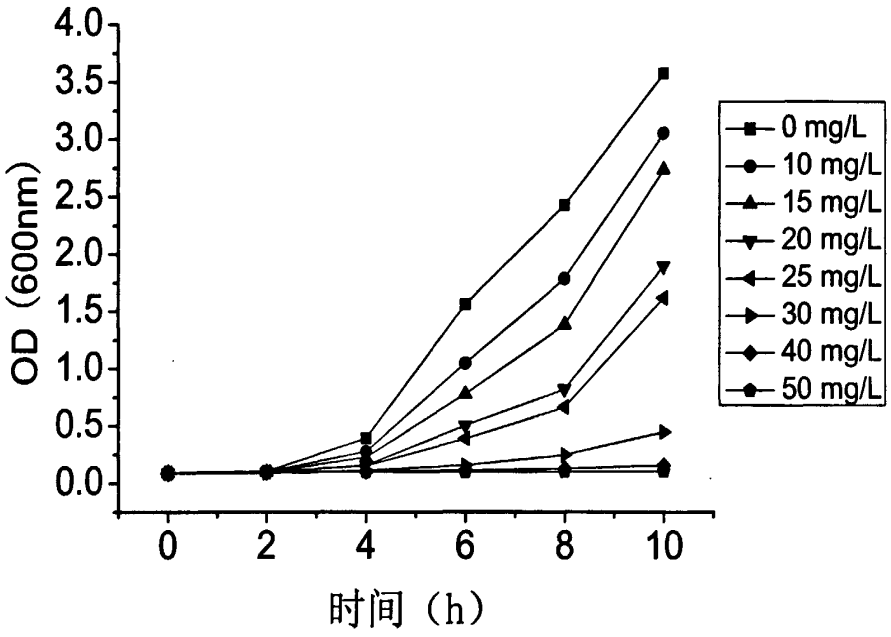


图 3-2 槐糖脂对金黄色葡萄球菌的抑制曲线

Fig. 3-2 Inhibition of sophorolipids to *S. aureus*. Concentration of sophorolipids were 0, 10, 15, 20, 25, 30, 40, 50 mg/L

3.2.1.3 槐糖脂对蜡状芽孢杆菌的抑制曲线

蜡状芽孢杆菌也是一种常见的革兰氏阳性菌，是食品和化妆品中常见的污染菌，能引起食物中毒。槐糖脂对其抑制作用如图 3-3 中所示。可以看出，槐糖脂对蜡状芽孢杆菌有比金黄色葡萄球菌更强的抑制作用。随着培养基中槐糖脂浓度的增加，菌体的浓度不断的减少。当槐糖脂浓度为 10 mg/L 时就能够抑制一部分

菌体的生长，当槐糖脂浓度达到 15 mg/L 时就能够完全抑制蜡状芽孢杆菌的生长。与对金黄色葡萄球菌的最低抑制浓度 40 mg/L 相比，槐糖脂对蜡状芽孢杆菌的最低抑制浓度更小。

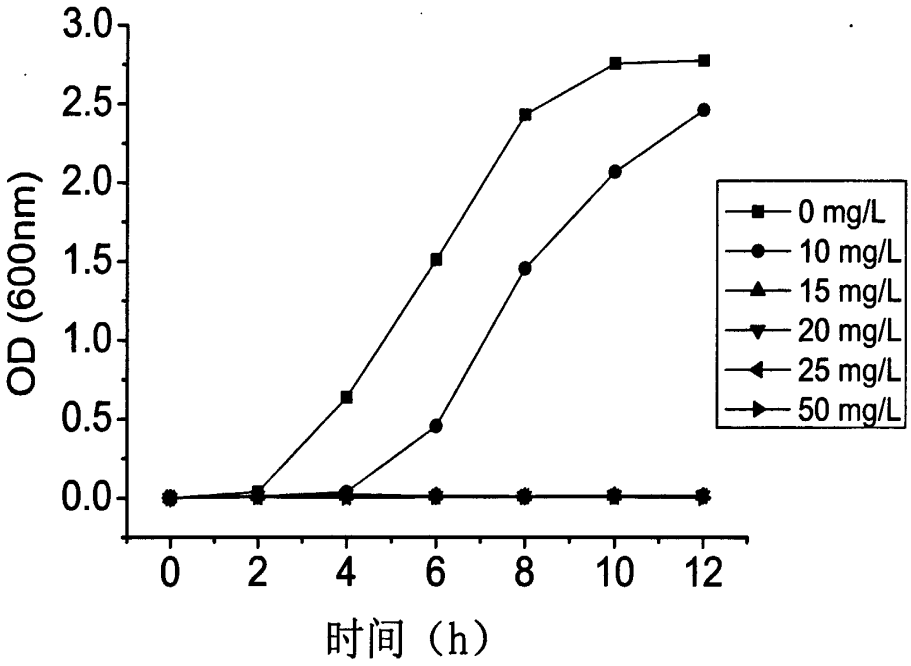


图 3-3 槐糖脂对蜡状芽孢杆菌的抑制曲线

Fig. 3-3 Inhibition of sophorolipids to *B. cereus*. Concentration of sophorolipids were 0, 10, 15, 20, 25, 50 mg/L

3.2.1.4 槐糖脂对变形链球菌的抑制曲线

变形链球菌是一种兼性厌氧的革兰氏阳性球菌，是口腔天然菌群里比例最大的链球菌属里中的一种。它具有很强的致龋性，能产生胞外葡聚糖、果聚糖和胞内多糖，其中葡聚糖介导细菌粘附，是重要的致龋毒力因子。变形链球菌不仅是牙冠部龋病的主要致病菌，也是根面龋的主要致病菌。从图 3-4 中可以看出，槐糖脂对变形链球菌也有很强的抑制作用。随着培养基中槐糖脂浓度的增加，菌体的浓度不断的减少。当槐糖脂浓度为 10，25 mg/L 时就能够抑制一部分菌体的生长，当槐糖脂浓度达到 50 mg/L 时就能够完全抑制变形链球菌的生长。槐糖

脂能在很低的浓度下抑制变形链球菌的生长，将其添加到牙膏或漱口水中可以有效的防治龋齿的发生。

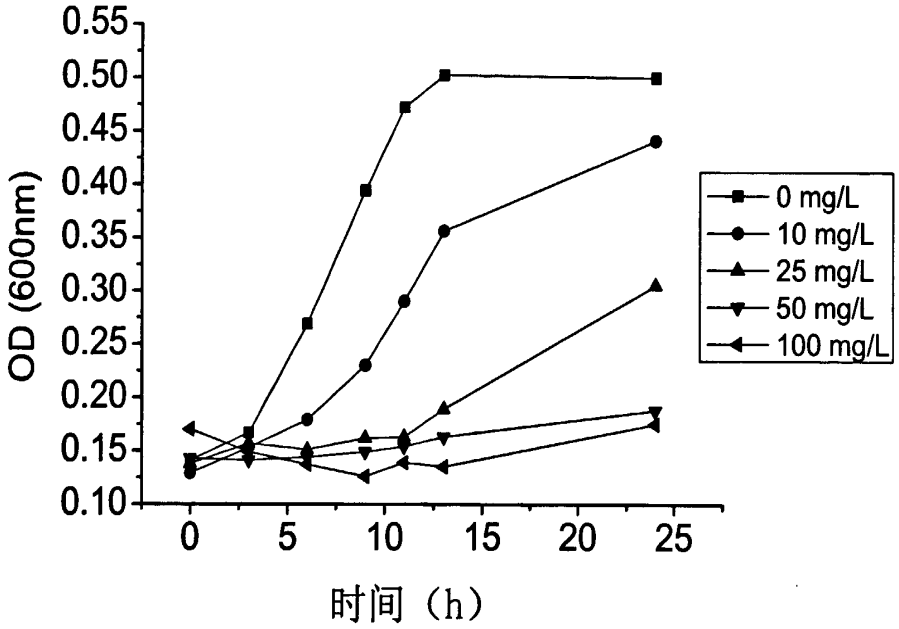


图 3-4 槐糖脂对变形链球菌的抑制曲线

Fig. 3-4 Inhibition of sophorolipids to *Streptococcus mutans*. Concentration of sophorolipids were 0, 10, 25, 50, 100 mg/L

3.2.2 槐糖脂对皮肤癣菌的抑制作用

3.2.2.1 内酯型槐糖脂对红色毛癣菌的抑制作用

如图 3-5 所示，内酯型槐糖脂对红色毛癣菌具有很好的抑制作用。培养 5 天后，没有加入槐糖脂溶液和只加入 0.25 mL 二甲亚砜溶剂的两组对照平板上红色毛癣菌生长状态良好。在含有 0.1 g/L 和 0.25 g/L 内酯型槐糖脂的平板上，与对照相比，红色毛癣菌的菌落数量大大减少，菌落大小也小了很多。说明内酯型槐糖脂既能减少菌落的数量，又能抑制菌丝的延伸。在含有 0.5 g/L 内酯型槐糖脂的平板上，完全没有菌落的生长，说明 0.5 g/L 内酯型槐糖脂能完全抑制红色毛

癣菌的生长。

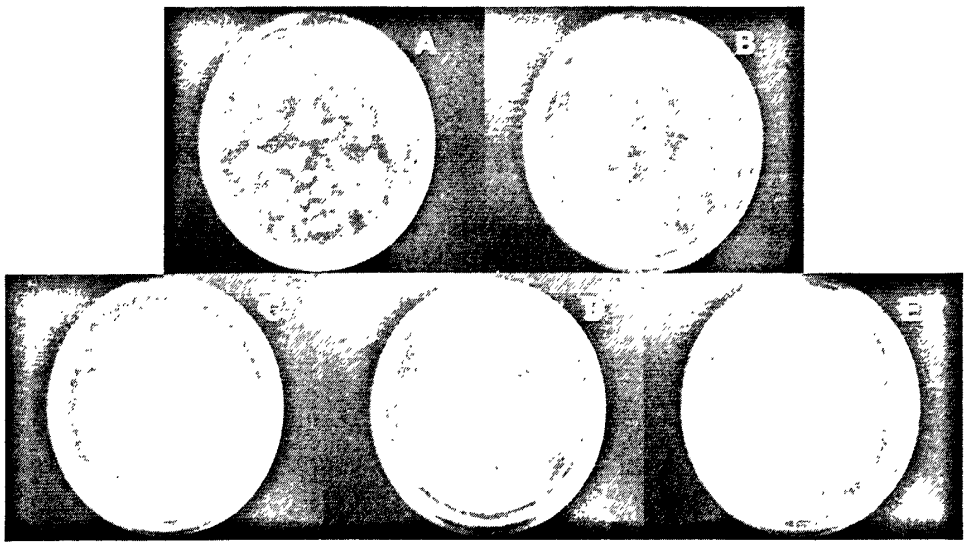


图 3-5 内酯型槐糖脂对红色毛癣菌的抑制作用

Fig. 3-5 Inhibition of lactonic sophorolipids to *Trichophyton rubrum*. A: medium without sophorolipids solutions; B: medium only with 0.25 mL dimethyl sulfoxide; C: medium with 0.1 g/L lactonic sophorolipids; D: medium with 0.25 g/L lactonic sophorolipids; E: medium with 0.5 g/L lactonic sophorolipids.

3.2.2.2 内酯型槐糖脂对石膏样毛癣菌的抑制作用

从图 3-6 可以看出，内酯型槐糖脂对石膏样毛癣菌也具有很强的抑制作用。培养 5 天后，没有加入槐糖脂溶液和只加入 0.25 mL 二甲亚砜溶剂的两组对照平板上红色毛癣菌生长状态良好。在含有 0.1 g/L、0.25 g/L 和 0.5 g/L 内酯型槐糖脂的平板上，都完全没有石膏样毛癣菌菌落的生长，说明在培养 5 天时 0.1 g/L 内酯型槐糖脂就能完全抑制住石膏样毛癣菌的生长，与红色毛癣菌相比，内酯型槐糖脂对石膏样毛癣菌具有更强的抑制作用。

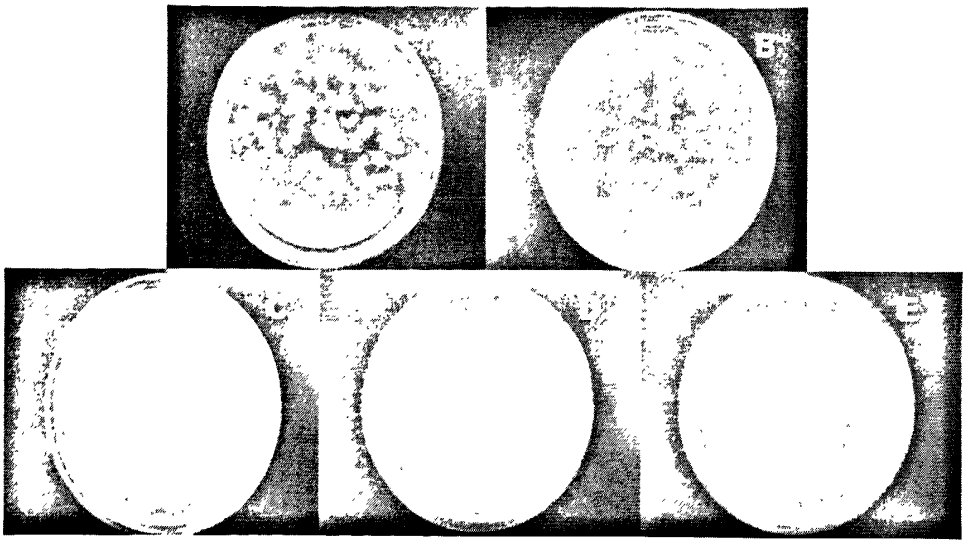


图 3-6 内酯型槐糖脂对石膏样毛癣菌的抑制作用

Fig. 3-6 Inhibition of lactonic sophorolipids to *Trichophyton gypseum*. A: medium without sophorolipids solutions; B: medium only with 0.25 mL dimethyl sulfoxide; C: medium with 0.1 g/L lactonic sophorolipids; D: medium with 0.25 g/L lactonic sophorolipids; E: medium with 0.5 g/L lactonic sophorolipids.

3.2.2.3 内酯型槐糖脂对犬小孢子菌的抑制作用

从图 3-7 可以看出，与石膏样毛癣菌一样，内酯型槐糖脂对犬小孢子菌也具有很强的抑制作用。培养 5 天后，没有加入槐糖脂溶液和只加入 0.25 mL 二甲亚砜溶剂的两组对照平板上犬小孢子菌生长状态良好。在含有 0.1 g/L、0.25 g/L 和 0.5 g/L 内酯型槐糖脂的平板上，都完全没有犬小孢子菌菌落的生长，说明在培养 5 天时 0.1 g/L 内酯型槐糖脂就能完全抑制住犬小孢子菌的生长，与红色毛癣菌相比，内酯型槐糖脂对犬小孢子菌具有更强的抑制作用。

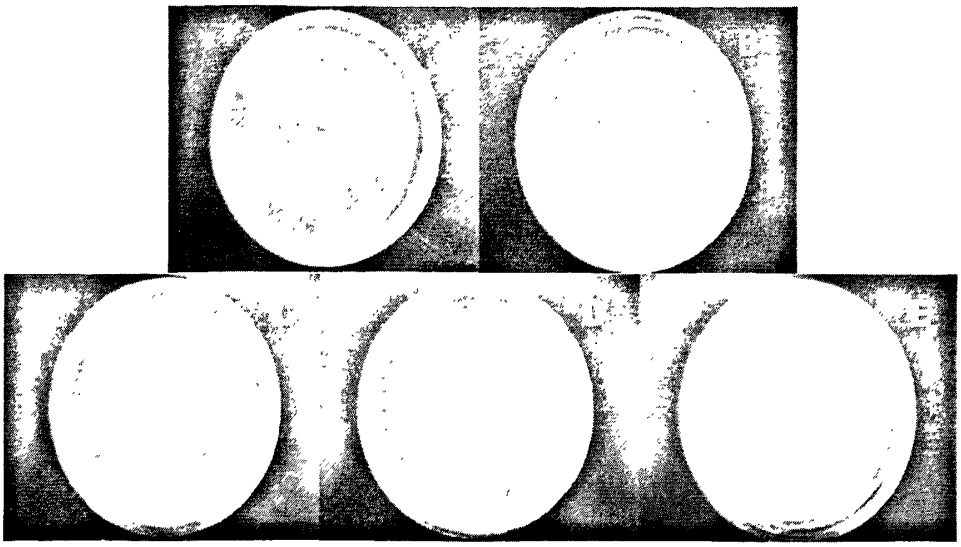


图 3-7 内酯型槐糖脂对犬小孢子菌的抑制作用

Fig. 3-7 Inhibition of lactonic sophorolipids to *Microsporum canis*. A: medium without sophorolipids solutions; B: medium only with 0.25 mL dimethyl sulfoxide; C: medium with 0.1 g/L lactonic sophorolipids; D: medium with 0.25 g/L lactonic sophorolipids; E: medium with 0.5 g/L lactonic sophorolipids.

3.2.2.4 酸型槐糖脂对红色毛癣菌的抑制作用

如图 3-8 所示，酸型槐糖脂对红色毛癣菌也具有很好的抑制作用。培养 5 天后，与菌落生长好的对照平板相比，随着培养基中酸型槐糖脂浓度的增大，平板上菌落的数量不断减少。在含有 0.5 g/L 酸型槐糖脂的平板上，几乎没有菌落的生长，说明在培养 5 天时，0.5 g/L 酸型槐糖脂几乎能完全抑制红色毛癣菌的生长。

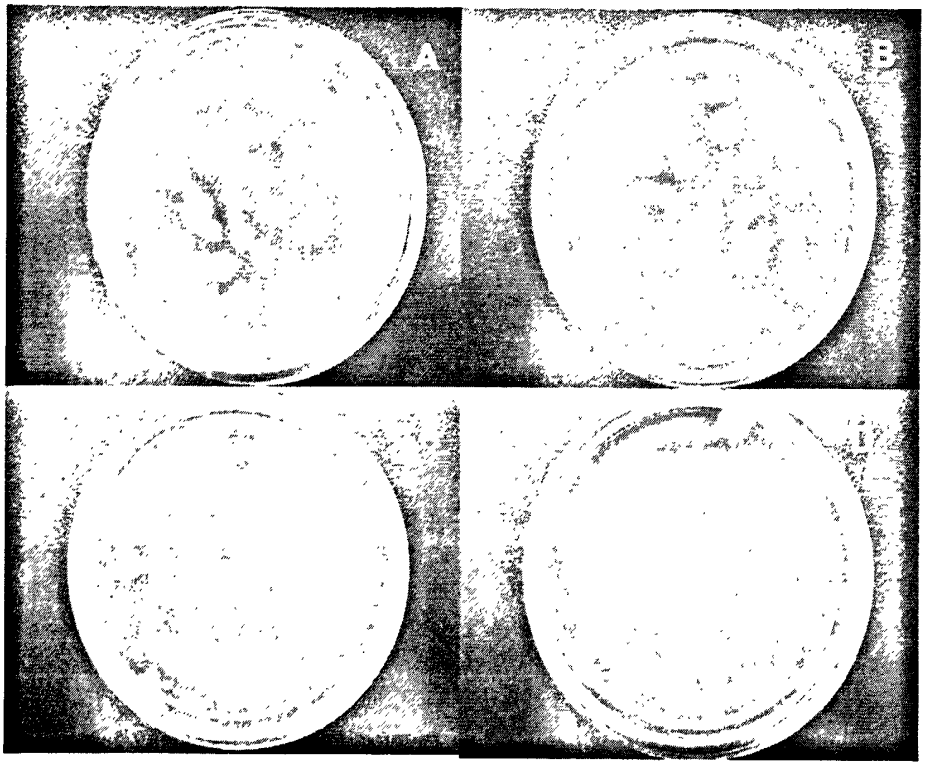


图 3-8 酸型槐糖脂对红色毛癣菌的抑制作用

Fig. 3-8 Inhibition of acid sophorolipids to *Trichophyton rubrum*. A: medium without sophorolipids solutions; B: medium with 0.1 g/L acid sophorolipids; C: medium with 0.25 g/L acid sophorolipids; D: medium with 0.5 g/L acid sophorolipids.

3.2.2.5 酸型槐糖脂对石膏样毛癣菌的抑制作用

由图 3-9 可以看出，与红色毛癣菌相比，酸型槐糖脂对石膏样毛癣菌具有强的抑制作用。培养 5 天后，对照平板上石膏样毛癣菌菌落生长旺盛。在含有 0.1 g/L 酸型槐糖脂平板上，与对照相比，石膏样毛癣菌的菌落数量大大减少，菌落大小也小了很多。说明酸型槐糖脂也能减少菌落的数量和抑制菌丝的延伸。在含有 0.25 g/L 和 0.5 g/L 酸型槐糖脂的平板上，完全没有菌落的生长，说明培养 5 天时，0.25 g/L 酸型槐糖脂就能完全抑制石膏样毛癣菌的生长。

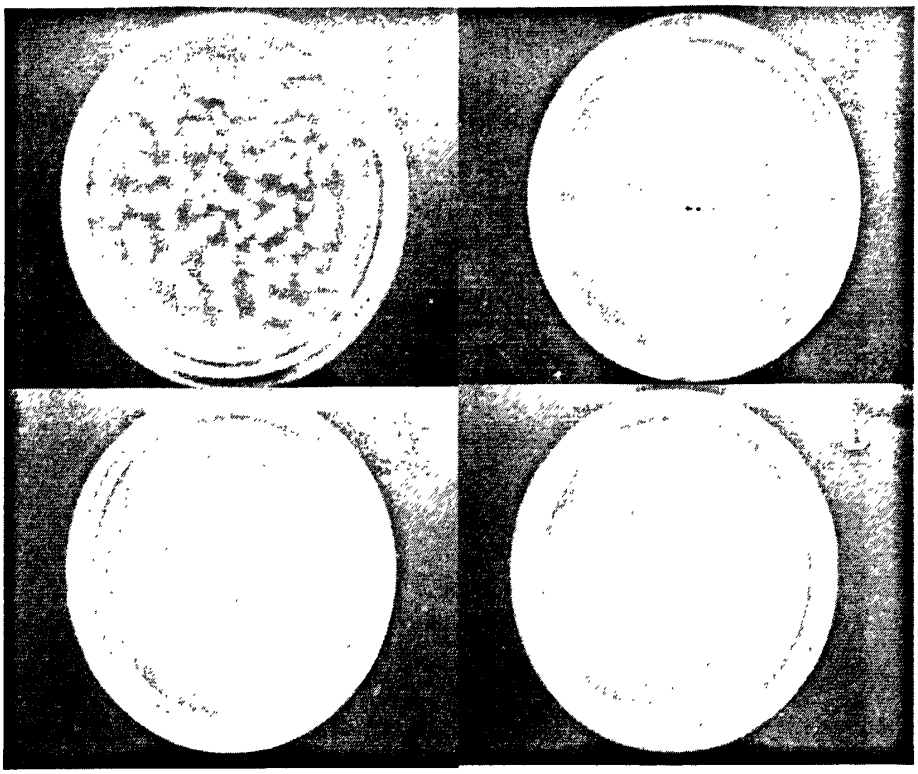


图 3-9 酸型槐糖脂对石膏样毛癣菌抑制作用

Fig. 3-9 Inhibition of acid sophorolipids to *Trichophyton gypseum*. A: medium without sophorolipids solutions; B: medium with 0.1 g/L acid sophorolipids; C: medium with 0.25 g/L acid sophorolipids; D: medium with 0.5 g/L acid sophorolipids.

3.2.2.5 酸型槐糖脂对犬小孢子菌的抑制作用

如图 3-10 所示，酸型槐糖脂对犬小孢子菌也具有较强的抑制作用。培养 5 天后，与菌落生长好的对照平板相比，随着培养基中酸型槐糖脂浓度的增大，平板上菌落的数量不断减少。在含有 0.5 g/L 酸型槐糖脂的平板上，几乎没有菌落的生长，说明在培养 5 天时，0.5 g/L 酸型槐糖脂几乎能完全抑制犬小孢子菌的生长。

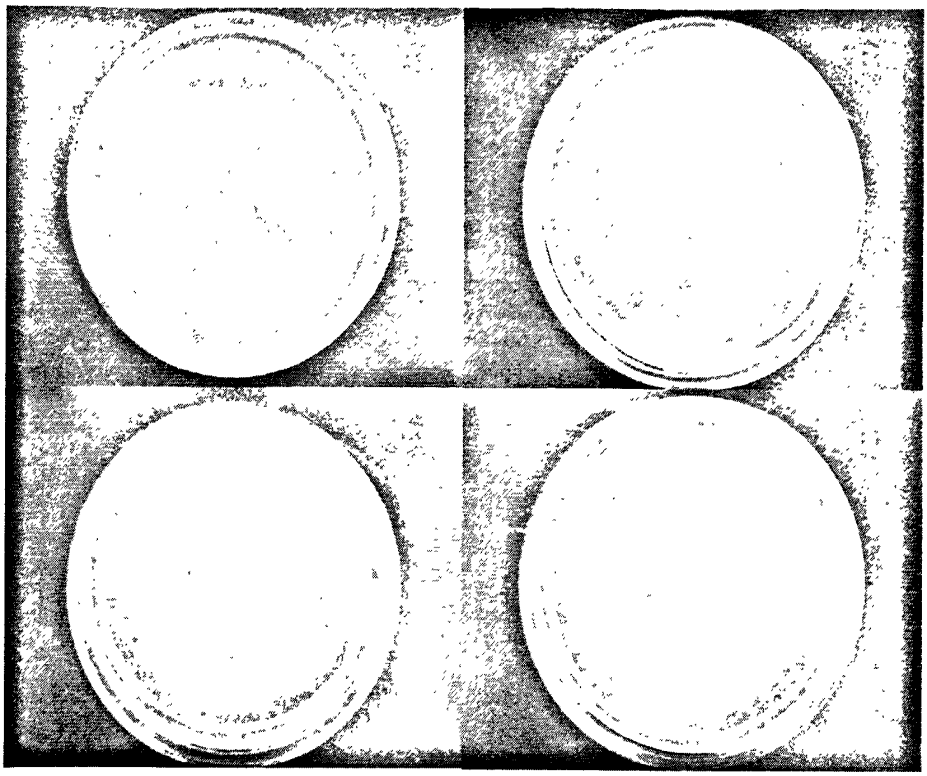


图 3-10 酸型槐糖脂对犬小孢子菌的抑制作用

Fig. 3-10 Inhibition of acid sophorolipids to *Microsporum canis*. A: medium without sophorolipids solutions; B: medium with 0.1 g/L acid sophorolipids; C: medium with 0.25 g/L acid sophorolipids; D: medium with 0.5 g/L acid sophorolipids.

3.2.2.6 内酯型槐糖脂与酸型槐糖脂对三种皮肤癣菌抑制作用的比较

我们比较了内酯型槐糖脂和酸型槐糖脂对红色毛癣菌、石膏样毛癣菌和犬小孢子菌三种皮肤癣菌的抑制作用。对于红色毛癣菌和犬小孢子菌我们观察了培养 5 天到 7 天的情况，对于石膏样毛癣菌，我们观察了培养 5 天到 10 天的情况。表 3-1 中只列出了培养第 7 天时，三种皮肤癣菌在不同浓度内酯型槐糖脂和酸型槐糖脂平板上的生长情况。由表中可以看出，酸型槐糖脂对石膏样毛癣菌的抑制效果要强于红色毛癣菌和犬小孢子菌，培养 7 天时，在含有 0.25 g/L 酸型槐糖脂的平板上，没有石膏样毛癣菌的菌落生长，而在含有 0.5 g/L 酸型槐糖脂的平板上，红色毛癣菌和犬小孢子菌都有一些菌落生长。在这三种皮肤癣菌中，内酯型

槐糖脂对犬小孢子菌具有最强的抑制作用，培养 7 天时，0.1 g/L 内酯型槐糖脂就能完全抑制犬小孢子菌菌落的生长。内酯型槐糖脂对石膏样毛癣菌也具有较强的抑制作用，在含有 0.25 g/L 和 0.5 g/L 内酯型槐糖脂的平板上都没有菌落长出。而对于红色毛癣菌来说，内酯型槐糖脂仅有一定的抑制作用，在含有 0.5 g/L 内酯型槐糖脂的平板上仍然能观察到一些菌落的生长。

对于红色毛癣菌来说，内酯型槐糖脂的抑制作用要比酸型槐糖脂的抑制效果强一点。随着内酯型和酸型槐糖脂浓度的增加，红色毛癣菌的菌落数量越来越少，当槐糖脂的浓度达到 0.5 g/L，培养第五天时，没有菌落生长，随着培养时间的延长，菌落慢慢的生长出来。在浓度相同的情况下，含有内酯型槐糖脂的平板上菌落的数量要少于含有酸型槐糖脂的平板上的菌落数量。

酸型槐糖脂和内酯型槐糖脂对石膏样毛癣菌都具有很强的抑制作用。不仅能抑制菌落的数量和大小，而且能在很长时间内抑制石膏样毛癣菌的生长。在低浓度的条件下，内酯型槐糖脂的作用效果要好于酸型槐糖脂，培养 5 天时，0.05 g/L 的内酯型槐糖脂就能完全抑制石膏样毛癣菌的生长，而含有 0.1 g/L 的酸型槐糖脂的平板上还有少量菌落生长。在高浓度条件下，酸型槐糖脂的作用效果要好于内酯型槐糖脂，培养 10 天后，0.5 g/L 酸型槐糖脂能完全抑制石膏样癣菌的生长，而含有 0.5 g/L 的内酯型槐糖脂的平板上还有少量菌落生长。我们猜测，对于酸型槐糖脂来说有一个临界浓度，当浓度高于这个临界浓度时，酸型槐糖脂就能完全抑制石膏样毛癣菌的生长。

对于犬小孢子菌，内酯型槐糖脂的抑制作用要比酸型槐糖脂的抑制效果强很多。在低浓度情况下，酸型槐糖脂对犬小孢子菌只有一点抑制作用，在高浓度条件下（0.5 g/L），酸型槐糖脂对犬小孢子菌有较强的抑制作用，能抑制菌体的生长到第 5 天。培养到 7 天后，在含有 0.1 g/L 内酯型槐糖脂的平板上都没有菌落的生长，说明内酯型槐糖脂对犬小孢子菌具有很强的持续抑制作用。

表 3-1 培养 7 天后内酯型槐糖脂与酸型槐糖脂对

红色毛癣菌、石膏样毛癣菌和犬小孢子菌抑制作用的比较

Table 3-1 Comparison of the inhibition of acidic sophorolipids with that of lactonic sophorolipids to *Trichophyton rubrum*, *Trichophyton gypseum* and *Microsporum canis* at the seventh day of cultivation

Sophorolipids concentration	*Trichophyton rubrum*	*Trichophyton gypseum*	*Microsporum canis*
Control*	+++++	+++++	+++++
0.1 g/L ASLs	+++++	++++	+++++
0.25 g/L ASLs	+++++	—	+++++
0.5 g/L ASLs	+++	—	+++
DSMO*	+++++	++++	+++++
0.05 g/L LSLs	+++++	++	+
0.1 g/L LSLs	++++	+	—
0.25 g/L LSLs	+++	—	—
0.5 g/L LSLs	++	—	—

Control* was medium without sophorolipids; DSMO* was medium only with 0.25 mL dimethyl sulfoxide; + showed the amount of the colonies; — indicated that no colonies was grown; ASLs was acidic sophorolipids; LSLs was lactonic sophorolipids

3.2.2.7 槐糖脂对三种皮肤癣菌的菌丝延伸抑制率

由表 3-2 可以看出，内酯型槐糖脂比酸型槐糖脂能更好的抑制三种皮肤癣菌的菌丝延伸。当酸型槐糖脂浓度为 0.5 g/L 时，红色毛癣菌、石膏样毛癣菌和犬小孢子菌的菌丝延伸抑制率分别只有 19.2%，12.5% 和 9.09%。随着内酯型槐糖脂浓度的增加，三种皮肤癣菌的菌落直径减小，菌丝延伸抑制率增大。当内酯型槐糖脂浓度为 0.5 g/L 时，红色毛癣菌、石膏样毛癣菌和犬小孢子菌的菌丝延伸

抑制率分别为53.8%，62.5% 和 68.2%。因为内酯型槐糖脂难溶于水，当内酯型槐糖脂浓度为0.5 g/L时，培养基是一种浑浊的状态，菌饼在平板上培养后，在菌饼的周围出现了透明圈的现象，说明槐糖脂在培养过程中进入了菌丝之中，在菌丝的内部起了作用，从而抑制了菌丝的生长延伸。

表3-2 培养5天后槐糖脂对三种皮肤癣菌的菌丝延伸抑制率

Table 3-2. Inhibition ratio of hypha extention at the fifth day of cultivation

Sophorolipids concentration	*Trichophyton rubrum*		*Trichophyton gypseum*		*Microsporum canis*	
	Diameter (mm)	Inhibition ratio (%)	Diameter (mm)	Inhibition ratio (%)	Diameter (mm)	Inhibition ratio (%)
Control	26	0.0	24	0.00	22	0.00
0.0625 g/L ASLs	25	3.84	23	4.17	22	0.00
0.125 g/L ASLs	24	7.69	22	8.33	21	4.55
0.25 g/L ASLs	24	7.69	21	12.5	20	9.09
0.50 g/L ASLs	21	19.2	21	12.5	20	9.09
DMSO*	23	11.5	22	8.33	20	9.09
0.0625 g/L LSLs	16	38.5	16	33.3	15	31.8
0.125 g/L LSLs	15	42.3	12	50.0	13	40.9
0.25 g/L LSLs	14	46.2	11	54.1	9	59.1
0.50 g/L LSLs	12	53.8	9	62.5	7	68.2

Control* was medium without sophorolipids; DSMO* was medium only with 0.2 mL dimethyl sulfoxide; ASLs was acidic sophorolipids; LSLs was lactonic sophorolipids

3.2.2.8 最小抑制浓度 (MIC)和最小杀灭浓度 (MFC)

在液体培养基中，酸型槐糖脂对三种皮肤癣菌几乎没有抑制作用，所以我们只研究了内酯型槐糖脂对三种皮肤癣菌的最小抑制浓度 (MIC)和最小杀灭浓度 (MFC)。由表 3-3 可以看出，内酯型槐糖脂对三种皮肤癣菌的 MIC 范围是0.0625-0.5 mg/mL。对红色毛癣菌、石膏样毛癣菌和犬小孢子菌的 MIC_{50} 值分别

是 0.0625，0.125，0.0625 mg/mL，MIC_{90} 值分别是 0.125，0.25，0.125 mg/mL，MFC 值分别是 0.5，0.5，0.25 mg/mL。

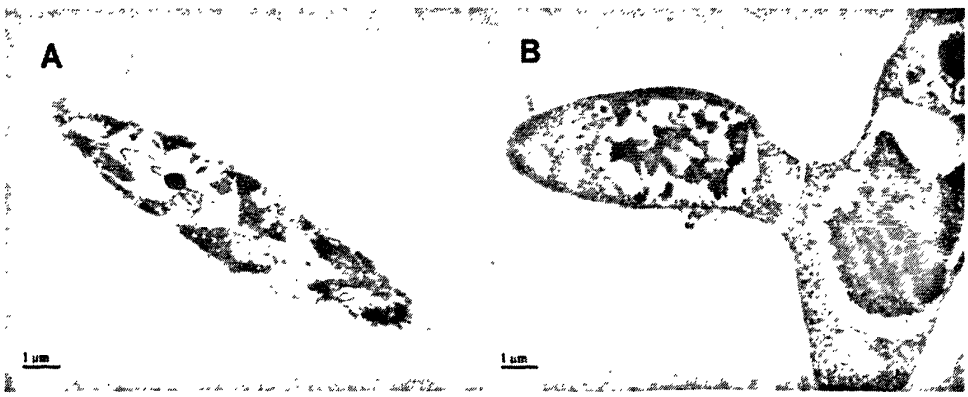
表 3-3 内酯型槐糖脂对三种皮肤癣菌的最小抑制浓度 (MIC)和最小杀灭浓度 (MFC)

Table 3-3 Minimum Inhibitive Consistency (MIC) and Minimal Fungicidal Consistency (MFC) of lactonic sophorolipids to three dermatophytes

Dermatophytes	MIC (mg/mL)			MFC(mg/mL)
	MIC range	MIC_{50}	MIC_{90}	
Trichophyton rubrum	0.0625-0.5	0.0625	0.125	0.5
Trichophyton gypseum	0.0625-0.5	0.125	0.25	0.5
Microsporum canis	0.0625-0.5	0.0625	0.125	0.25

3.2.2.9 透射电镜分析槐糖脂处理前后皮肤癣菌的形态变化

为了研究槐糖脂对三种皮肤癣菌的作用机制，我们利用透射电镜观察了内酯型槐糖脂处理前后三种皮肤癣菌显微结构的变化。如图 3-11 所示，槐糖脂处理前，三种皮肤癣菌菌丝的细胞壁完整且厚度均匀，细胞质均一，细胞器和液泡明显，细胞器和液泡的膜完整，细胞核的核区清晰，核膜完整。经过内酯型槐糖脂处理后，红色毛癣菌、石膏样毛癣菌和犬小孢子菌的细胞都发生了明显的显微形态改变。细胞壁变厚而且松散，细胞质凝集，形成高电子密度的团块，细胞器的膜消失，细胞器被损坏，细胞质中没有完整的细胞器和核区。这些变化的原因可能是由于槐糖脂具有的表面活性，也可能跟槐糖脂能引起细胞凋亡有关。



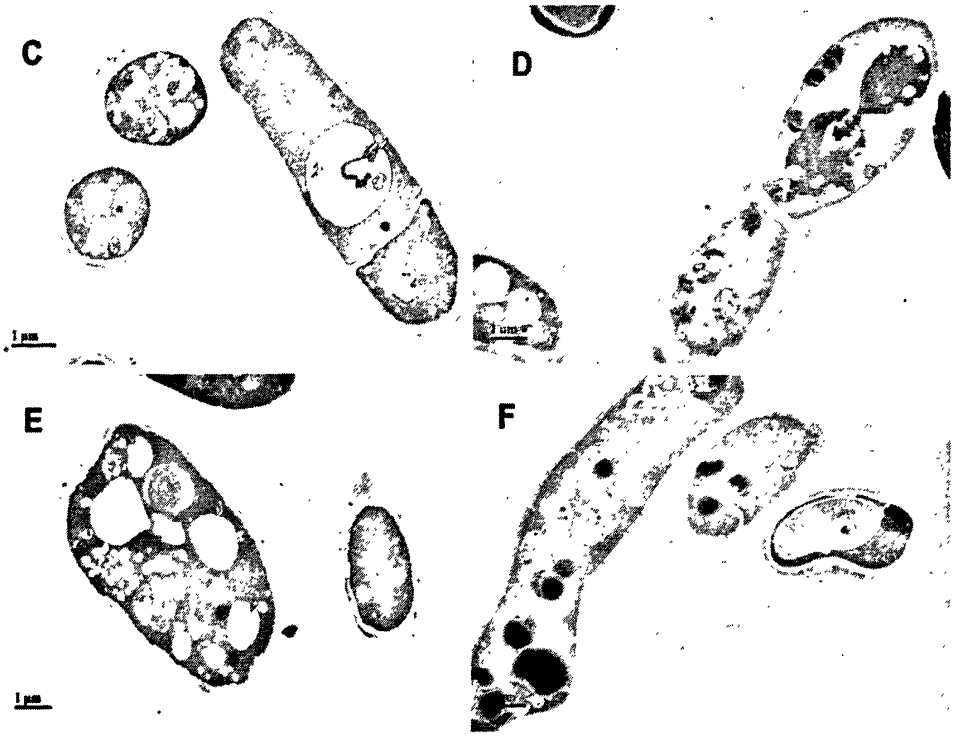


图 3-11 透射电镜分析槐糖脂处理前后红色毛癣菌、石膏样毛癣菌和犬小孢子菌的形态变化

Fig. 3-11 TEM observation of *Trichophyton gypseum*, *Trichophyton rubrum* and *Microsporum canis* with sophorolipids treatment. A: *Trichophyton gypseum* without being treated by sophorolipids; B: *Trichophyton gypseum* treated by 0.5 g/L sophorolipids for 48h; C: *Trichophyton rubrum* without being treated by sophorolipids; D: *Trichophyton rubrum* treated by 0.5 g/L sophorolipids for 48 h; E: *Microsporum canis* without being treated by sophorolipids; F: *Microsporum canis* treated by 0.5 g/L sophorolipids for 48h

3.3 本章小结

（1）槐糖脂对革兰氏阳性菌有明显的抑制作用，对大肠杆菌几乎没有抑制作用。首次报道了槐糖脂对引起龋齿的变形链球菌具有明显的抑制作用。

（2）首次报道了槐糖脂对红色毛癣菌、石膏样毛癣菌和犬小孢子菌三种常见的皮肤病致病菌的抑制作用，证明槐糖脂对这三种皮肤癣菌都有很强的抑制作用。

（3）比较了内酯型槐糖脂和酸型槐糖脂对这三种皮肤癣菌的作用效果的不

同。对红色毛癣菌来说，内酯型槐糖脂的抑制效果比酸型槐糖脂的抑制效果好一点。对石膏样毛癣菌来说，酸型槐糖脂和内酯型槐糖脂具有很强的抑制作用，不仅能抑制菌落的数量和大小，而且能在很长时间内抑制石膏样毛癣菌的生长。对犬小孢子菌来说，内酯型槐糖脂的抑制效果比酸型槐糖脂的抑制效果好很多。

（4）内酯型槐糖脂比酸型槐糖脂能更好的抑制三种皮肤癣菌的菌丝延伸。在液体培养基中，酸型槐糖脂对皮肤癣菌几乎没有抑制作用。因此，内酯型槐糖脂具有更好的潜力应用于临床的皮肤病治疗。

（5）通过透射电镜观察，发现经过内酯型槐糖脂处理后，红色毛癣菌、石膏样毛癣菌和犬小孢子菌的细胞的显微结构都发生了明显改变。细胞壁变厚而且松散，细胞质凝集，细胞器的膜消失，细胞质中没有完整的细胞器和核区。

第四章 槐糖脂的分离纯化、结构鉴定及抗肿瘤活性

引言

天然合成的槐糖脂是由很多槐糖脂分子所组成的混合物。这些槐糖脂分子有一个共同的特征，分子由亲水性和疏水性两部分组成。亲水性部分是槐糖（两个葡萄糖分子以β-1, 2 糖苷键结合），疏水性部分是饱和或不饱和的长链ω-（或ω-1）羟基脂肪酸，这两部分以糖苷键相连。而它们的区别在于它们所含脂肪酸碳链的长度不同、饱和或不饱和的程度不同、槐糖部分乙酰化的程度以及乙酰化位置不同，以及是否存在内酯化作用（Asmer, 1988）。

由不同的底物发酵生产得到的槐糖脂的组分也不相同，尤其是第二碳源的组成对槐糖脂的组分结构影响更大。在以葡萄糖和脂肪酸为底物时，槐糖脂的合成途径为：部分葡萄糖经过糖酵解途径，分解为丙酮酸，然后在丙酮酸脱羧酶的作用下，生成乙酰 CoA，乙酰 CoA 进入三羧酸循环，为菌体的生长代谢提供能量。脂肪酸在单加氧酶细胞色素 450 (Cyt P450)的作用下生成 ω-/ω-1 羟基脂肪酸，在糖基转移酶 1 的作用下，将一个葡萄糖分子转移到 ω-/ω-1 羟基脂肪酸，生成葡萄糖脂；在糖基转移酶 2 的作用下将另一个葡萄糖分子转移到葡萄糖脂上，生成槐糖脂；在乙酰转移酶作用下，生成乙酰化的槐糖脂。当所用油脂底物主要成分为 16 碳或 18 碳脂肪酸时，脂肪酸会被直接利用合成槐糖脂，所合成的槐糖脂组分的结构被脂肪酸底物的结构影响。当所用油脂底物主要成分为不是 16 碳或 18 碳脂肪酸时，槐糖脂合成过程中首先会通过 β-氧化过程将脂肪酸变为 16 碳或 18 碳脂肪酸，然后再合成槐糖脂，所以天然合成的槐糖脂脂肪酸部分主要为 16 碳或 18 碳。

我们将 *Wickerhamiella domercqiae var. sophorolipid* 利用菜籽油为第二碳源发酵得到的天然槐糖脂混合物通过 HPLC 进行分离纯化并鉴定每一个组分的结构，以了解我们发酵得到的槐糖脂粗品的组成成分。

近年来，槐糖脂在医药领域的应用得到了广泛的关注。槐糖脂除了具有抗微生物活性，抗病毒活性和抗炎症反应等，还有抗肿瘤的生物活性。1997 年，Isoda 等发现粗品槐糖脂能够诱导人早幼粒白血病细胞 HL60 的分化，10 μg/mL 的粗品

槐糖脂就能将 HL60 细胞诱导为单核细胞。但是该研究没有弄清楚到底是那种槐糖脂发挥的作用（Isoda *et al.*，1997）。1998 年，Scholz 等纯化得到内酯型槐糖脂，然后通过醇解和脂肪酶催化的方法制备了一些槐糖脂的衍生物。用内酯型槐糖脂及其衍生物作用于两种肿瘤细胞，悬浮细胞 Leukemic Jurkat 和贴壁细胞 Head and neck cancer Tu 138。结果发现，槐糖脂粗品、乙酰化的槐糖脂乙酯和乙酰化的槐糖脂丁酯对这两种细胞都具有很强的抑制作用，而脱乙酰化的槐糖脂乙酯和丁酯对细胞生长基本没有抑制作用。因此作者得出槐糖脂的抗肿瘤活性与槐糖脂分子上的乙酰基团有关（Scholz *et al.*, 1998）。

2005 年，我们实验室报道了脂肪酸部分为油酸的双乙酰基内酯型槐糖脂对四种肿瘤细胞 H7402（人肝癌细胞），A549 （人肺腺癌细胞）， HL60（人急性早幼粒白血病细胞）和 K562（人慢性粒细胞白血病细胞）具有很强的抑制作用（Chen *et al.*, 2005）。随后又进一步研究了脂肪酸部分为油酸的双乙酰基内酯型槐糖脂对人肝癌细胞 H7402 的作用机制，发现槐糖脂的抗肿瘤机制是引起细胞的凋亡（Chen *et al.*, 2006）。2008 年，Fu 等报道了天然槐糖脂混合物以及槐糖脂衍生物（包括槐糖脂甲酯、槐糖脂乙酯、单乙酰基槐糖脂乙酯、双乙酰基槐糖脂乙酯、酸型槐糖脂、双乙酰基内酯型槐糖脂）对胰腺癌细胞的抑制作用（Fu *et al.*，2008）。

但是目前还没有关于不同结构的天然槐糖脂组分的抗肿瘤活性的研究。因为天然槐糖脂分子的结构主要区别在于槐糖部分乙酰化程度的不同，脂肪酸部分饱和程度不同以及是否内酯化，我们将分离纯化得到的几种不同结构的槐糖脂组分，用于对食管癌细胞 KYSE 109 和 KYSE 450 的抗肿瘤实验中，通过分析天然槐糖脂组分结构与抗肿瘤活性的关系，可以进一步揭示槐糖脂抗肿瘤活性的机制，并有助于对槐糖脂进行修饰以得到生物活性更好的槐糖脂衍生物。

4.1 材料和方法

4.1.1 仪器和试剂

所用菜籽油为食用级，其他试剂均为分析纯或色谱纯；LD2X-40B1 型立式

自动电热压力蒸汽灭菌器（上海申安医疗器械厂）；HZQ-Q 全温振荡器（哈尔滨东联电子技术开发有限公司）；旋转蒸发仪（上海申胜生物技术有限公司）；美国 Agilent 公司 1100 型高压液相色谱仪；美国 Applied Biosystems 公司的 API4000 型质谱仪。

抗肿瘤活性研究所用器皿和仪器：

(1) 器皿：血清瓶，细胞培养瓶，4 孔、6 孔、24 孔、96 孔细胞培养板，10 μL、100 μL、1 mL、5 mL 枪尖；

(2) 仪器：Spectra Max 190 微板光谱仪（Molecular Devices Co. USA），BNA-310 型二氧化碳培养箱（上海福玛实验设备有限公司）。

细胞培养用试剂：

(1) 新生牛血清，杭州四季青生物工程材料有限公司；

(2) RPMI 1640 培养基，HyClone 公司；

(3) MTT，[3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide]，四甲基噻唑蓝，AMRESCO 产品；

(4) $NaHCO_3$，分析纯，上海虹光化工厂；

(5) 二甲基亚砜，分析纯，中国华东化工学院江南化工二厂；

(6) 谷氨酰胺，北京经科公司；

(7) 硫酸链霉素，streptomycin sulfate，AMRESCO 公司；

(8) 青霉素钠，80 万单位，山东鲁抗医药股份有限公司；

(9) 胰蛋白酶，Trypsin，1:250，AMRESCO 公司；

(10) SDS，BBI 产品。

4.1.2 槐糖脂生产菌株

拟威克酵母 *Wickerhamiella domercqiae var. sophorolipid*，由本实验室自污水中分离。

4.1.3 抗肿瘤研究所用的肿瘤细胞株

KYSE109 和 KYSE450：人食管癌细胞，贴壁细胞，山东省齐鲁医院提供。

4.1.4 培养基

4.1.4.1 斜面及种子 YEPD 培养基

葡萄糖 2.0 %，蛋白胨 2.0 %，酵母粉 1.0 %，琼脂粉 2.0 %（w/v, 液体种子培养时不加）。

4.1.4.2 槐糖脂发酵培养基

葡萄糖 8.0%，菜籽油 6.0%，KH_2PO_4 0.10 %，$Na_2HPO_4 \cdot 12H_2O$ 0.10 %，$MgSO_4 \cdot 7H_2O$ 0.05 %（w/v）。

4.1.4.3 细胞培养用培养基及培养用液的配制

(1) RPMI 1640 基本培养基

取 RPMI 1640 一袋，加 $NaHCO_3$ 2 g，加入 1000 mL 三蒸水中溶解，用 HCl 调 pH 至 7.2，过滤除菌，4 ℃冰箱保存。

(2) 双抗溶液

1. 准确称取硫酸链霉素 0.8 g，用 2 mL 无菌三蒸水溶解，过滤后取 1 mL；
2. 称取青霉素钠，80 万单位，用 2 mL 无菌三蒸水溶解，过滤后取 1 mL；
3. 加入无菌三蒸水 38 mL，使总体积为 40 mL，每毫升含青霉素 10000 U，链霉素 10000 μg；
4. 分装于 1.5 mL（内含 1mL）小离心管中，-20℃冰箱保存。

(3) RPMI1640 完全培养基

RPMI 1640 基本培养基 89 mL，新生牛血清 10 mL，双抗溶液 1 mL，混匀。

(4) MTT 溶液（5 mg/mL）

准确称取 100 mg MTT，加入 pH 7.2 的 PBS 液 20 mL，溶解后过滤除菌，分装于

离心管中，-20 ℃冰箱保存。

(5) NaHCO3 溶液（pH 调整液）

准确称取 5.6 g $NaHCO_3$，溶解于 100 mL 三蒸水中，终浓度为 5.6%，过滤除菌后，分装于离心管中。

(6) PBS 液（pH 7.2）

NaCl（A.R.） 8.5 g

KCl（A.R.） 0.2 g

$Na_2HPO_4 \cdot 12H_2O$ 2.85 g

KH_2PO_4 0.27 g

溶于 1000 mL 三蒸水中，调节 pH 为 7.2，经 121 ℃灭菌 15 min 后，室温保存。

(7) 0.25% 胰蛋白酶消化液

准确称取 0.25 g 胰蛋白酶，用 pH 7.2 的 PBS 溶解，搅拌均匀，放置在室温 4 h 或冰箱内过夜，并不时搅拌，然后过滤除菌，分装与离心管中，-20 ℃冰箱保存。

(8) 10% SDS 溶液

准确称取 10 g SDS，溶解于 100 mL 三蒸水中，终浓度为 10 %。用于细胞裂解。

4.1.5 槐糖脂粗品的提取

发酵液经等体积的乙酸乙酯萃取后，取上层有机相 50 ℃减压干燥，用正己烷洗涤 2 遍，去除剩余的菜籽油，再经 50 ℃减压干燥，得到淡黄色块状固体，即为粗提槐糖脂样品。

4.1.6 粗提槐糖脂样品的分离纯化与制备

槐糖脂粗提样品首先经分析型 HPLC 分离。条件：柱子为 KromasiL C_{18}，5 μm

(250mm×4.6mm，Agela Technologies Inc., USA)的分析柱，流动相为乙腈-水进行梯度洗脱（体积比），洗脱方案为：0 min~15 min，乙腈含量从 40 %升高至 60 %；15 min~30 min，乙腈含量从 60 %升高至 70 %；30 min~40 min，乙腈含量从 70 %升高至 90 %；40 min~55 min，乙腈含量维持在 90 %。进样体积 5 μL，流速 1 mL/min，207 nm 检测。制备型 HPLC 条件：PrepHT XDB C_{18} column (250 mm×21.2 mm, Agela Technologies Inc., USA)制备柱，进样体积 500 μL，流速 15 mL/min，洗脱条件与分析型 HPLC 相同。

4.1.7 MS 分析

经制备型 HPLC 制备的槐糖脂纯品用 API4000 质谱仪（Applied Biosystems, USA）分析。离子源：ESI；喷雾电压：-4500 V；离子源温度：100 ℃；雾化气：25 psi；辅助气：20 psi。

4.1.8 细胞传代培养方法

1. 当细胞长满细胞培养瓶的瓶壁以后，倒出培养液；
2. 用 3 mL pH 7.2 的 PBS 洗涤两次；
3. 加入 1 mL 0.25 %的胰蛋白酶消化液，消化 1 min；
4. 加入 3 mL 的 RPMI 1640 完全培养基；
5. 用枪尖吹打混匀后将细胞分到 2 个细胞培养瓶中；
6. 放置于 37 ℃，5% CO_2 培养箱中培养。隔两天传代一次。

4.1.9 槐糖脂样品的稀释

槐糖脂样品用乙醇溶解，然后用 RPMI 1640 完全培养基稀释至不同浓度（乙醇浓度小于 0.02 %，乙醇浓度为 0.1 %时对细胞存活率无影响），用 0.22 μm 膜过滤除菌。

4.1.10 MTT 法分析细胞存活率（Price & Mcmillan, 1990）

1. 收集细胞：将处于对数生长期的肿瘤细胞 1000 r/min 离心 5min，弃上清，用 RPMI 1640 完全培养基稀释成细胞悬液，使细胞浓度为 1.0×10^5 cells/mL；
2. 槐糖脂样品处理细胞：取 50 μL 细胞悬液加入 96 孔细胞培养板中，培养 24 h 后再加入 50 μL 槐糖脂样品，每组样本 6 个复孔，并设细胞对照组（只加细胞）和空白对照组（只加培养基，无细胞）；
3. 放置于 37℃，5 % CO_2 孵箱中培养 24；
4. 加入 10 μL MTT 溶液（5 mg/mL），继续培养 4 h；
5. 加入 100 μL SDS 溶液，于 37℃，5% CO_2 孵箱中培养过夜，使甲臜结晶充分溶解，570 nm 下测 OD 值；
6. 按照下列公式计算细胞存活率

$$\text{细胞存活率} = \frac{\text{样品组 OD 值—空白组 OD}}{\text{细胞对照组 OD 值—空白组 OD}} \times 100\%$$

4.1.11 数据分析

每个值为平均值±标准差，采用 *t* 检验。

4.2 结果

4.2.1 HPLC 分离纯化槐糖脂

Wickerhamiella domercqiae var. sophorolipid 利用菜籽油为第二碳源发酵得到的槐糖脂粗品经 HPLC 分析，各种组分如图 4-1 所示。从图中可以看出，槐糖脂粗品中含有多达十几种以上的组分。将其中含量较高的 10 种组分用制备型 HPLC 进行了制备。

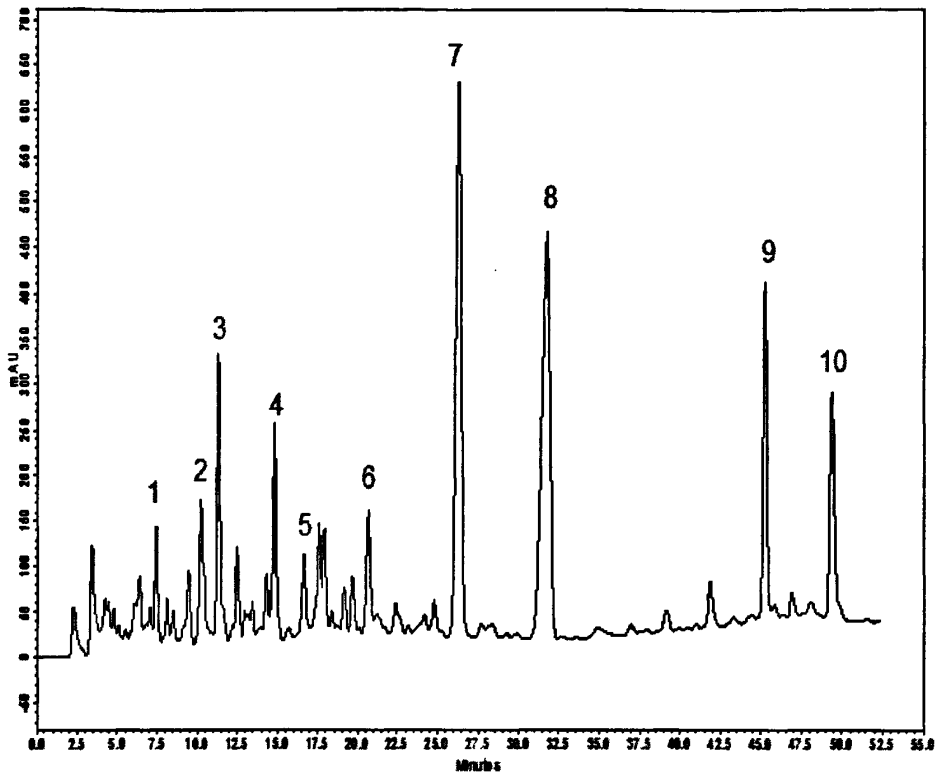


图 4-1 槐糖脂粗品组分的 HPLC 分析

Fig. 4-1 HPLC chromatogram of crude sophorolipids produced by *Wickerhamiella domercqiae var. sophorolipid*. Ten sophorolipid molecules were separately collected

4.2.2 各槐糖脂组分结构鉴定

将制备得到的 10 种槐糖脂组分进行了 MS 分析。每种组分的出峰时间、分子离子峰，以及具体结构如表 4-1 所示。由表中的结果可以看出，我们分离得到的槐糖脂组分的脂肪酸部分都是 18 碳脂肪酸，而它们的结构的区别在于脂肪酸部分的不饱和程度不同（无双键、一个双键或两个双键），槐糖脂部分的乙酰化程度不同（单乙酰基、双乙酰基、非乙酰化），以及是否存在内酯化（内酯型、酸型）。

峰 1 到峰 5 出峰时间较早，极性较大，为酸型槐糖脂，峰 6 到峰 9 出峰时间较晚，极性较小，为内酯型槐糖脂，其中内酯型槐糖脂所占比例较高。在 5 种酸型槐糖脂中，峰 1 的槐糖脂部分没有乙酰化，脂肪酸部分为 C18:2，峰 2 和峰 3

的槐糖部分为单乙酰化，脂肪酸部分分别为 C18:2 和 C18:1，峰 4 和峰 5 的槐糖部分为双乙酰化，脂肪酸部分分别为 C18:2 和 C18:1。

在 4 种内酯型槐糖脂中，峰 6 的槐糖部分为单乙酰基，脂肪酸部分为 C18:1，峰 7、峰 8 和峰 9 的槐糖部分为双乙酰基，脂肪酸部分分别为 C18:2、C18:1 和 C18:0。乙酰化程度越大，槐糖脂的极性减小，所以槐糖脂的出峰时间随着乙酰化程度增加而增加。在乙酰化程度相同的情况下，脂肪酸部分所含双键越少，槐糖脂的极性越小，所以槐糖脂的出峰时间随着脂肪酸部分饱和程度增加而增加。峰 10 的分子离子峰为 313，根据这个结果不能分析出任何的槐糖脂结构，这可能是槐糖脂分子分裂所得到的峰。

表 4-1 槐糖脂组分的结构鉴定

Table 4-1 Stucture elucidation of sophorolipid molecules based On m/z peaks of protonated molecular ion [M+H$^+$]

No. as assigned in Fig. 2	Retention time (min)	m/z peak of protonated molecular ions	Sophorolipid structure	Abbreviation
1	7.42	621	unacetylated acidic SL with a C18 diunsaturated fatty acid	C18:2 NASL
2	10.21	663	monoacetylated acidic SL with a C18 diunsaturated fatty acid	C18:2 MASL
3	11.35	665	monoacetylated acidic SL with a C18 monounsaturated fatty acid	C18:1 MASL
4	14.81	705	diacetylated acidic SL with a C18 diunsaturated fatty acid	C18:2 DASL
5	16.64	707	diacetylated acidic SL with a C18 monounsaturated fatty acid	C18:1 DASL
6	20.68	647	monoacetylated lactonic SL with a C18 momounsaturated fatty acid	C18:1 MLSL
7	26.31	687	diacetylated lactonic SL with a C18 diunsaturated fatty acid	C18:2 DLSL
8	31.71	689	diacetylated lactonic SL with a C18 momounsaturated fatty acid	C18:1 DLSL
9	45.21	691	diacetylated lactonic SL with a C18 saturated fatty acid	C18:0 DLSL
10	49.35	313	unknown	

4.2.3 乙酰基数量对槐糖脂抑制食管癌细胞作用的影响

从结果可以看出（图 4-2），双乙酰基内酯型槐糖脂对这两株细胞抑制效果最强，30 μg/mL 的浓度就可以完全杀灭细胞；单乙酰内酯型槐糖脂的抑制效果次之， 60 μg/mL 的浓度下可以完全杀灭细胞。

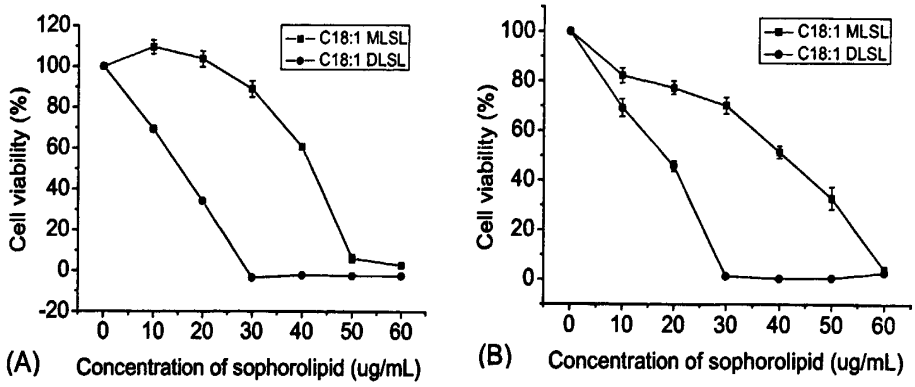


图 4-2 不同乙酰化程度的内酯型槐糖脂对 KYSE109 (A) 和 KYSE450 (B) 的抑制作用

Fig. 4-2 Effects of lactonic sophorolipid with different acetylation degree of sophorose on cell viabilities of KYSE109 (A) and KYSE450 (B). Cell viability was determined by MTT assay. C18:1 MLSL= monoacetylated lactonic SL with a C18 momounsaturated fatty acid; C18:1 DLSL= diacetylated lactonic SL with a C18 momounsaturated fatty acid. The data represent mean±S.D. n = 6 in all groups. $p < 0.01$ by comparison with the control

4.2.4 脂肪酸不饱和程度对槐糖脂抑制食管癌细胞作用的影响

从结果可以看出（图4-3），没有双键的双乙酰内酯型槐糖脂对KYSE109和KYSE450抑制效果不明显，60 μg/mL 的浓度处理后两种细胞的存活率分别还有77%和76%；含有一个双键的双乙酰内酯型槐糖脂对这两种细胞抑制效果最强，30 μg/mL 的浓度就可以完全杀死细胞；含有两个双键的双乙酰内酯型槐糖脂对这两种细胞的抑制效果次之，分别需要50 μg/mL 和60 μg/mL 的浓度才可以完全杀死细胞。

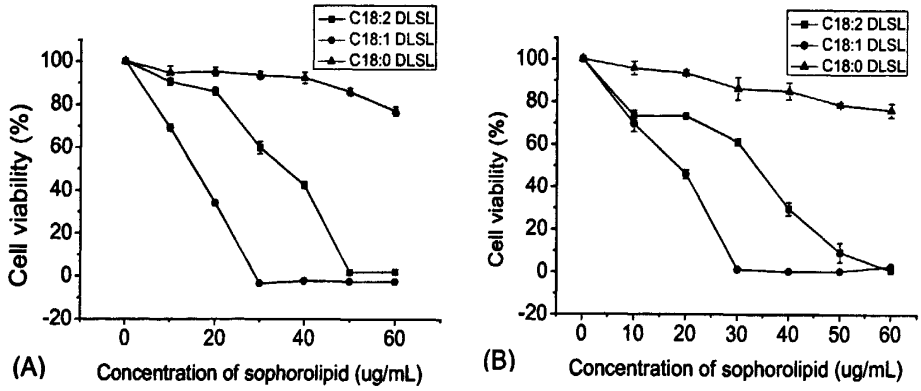


图 4-3 不同不饱和程度的内酯型槐糖脂对 KYSE109 (A) 和 KYSE450 (B) 的抑制作用

Fig. 4-3 Effects of lactonic sophorolipid different in unsaturation degree of hydroxyl fatty acid on KYSE109 (A) and KYSE450 (B) cell viability. Cell viability was determined by MTT assay. C18:2 DLSL= diacetylated lactonic SL with a C18 diunsaturated fatty acid; C18:1 DLSL= diacetylated lactonic SL with a C18 momounsaturated fatty acid; C18:0 DLSL= diacetylated lactonic SL with a C18 saturated fatty acid. Values represent mean±S.D. n = 6 in all groups. $p < 0.01$ by comparison with the control.

4.2.5 酸型槐糖脂对食管癌细胞的抑制作用

由结果可以看出（图 4-4），不论槐糖部分是双乙酰基还是单乙酰基，脂肪酸部分是一个双键还是两个双键，酸型槐糖脂对食管癌细胞 KYSE109 和 KYSE450 都是基本没有抑制作用。槐糖脂浓度达到 60 μg/mL 时，细胞的存活率仍然接近 100%。

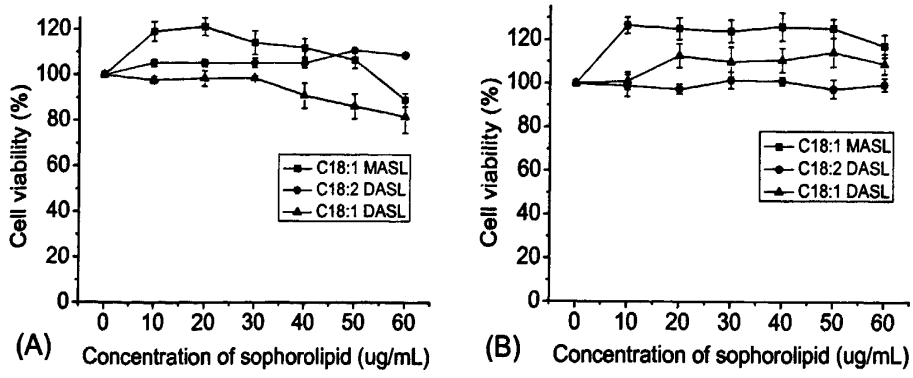


图 4-4 酸型槐糖脂对 KYSE109 (A) 和 KYSE450 (B) 的抑制作用

Fig. 4-4 Effects of acidic sophorolipid on the cell viabilities of KYSE109 (A) and KYSE450 (B) Cell viability was determined by MTT assay. C18:1 MASL= monoacetylated acidic SL with a C18 monounsaturated fatty acid; C18:2 DASL= diacetylated acidic SL with a C18 diunsaturated fatty acid; C18:1 DASL= diacetylated acidic SL with a C18 monounsaturated fatty acid. Values represent mean±S.D. n = 6 in all groups. $p < 0.01$ by comparison with the control.

4.3 讨论

本章中我们将拟威克酵母利用菜籽油为第二碳源发酵得到的槐糖脂粗品经HPLC分析，结果发现槐糖脂粗品中含有多达10几种以上的组分。将其中含量较高的10种组分用制备型HPLC进行了制备，并用质谱分析10种槐糖脂组分的结构。这些槐糖脂分子的结构区别在于在于脂肪酸部分的不饱和程度不同（无双键、一个双键或两个双键），槐糖脂部分的乙酰化程度不同（单乙酰基、双乙酰基、非乙酰化），以及是否存在内酯化（内酯型、酸型）。将这些不同结构的槐糖脂分子用于对食管癌细胞KYSE 109 和KYSE 450的抗肿瘤实验中，研究天然槐糖脂组分结构与抗肿瘤活性的关系。

1998年，Scholz 等报道了槐糖脂衍生物乙酰化的槐糖脂乙酯和乙酰化的槐糖脂丁酯对悬浮细胞Leukemic Jurkat和贴壁细胞Head and neck cancer Tu 138具有很强的抑制作用，没有乙酰化的槐糖脂乙酯和没有乙酰化的槐糖脂丁酯对 这两种肿瘤细胞的生长基本没有抑制作用。因此作者提出槐糖脂的抗肿瘤活性与槐糖脂分子上的乙酰基团有关（Scholz *et al.*, 1998）。我们的研究结果显示双乙酰化的内酯型槐糖脂对食管癌细胞的抑制作用要强于单乙酰化的内酯型槐糖脂，这也印证了之前关于槐糖脂活性与槐糖脂槐糖部分乙酰化程度有关的报道。

实验结果显示， 脂肪酸部分饱和程度不同的内酯型槐糖脂分子对食管癌细胞的抑制作用也有所不同。脂肪酸部分含有1个双键的内酯型槐糖脂分子对食管癌细胞具有最强的细胞毒性（30 μg/mL 的浓度就可以完全杀死细胞），脂肪酸部分含有2个双键的内酯型槐糖脂分子对食管癌细胞的抑制作用稍弱（60 μg/mL

的浓度能够完全杀死细胞），脂肪酸部分没有双键的内酯型槐糖脂分子对食管癌细胞的细胞毒性最弱（60 μg/mL 的浓度只能够抑制 20%的细胞生长）。这是对于内酯型槐糖脂的抗肿瘤活性与脂肪酸部分的不饱和程度关系的首次报道。

有趣的是，不论酸型槐糖脂的槐糖部分是单乙酰化还是双乙酰化，脂肪酸部分是一个双键还是两个双键，酸型槐糖脂对于食管癌细胞都几乎没有抑制作用。这个结果与之前的关于酸型槐糖脂具有较弱的生物活性的报道相符合（Shah *et al*, 2005）。最近，Fu 等报道了酸型槐糖脂（100 μg/mL 具有 35.5% 细胞毒性）比内酯型槐糖脂（100 μg/mL 具有 26.2% 细胞毒性）对胰腺癌细胞有较强的抑制作用（Fu *et al.*, 2008）。这个结果与我们不同可能是因为槐糖脂对胰腺癌细胞的作用机制可能与其他细胞有所不同。

我们研究了槐糖脂的抗肿瘤活性与其结构之间的关系，这对于进一步揭示槐糖脂的作用机制以及对槐糖脂进行结构修饰得到具有更好生物活性的新型槐糖脂分子都有重要的意义。

4.4 本章小结

（1） 将拟威克酵母利用菜籽油为第二碳源发酵得到的槐糖脂粗品经 HPLC 分析，结果发现槐糖脂粗品中含有多达 10 几种以上的组分。将其中含量较高的 10 种组分用制备型 HPLC 进行了制备。

（2） 用质谱分析 10 种槐糖脂组分的结构。结果发现我们分离得到的槐糖脂组分的脂肪酸部分都是 18 碳脂肪酸，而它们的结构的区别在于脂肪酸部分的不饱和程度不同（无双键、一个双键或两个双键），槐糖脂部分的乙酰化程度不同（单乙酰基、双乙酰基、非乙酰化），以及是否存在内酯化（内酯型、酸型）。

（3） 内酯型槐糖脂的抗肿瘤作用与槐糖部分的乙酰基的数量有关，双乙酰基内酯型槐糖脂对食管癌细胞的抑制效果好于单乙酰基内酯型槐糖脂。

（4）首次报道了内酯型槐糖脂的抗肿瘤作用与脂肪酸部分的不饱和程度有关，没有双键的双乙酰内酯型槐糖脂对食管癌细胞抑制效果不明显；含有一个双

键的双乙酰内酯型槐糖脂对食管癌细胞抑制效果最强；含有两个双键的双乙酰内酯型槐糖脂对这两种细胞的抑制效果次之。

（5）酸型槐糖脂无论槐糖部分是双乙酰基还是单乙酰基，脂肪酸部分是一个双键还是两个双键，其对食管癌细胞都基本没有抑制作用。

第五章 不同结构的槐糖脂诱导食管癌细胞系 KYSE450 细胞凋亡的研究

引言

关于槐糖脂对肿瘤细胞的抑制机理，研究者们一直以来都在不断地探索。1997 年， Isoda 发现粗品槐糖脂能够诱导人早幼粒白血病细胞 HL60 的分化，但是该研究并没有弄清楚到底是那种槐糖脂发挥的作用，也没有对其抑制机制有进一步的研究（Isoda *et al.*，1997）。1998 年，Scholz 等纯化得到内酯型槐糖脂，然后通过醇解和脂肪酶催化的方法制备了一些槐糖脂的衍生物。用内酯型槐糖脂及其衍生物作用于悬浮细胞 Leukemic Jurkat 和贴壁细胞 Head and neck cancer Tu 138 两种肿瘤细胞。结果发现，槐糖脂粗品、乙酰化的槐糖脂乙酯和乙酰化的槐糖脂丁酯对这两种细胞都具有很强的抑制作用，而脱乙酰化的槐糖脂乙酯和丁酯对细胞生长基本没有抑制作用。因此作者得出槐糖脂的抗肿瘤活性与槐糖脂分子上的乙酰基团有关（Scholz *et al.*, 1998）。2006 年，我们实验室研究了脂肪酸部分为油酸的双乙酰基内酯型槐糖脂对人肝癌细胞 H7402 的作用机制，发现槐糖脂的抗肿瘤机制是引起细胞的凋亡（Chen *et al.*, 2006）。但是槐糖脂对于不同的肿瘤细胞的抗肿瘤机制是否相同，不同结构的槐糖脂对于同一类肿瘤细胞的作用是否有差异？这些都是需要进一步深入研究的课题。

本章选取了 C18:1 MLSL 和 C18:1 DLSL 两种槐糖脂，以食管癌细胞 KYSE450 作为研究对象，采用多种检测方法，细胞形态学观察（如光学显微镜观察、电子显微镜观察、荧光显微镜观察），流式细胞技术检测细胞周期分布及凋亡率以及 DNA 裂解的原位检测对不同结构槐糖脂对于食管癌细胞 KYSE450 的抗肿瘤作用和机制进行了研究。

5.1 材料和方法

5.1.1 仪器和试剂

(1) 主要仪器

超净工作台(中国苏州净化设备厂);BNA-310 型二氧化碳培养箱(日本 Tabai 公司);倒置显微镜(日本 Nikon 公司);LSM510 激光扫描共聚焦显微镜(德国 Zeiss 公司);EM-1200ES 透射电镜(日本日立公司);JCytomics^{TM} FC500 流式细胞仪(Beckman),Modifit-3 program 分析系统;Spectra Max 190 微板光谱仪(USA)。

(2) 试剂

1. 细胞培养用培养基及试剂的配制

同 4.1.1,4.1.4.3。

2. Giemsa 染色所用试剂的配制

母液:准确称取 0.75 g Giemsa 试剂(上海试剂三厂),加入甘油 25 mL,甲醇 25 mL,研磨溶解,放置一个月后使用。

工作液:用 pH7.2 的 PBS 稀释 10 倍后使用。

方法:将 Giemsa 粉末放入研钵中,边研磨边加入甘油,完全溶解后,再加入甲醇,混匀后,放入棕色瓶中(薛庆善, 2001)。

3. 吖啶橙染色所用试剂的配制

母液:准确称取 0.5 g 吖啶橙试剂(AO,Sigma 公司产品),加入 50 mL 生理盐水溶解,即为 1 %的母液。

工作液:使用时用 pH 7.2 的 PBS 稀释 10 倍后(0.1 %的工作液)应用(薛庆善, 2001)。

4. 透射电镜所用试剂

戊二醛,Sigma 公司产品,用 0.1mol/L,pH 7.4 的二甲砷酸钠配成 4%的溶

液。四氧化锇、Epon812、Sigma 公司产品。

5. TUNEL 检测试剂盒

DeadEnd ™ colorimetric TUNEL system, Promega，华美公司，USA。

4%多聚甲醛的配制：

称取 4g 多聚甲醛溶解于 pH7.2 的 PBS 中，定容到 100 mL， 65℃水浴中溶解，4℃保存（两周内使用）。

6. 流式细胞技术所用试剂

PI（propidium iodide, 碘化丙啶，Sigma 公司产品）染色液：50 mg/mL PI, 0.1 mol/L $EDTA(Na)_2$, 1.0% Triton X-100, 50 μg/mL RnaseA。**5.1.2 槐糖脂样品的稀释**

同 4.1.9。

5.1.3 细胞株

KYSE450：人食管癌细胞，贴壁细胞，山东省齐鲁医院提供。

5.1.4 细胞传代培养方法

同 4.1.8。

5.1.5 细胞的爬片

按 4.1.8 的方法，收集处于指数生长期的细胞，稀释细胞浓度为 1.0×10^5 cells/mL，取 0.5 mL 加入到铺有盖玻片的 6 孔细胞培养板中，继续培养 24 h；加入槐糖脂样品处理 24 h，处理结束后，取处爬片（鲁藜等, 2004），室温晾干。

5.1.6 阴性对照组和空白对照组的设立

以正常细胞为阴性对照组，以无细胞体系为空白对照组，以下各实验都设有阴性和空白对照组。

5.1.7 倒置相差显微镜观察槐糖脂作用后的细胞形态

收集处于指数生长期的细胞，稀释细胞浓度为1.0×10^5 cells/mL，取1 mL加入6孔细胞培养板中，继续培养24 h；加入槐糖脂样品使其终浓度为40 μg/mL，处理24 h，用倒置相差显微镜观察槐糖脂处理前后细胞形态的变化。

5.1.8 Giemsa 染色观察槐糖脂作用后的细胞形态

按照 5.1.5 的方法，终浓度为 40 μg/mL 的槐糖脂样品作用 24 h 后，取出爬片，室温晾干；甲醇固定 5 min，晾干；Giema 染液室温染色 15 min，pH 7.2 的 PBS 冲洗，晾干；甲醛树胶封固；光学显微镜观察。

5.1.9 吖啶橙染色观察槐糖脂作用后的细胞形态

按照 5.1.5 的方法，终浓度为 40 μg/mL 的槐糖脂样品作用 24 h 后，取出爬片，室温晾干；乙醇固定 15 min，晾干；吖啶橙染液室温染色 15 min，pH 7.2 的 PBS 冲洗，晾干；荧光显微镜观察。选择蓝光激发(488 nm)，发射波长 515 nm（DNA, 绿色）。

5.1.10 透射电镜观察槐糖脂作用后的细胞形态

终浓度为 40 μg/ml 的样品作用 24 h 后，用细胞刮刀收集细胞，用 pH 7.2 的 PBS 洗涤 2 次；细胞沉淀用 2.5%的戊二醛固定液固定，4℃过夜；pH 7.2 的 PBS 洗 3 次；1%的锇酸 4℃固定 2 h；梯度丙酮脱水(25%-100% 丙酮)；包埋剂包埋；LKBV 型超薄切片机切片，JEM-1200EX 透射电镜观察并拍照(Catelas *et al*, 2005; 郭延奎, 2002)。

5.1.11 TUNEL (TdT-mediated dUTP nick end labeling) 检测细胞凋亡

按试剂盒操作手册进行。

1. 固定：将爬片浸在 4%多聚甲醛中 25 min；
2. 洗涤：将爬片浸在 pH 7.2 的 PBS 中洗涤两次，每次 5 min；
3. 渗透：将爬片浸在 0.2% Triton X-100 中 5 min；
4. 洗涤：将爬片浸在 pH 7.2 的 PBS 中洗涤两次，每次 5 min；

5. 平衡：加 100 μL 平衡缓冲液，室温平衡 5-10 min；
6. 标记：加 100 μL TdT 反应混合液到爬片上。不能让细胞完全干燥，盖上盖玻片保证反应液分布均匀，然后放在 37℃潮湿的温箱中 60 min；
7. 终止反应：移去盖玻片，浸倒 2×SSC 中反应 15 min；
8. 洗涤：PBS 中洗涤 3 次，每次 5 min；
9. 封闭：浸入 0.3%过氧化氢中 3-5 min，以终止内源性过氧化物酶作用；
10. 洗涤：PBS 中洗涤 3 次，每次 5 min；
11. 结合：加 100 μL 链霉抗生物素（1: 500 PBS 稀释），室温孵育 30 min；
12. 洗涤：PBS 中洗涤 3 次，每次 5 min；
13. 染色：加 100 μL DAB（用前准备），直到浅的褐色背景出现，不让背景变得很深，DAB 要避光保存，并在 30 min 内使用；
14. 洗涤：用去离子水洗几次；
15. 观察：用甲醛树胶封固，在光学显微镜下观察。

5.1.12 流式细胞技术检测细胞凋亡率及周期分布

1. 分别收集各组细胞（细胞个数大于 1×10^6），pH 7.2 的 PBS 中洗涤两次；
2. 细胞沉淀中加入 1 mL 70% 的冰冷乙醇－20 ℃下固定过夜；
3. 在 pH 7.2 的 PBS 中洗涤两次，用 100 目的筛网过滤，离心后细胞沉淀加入 0.5 mL PI 染色液，混匀，室温避光 1 h；
4. 在 pH 7.2 的 PBS 中洗涤 2 次；
5. 流式细胞仪收集 5000 个细胞，并进行分析(Wang *et al.*，2003)。

5.2 结果

5.2.1 倒置相差显微镜观察槐糖脂作用后食管癌细胞形态的变化

倒置相差显微镜观察正常细胞成贴壁状态、多边形紧密排列、生长活跃；槐糖脂作用于 KYSE450 细胞后，细胞的生长受到抑制，细胞的形态发生了很大的

变化。当细胞用 40 μg/mL 的 C18:1 MLSL 处理后，细胞数量减少，但是细胞形态变化不大。当细胞用 40 μg/mL 的 C18:1 DLSL 处理后，细胞收缩、变圆、体积变小、膜泡突出并且出现一些小体结构，从培养板壁脱离下来（图 5-1）。

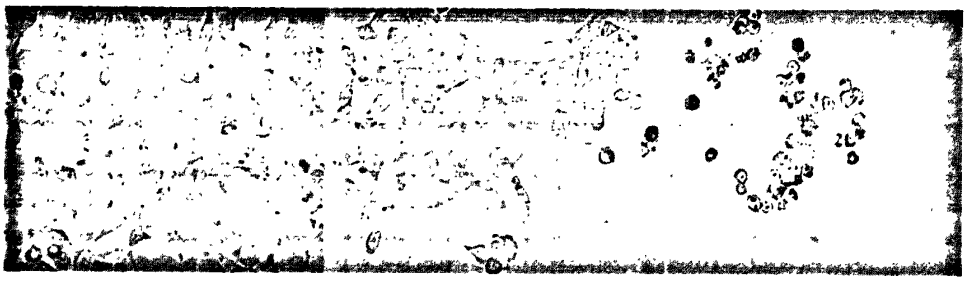


图 5-1 C18:1 MLSL 和 C18:1 DLSL 作用于 KYSE450 细胞后细胞形态变化

KYSE450 正常细胞（A）；40 μg/mL 的 C18:1 MLSL（B）和 40 μg/mL 的 C18:1 DLSL（C）

Fig.5-1 Effects of C18:1 MLSL and C18:1 DLSL on morphological changes of KYSE450 cells（×400）. Control, untreated cells (A); Cells treated with 40μg/mL C18:1 MLSL (B) and 40 μg/mL C18:1 DLSL (C) for 24h.

5.2.2 Giemsa 染色观察槐糖脂作用后食管癌细胞形态的变化

在光镜下观察，正常的 KYSE450 细胞，细胞体积大，核浆比值高，核圆形至椭圆形，核仁多个，深蓝色，细胞质呈淡蓝色。当细胞用 40 μg/mL 的 C18:1 MLSL 作用后，细胞排列稀疏，部分细胞核染色质凝集，凝聚于核膜内侧，发生边缘化，有的细胞核碎裂成多个块状。当细胞用 40 μg/mL 的 C18:1 DLSL 作用后，细胞变圆，大部分细胞不再贴壁，细胞核凝集，细胞内部结构完全破坏，并出现膜包绕的小体样结构。（图 5-2）。

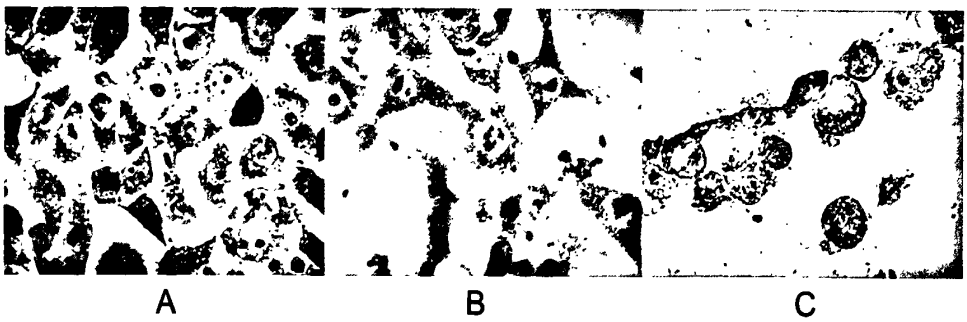


图 5-2 Giemsa 染色观察 KYSE450 细胞形态变化

KYSE450 正常细胞（A）；40 μg /mL 的 C18:1 MLSL（B）和 40 μg/mL 的 C18:1 DLSL（C）

Fig. 5-2 Morphological changes of KYSE450 by Giemsa stain（×400） Control, untreated cells (A); Cells treated with 40 μg/mL C18:1 MLSL (B) and 40 μg/mL C18:1 DLSL (C) for 24h.

5.2.3 吖啶橙染色观察槐糖脂作用后食管癌细胞形态的变化

荧光显微镜下观察，正常的KYSE450细胞核成弥散均匀荧光，圆形至椭圆形，核仁多个；当细胞用40 μg/mL 的C18:1 MLSL作用后，部分细胞核发生凝聚边缘化；当细胞用40 μg/mL 的C18:1 DLSL作用后，细胞聚集成团，细胞核凝集形成明亮的块状或雪花状荧光斑点。（图6-3）。

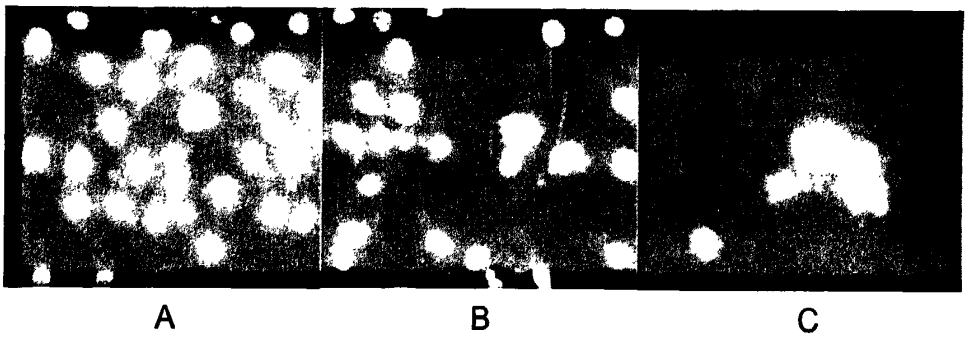


图5-3 吖啶橙染色观察KYSE450细胞形态变化

KYSE450正常细胞（A）；40 μg/mL的C18:1 MLSL（B）和40 μg/mL的C18:1 DLSL（C）

Fig. 5-3 Morphological changes of KYSE450 by acridine orange stain（×400）; Control, untreated cells (A); Cells treated with 40 μg/mL C18:1 MLSL (B) and 40 μg/mL C18:1 DLSL (C) for 24h.

5.2.4 透射电镜观察槐糖脂作用后食管癌细胞细胞核形态的变化

以上结果已经表明C18:1 MLSL和C18:1 DLSL能够诱导KYSE450细胞死亡，但是到底是细胞凋亡还是坏死引起的还不是很清楚，为了更清楚地说明这一问题进行了透射电镜观察。

透射电镜是检验细胞死亡方式最有效的一种方法，被称为检验细胞凋亡的“金标准”(Zakeri *et al*，1995; Du *et al*，2005)。

槐糖脂处理前的正常的KYSE450细胞，细胞核浆比值大，核常染色质，核

仁大，核膜完整。40 μg/mL 的C18:1 MLSL 作用后，细胞核染色质凝集，分散成块状，核膜消失，无明显核区和核仁。40 μg/mL 的C18:1 DLSL 作用后，细胞体积缩小，细胞浆浓缩，细胞核染色质凝集，凝聚于核膜内侧，并成块状或新月状边缘化；胞浆内空泡增多，细胞膜完整，表面微绒毛和伪足减少；并可见膜包裹细胞核碎片的凋亡小体（图 5-4）。

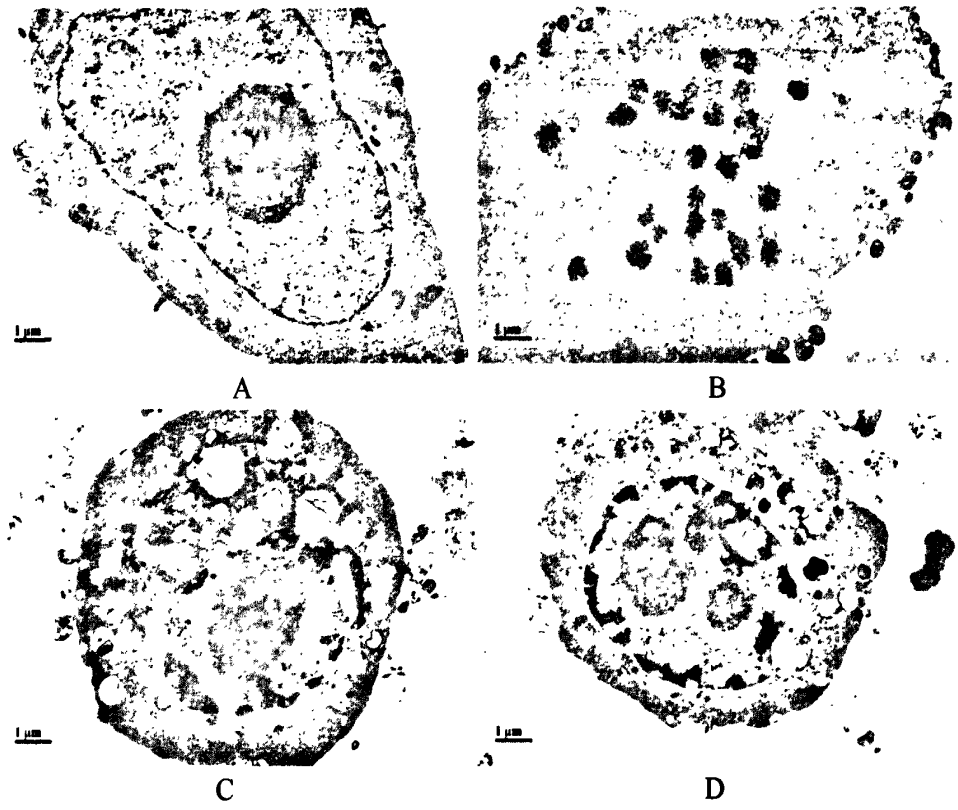


图 5-4 透射电镜观察 C18:1 MLSL 和 C18:1 DLSL 作用后 KYSE450 细胞核变化

KYSE450 正常细胞（A）；40 μg/mL 的 C18:1 MLSL（B）和 40 μg/mL 的 C18:1 DLSL（C,D）

Fig.5-4 Effects of sophorolipid on nuclear fragmentation of KYSE450 cells by transmission electron microscopy (×4000). Control, untreated cells (A); Cells treated with 40 μg/mL C18:1 MLSL (B) and 40 μg/mL C18:1 DLSL (C, D) for 24h.

5.2.5 TUNEL (TdT-mediated dUTP nick end labeling) 检测食管癌细胞的凋亡

细胞凋亡的典型特征就是依赖Ca/Mg核酸内切酶的激活从而导致DNA的片断化。有活性的核酸内切酶水解 DNA，在 DNA 链上产生缺口，然后 DNA 发生断裂。

TUNEL 法就是利用末端脱氧核苷酸转移酶(TdT)将生物素标记的脱氧核苷酸（biotin-dUTP）转移到已经产生缺口的 DNA 链上的原位标记技术。生物素标记的脱氧核苷酸连接到 DNA 链上后，链酶抗生物素-辣根过氧化物酶（Streptavidin-HRP）与生物素进行抗原抗体结合，辣根过氧化物酶水解过氧化氢释放出氧气使二氨基苯肼（DAB）显色（Gavrieli et al 1992）。

由图 5-5 可以看出，未经槐糖脂处理的正常细胞颜色很浅。细胞形状规则，大小颜色均一，呈贴壁生长，经槐糖脂样品处理后的细胞的细胞核显示出棕色和深棕色，细胞形态大小发生了明显的变化，尤其是经双乙酰的内酯型槐糖脂处理后的细胞，其细胞核的颜色呈深棕色，细胞已完全不呈贴壁生长，看不出完整的细胞形态。以上结果表明经两种内酯型槐糖脂作用后，KYSE450 细胞的核酸内切酶被激活，细胞都发生了凋亡，而双乙酰的内酯型槐糖脂比单乙酰的内酯型槐糖脂对于 KYSE450 细胞的抑制作用更强。

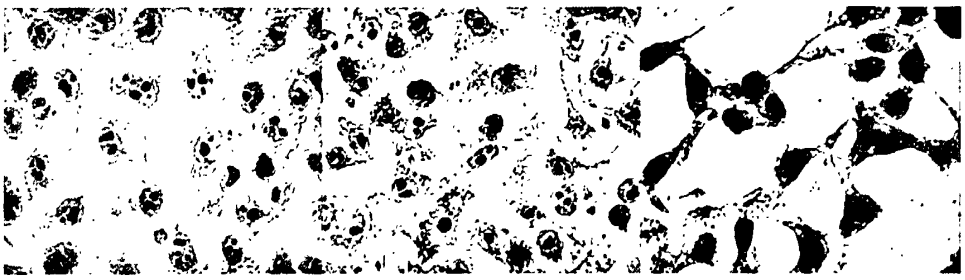


图 5-5 TUNEL 原位检测 C18:1 MLSL 和 C18:1 DLSL 作用后 KYSE450 细胞核的断裂

KYSE450 正常细胞（A）；40 μg/mL 的 C18:1 MLSL（B）和 40 μg/mL 的 C18:1 DLSL（C）

Fig.5-5 Apoptotic KYSE450 cells as demonstrated by analysis using TdT-mediated dUTP nick end labeling. Untreated cells, no apoptotic sign was detected (A); Cells treated with 40 μg/mL C18:1 MLSL (B) and 40 μg/mL C18:1 DLSL (C) for 24h.

5.2.6 流式细胞技术检测槐糖脂作用后食管癌细胞的凋亡率及周期分布

从图5-6，表5-1的结果可以看出，与正常细胞相比，C18:1 MLSL处理后，细胞G_2/M上升至20.25%，细胞S期下降至35.12%；C18:1 DLSL 处理后，细胞G_2/M上升至18.56%，细胞G_0/G_1下降为36.47%。这些数据表明C18:1 MLSL和C18:1 DLSL诱导后将细胞阻滞在G_2期，使细胞不能进入下一个细胞周期，从而发生凋亡。浓度为40 μg/mL的C18:1 MLSL和C18:1 DLSL诱导24 h，细胞的亚二倍体占

的比例分别为为10.17%和12.25%。

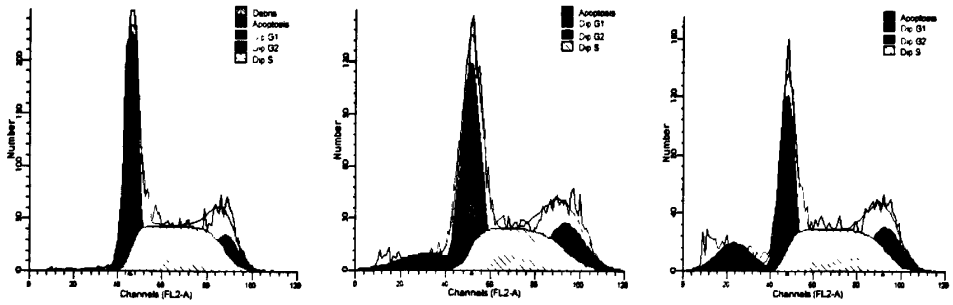


图 5-6 流式细胞技术检测 C18:1 MLSL 和 C18:1 DLSL 作用后细胞周期变化

Fig. 5-6 Cell cycle distribution in KYSE450 cells treated with C18:1 MLSL and C18:1 DLSL. Control, untreated cells (A); Cells treated with 40 μg/mL C18:1 MLSL (B) and 40 μg/mL C18:1 DLSL (C) for 24h.

表 5-1 槐糖脂对细胞周期分布的影响及细胞凋亡率

Table 5-1 Effects of C18:1 MLSL and C18:1 DLSL on cell cycle distribution and apoptosis rate at 24 h

	G_0/G_1 (%)	G_2/M (%)	S (%)	Sub-G1 (%)
control	42.53	13.13	44.35	0.02
C18:1 MLSL	44.63	20.25	35.12	10.17
C18:1 DLSL	36.47	18.56	44.97	12.25

5.3 讨论

实验室之前的研究证明，脂肪酸部分为 C18:1 的双乙酰内酯型槐糖脂抑制人肝癌细胞 H7402 的机制是引起细胞的凋亡（Chen *et al*，2006）。本研究采用多种检测方法，细胞形态学观察（如光学显微镜观察、电子显微镜观察、荧光显微镜观察），DNA 裂解的原位检测以及流式细胞技术检测细胞周期分布及凋亡率对 C18:1 MLSL 和 C18:1 DLSL 两种槐糖脂诱导食管癌细胞 KYSE450 的作用机制进行了研究。

通过形态学观察，槐糖脂作用后，肿瘤细胞收缩、细胞膜鼓泡、染色体凝聚和边缘化、形成凋亡小体以及核酸内切酶活化。通过流式细胞仪分析说明细胞周期的分布发生了变化，并且出现了一个非常明显的亚二倍体峰。以上都表明，这两种结构的槐糖脂都能够在一定浓度范围内引起食管癌细胞的凋亡，说明槐糖脂抑制肿瘤细胞的机制对于不同的肿瘤细胞具有一致性，都是引起细胞凋亡。

另外，不同结构的槐糖脂在相同浓度下引起肿瘤细胞凋亡的水平也不相同，无论从细胞形态观察还是从细胞周期的变化以及凋亡率的大小，都可以说明，相同浓度的 C18:1 DLSL 比 C18:1 MLSL 能诱导食管癌细胞 KYSE450 更大程度的发生凋亡。这一点也与上一章中双乙酰基的内酯型槐糖脂对食管癌细胞的抑制效果优于单乙酰基的内酯型槐糖脂的结果相符合。

5.4 本章小结

（1）通过对 C18:1 MLSL 和 C18:1 DLSL 两种槐糖脂对食管癌细胞 KYSE450 的抑制机制的研究，我们可以得出在一定浓度范围内，槐糖脂分子能够诱导食管癌细胞 KYSE450 凋亡。

（2）相同浓度（40 μg/mL）的 C18:1 MLSL 和 C18:1 DLSL 引起食管癌细胞 KYSE450 凋亡的水平不同，C18:1 DLSL 比 C18:1 MLSL 能诱导食管癌细胞 KYSE450 更大程度的发生凋亡。

全文总结与展望

总结

.本文综合运用了微生物学、分析化学、药理学、细胞生物学及医学等知识和方法，通过补料发酵的方法提高了拟威克酵母生产槐糖脂的产量，研究了槐糖脂的抗细菌、抗真菌活性，不同结构的槐糖脂的抗肿瘤活性，以及不同结构的槐糖脂对食管癌细胞的作用机制。具体来说，已经取得了以下结果：

1. 300 mL 摇瓶补料发酵，在发酵过程中补加三次 5%菜籽油后，槐糖脂产量为 43.1 g/L，比分批发酵的槐糖脂产量提高了 32%。在 5 L 发酵罐中扩大生产槐糖脂，补料发酵 288 h 后，槐糖脂产量为 68.2 g/L，比摇瓶分批发酵提高了 109.2%，比摇瓶补料发酵产量提高了 58.2%。将 5 L 发酵罐的转速从 400 rpm 提高到 500 rpm，发酵 196 h 后，槐糖脂产量达到 71.1 g/L，与提高转速之前相比，槐糖脂的产量略高一点，但是发酵周期大大缩短，由 288 h 缩短到 196 h， 提前了 96 h，槐糖脂的容积生产率由 0.24 g/L/h 提高到 0.37 g/L/h。

2. 研究发现槐糖脂对革兰氏阳性菌有明显的抑制作用，对大肠杆菌几乎没有抑制作用。首次报道了槐糖脂对引起龋齿的变形链球菌具有明显的抑制作用。首次报道了槐糖脂对红色毛癣菌、石膏样毛癣菌和犬小孢子菌这三种常见的皮肤病致病菌的抑制作用，证明槐糖脂对这三种皮肤癣菌都有很强的抑制作用，并比较了内酯型槐糖脂和酸型槐糖脂对这三种皮肤癣菌的作用效果的不同。

3. 将拟威克酵母利用菜籽油为第二碳源发酵得到的槐糖脂粗品经 HPLC 分析，其中含量较高的 10 种组分用制备型 HPLC 进行了制备，并用质谱分析 10 种槐糖脂组分的结构。结果发现我们分离得到的槐糖脂组分的脂肪酸部分都是 18 碳脂肪酸，而它们的结构的区别在于脂肪酸部分的不饱和程度不同（无双键、一个双键或两个双键），槐糖脂部分的乙酰化程度不同（单乙酰基、双乙酰基、非乙酰化），以及是否存在内酯化（内酯型、酸型）。

4. 研究了不同结构的天然槐糖脂组分对食管癌细胞 KYSE 109 和 KYSE

450的抗肿瘤活性，发现槐糖脂分子的乙酰化程度、脂肪酸部分饱和程度和是否内酯化都对其抗肿瘤活性有重要的影响。首次报道了槐糖脂分子脂肪酸部分饱和程度对抗肿瘤活性的影响。

5. 研究了C18:1 MLSL和C18:1 DLSL两种不同结构槐糖脂对于食管癌细胞KYSE450的抗肿瘤机制，证明槐糖脂对食管癌细胞的作用机制是引起细胞凋亡，且C18:1 MLSL和C18:1 DLSL两种槐糖脂都能引起肿瘤细胞的凋亡，在相同浓度下引起细胞凋亡的水平不同。

展望

本文取得了一定的研究成果，对槐糖脂的应用发展具有一定的促进作用。然而，本研究还存在不足之处，很多方面有待于进一步研究和探索。

1. 虽然通过补料发酵提高了拟威克酵母生产槐糖脂的产量，但是还没有达到文献报道的*C. bombicola*生产槐糖脂的水平，还需要进一步提高产量以适应应用的需求。

2. 通过基因工程的方法，对槐糖脂的代谢途径中的关键酶进行过量表达或者敲除，改变槐糖脂组分的结构或者提高槐糖脂的产量。

3. 开展动物实验，选择和建立合适的动物模型，确定体内抗肿瘤作用。进行急性和慢性毒性试验，确立用药的安全性。

参考文献

1 Asmer HJ, Lang S, Wagner F, Wray V. Microbial production, structure elucidation and bioconversion of sophorose lipids. JAOCS, 1988, 65: 1460-1466.

2 Ashby RD, Solaiman DKY, Foglia TA. The use of fatty acid esters to enhance free acid sophorolipid synthesis. Biotechnology Letters, 2006, 28: 253-260.

3 Azim A, Shah V, Doncel GF, Peterson N, Gao W, Gross R. Amino acid conjugated sophorolipids: a new family of biological active functionalized glycolipids. Bioconjug Chem, 2006,17: 1523-1529.

4 Banat I. Biosurfactants production and possible uses in microbial enhanced oil recovery and oil pollution remediation. A review. Bioresour Technol, 1995, 51: 1-12.

5 Banat I, Makkar RS, Cameotra SS. Potential commercial applications of microbial surfactants. Appl Microbiol Biotechnol, 2000, 53: 495-508.

6 Bento FM, Camargo FO, Okeke BC, Frankenberger WT. Diversity of biosurfactant producing microorganisms isolated from soils contaminated with diesel oil. Microbiological Research, 2005, 160: 249-255.

7 Bluth MH, Fu S L, Fu A, et al. Sophorolipids decrease asthma severity and ova-specific IgE production in a mouse asthma model. J Allergy Clin Immunol, 2008, 121: 2.

8 Catelas I, Petit A, Vali H, Fragiskatos C, Meilleur R, Zukor DJ, Antoniou J, Huk OL. Quantitative analysis of macrophage apoptosis vs necrosis induced by cobalt and chromium ions in vitro. Biomaterials, 2005, 26: 2441-2453.

9 Carr JA, Bisht KS. Enzyme-catalyzed regioselective transesterification of peracylated sophorolipids. Tetrahedron, 2003, 59: 7713-7724.

10 Cavalero DA, Cooper DG. The effect of medium composition on the structure

and physical state of sophorolipids produced by *Candida bombicola* ATCC 22214. Journal of Biotechnology, 2003, 103: 31-41.

11 Chen J, Song X, Zhang H, Qu YB, Miao JY. Production, structure elucidation and anticancer properties of sophorolipid from *Wickerhamiella domercqiae.* Enzyme Microb Technol, 2006a, 39: 501-506.

12 Chen J, Song X, Zhang H, Qu YB, Miao JY. Sophorolipid produced from the new yeast strain *Wickerhamiella domercqiae* induces apoptosis in H7402 human liver cancer cells. Appl Microbiol Biotechnol, 2006b, 72:52-59.

13 Daverey A, Pakshirajan K. Production, Characterization, and Properties of Sophorolipids from the Yeast Candida bombicola using a Low-cost Fermentative Medium. Appl Biochem Biotechnol, 2009, 158: 663-74.

14 Davila AM, Monin MN, Vandecasteele JP. Identification and determination of individual sophorolipids in fermentation products by gradient elution high-performance liquid chromatography with evaporative light-scattering detection. Journal of chromatography, 1993, 648: 139-149.

15 Davila AM, Marchal R, Vandecasteele JP. Sophorose lipid fermentation with differentiated substrate supply for growth and production phases. Applied Microbiology and Biotechnology, 1997, 47: 496-501.

16 Daniel HJ, Otto RT, Binder M, Reuss M, Syldatk C. Production of sophorolipids from whey: development of a two-stage process with *Cryptococcus curvatus*ATCC 20509 and *Candida bombicola*ATCC 22214 using deproteinized whey concentrates as substrates. Appl Microbiol Biotechnol, 1999, 51: 40-45.

17 Daniel HJ, Reuss M, Syldatk C. Production of sophorolipids in high concentration from deproteinized whey and rapeseed oil in a two stage fed batch process using *Candida bombicola* ATCC 22214 and Cryptococcus curvatus ATCC 20509. Biotechnology Letters, 1998, 20: 1153-1156.

18 Deslipande M, Daniels L. Evaluation of sophorolipid biosurfactant production by *Candida bombicola* using animal fat. Biosource technol, 1995, 54: 143-150.

19 Du AY, Zhao BX, Yin DL, Zhang SL, Miao JY. Discovery of a novel small molecule,1-ethoxy-3-(3,4-methylenedioxyphenyl)-2-propanol, that induces apoptosisin A549 human lung cancer cells. Bioorganic & Medicinal Chemistry, 2005, 13: 4176-4183.

20 Esders TW, Lights RJ. Glucosyl- and Acetyltransferases Involved in the Biosynthesis of Glycolipids from *Candida bogoriensis*. The Journal of Biological Chemistry, 1972, 247: 1375-1386.

21 Felse PA, Shah V, Chan J, Rao KJ, Gross RA. Sophorolipid biosynthesis by *Candida bombicola* from industrial fatty acid residues. Enzyme and Microbial Technology, 2007, 40: 316-323.

22 Fleurackers SJJ, Belgium ENV. On the use of waste frying oil in the synthesis of sophorolipids. Eur. J. Lipid Sci. Technol., 2006, 108: 5-12.

23 Fu SL, Wallner SR, et al. Sophorolipids and Their Derivatives Are Lethal Against Human Pancreatic Cancer Cells. Journal of Surgical Research, 2008, 148: 77-82.

24 Gavrieli Y, Sherman Y, Ben Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol, 1992, 119: 493-501.

25 Gorin PAJ, Spencer JFT, Tulloch AP. Hydroxy fatty acid glycosides of sophorose from Torulopsis magnoliae. Can J Chem, 1961, 39: 846-855.

26 Gross RA, Guilmanov V, Scholz C. Glycolipids from *Torulopsis bombicola*: Biosynthesis, lipase-selective modification and anti-cancer activity. 217th National Meeting of the American Chemical Society, Abstracts of Papers American Chemical Society, 1999, 217: 1-2.

27 郭延奎, 王洪海, 戴兴岐, 王军, 高慧英. 锌减轻顺铂诱发肝损伤的超微结

构研究. 电子显微学报, 2002, 21: 169-171.

28 Hagler M, Smith-Norowitz TA, Chice S, et a1. Sophorolipids decrease IgE production in U266 cells by downregulation of BSAP (Pax5), TLR-2, STAT3 and IL-6. J Allergy Clin Immunol, 2007, 119: S263.

29 Hardin R, Pierre J, et al. Sophorolipids Improve Sepsis Survival: Effects of Dosing and Derivatives. Journal of Surgical Research, 2007, 142: 314-319.

30 Healy MG., Devine CM, Murphy R. Microbial production of biosurfactants. Resources, Conservation and Recycling, 1996, 18: 41-57.

31 Hommel RK, Huse K. Regulation of sophorose lipid production by *Candida apicola*. Biotechnology letters, 1993, 15: 853-858.

32 Hommel RK, Weber L, Weiss A, et al. Production of sophorose lipid by *Candida apicola* grown on glucose. Journal of Biotechnology, 1994(a), 33: 147-155.

33 Hommel RK, Stegner S, Weber L, Kleber HP. Effect of ammonium ions on glycolipid production by *Candida* (*Torulopsis*) *apicola*. Appl Microbiol Biotechnol, 1994(b), 42: 192-197.

34 Hommel R, Stuwer O, Stuber W, et al. Production of water-soluble surface exolipids by *Torulopsis apicola*. Appl Microbiol Biotechnol, 1987, 26: 199-205.

35 Hu YM, Ju LK. Purification of lactonic sophorolipids by crystallization. Journal of Biotechnology, 2001b, 87: 263-272.

36 Hu YM, Ju LK. Sophorolipid production from different lipid precursors observed with LC-MS. Enzyme and Microbial Technology, 2001a, 29: 593-601.

37 Inoue S, Kiruma Y, Kinta M. Dehydrating purification process for a fermentation product. US patent, 1980, 4197166

38 Isoda H, Kitamoto D, Shinmoto H, Matsumoto M, Nakahara T. Microbial extracellular glycolipid induction of differentiation and inhibition of protein

kinase C activity of human promyelocytic leukemia cell line HL60. Biosci Biotechnol Biochem, 1997, 61: 609-614.

39 Kim KJ, Yoo D, Kim, YB, et al. Characteristics of sophorolipid as an antimicrobial agent. Journal of Microbiology and Biotechnology, 2002, 12: 235-241.

40 Kim HS, Yoon BD, Choung DH, et al. Characterization of a biosurfactant, mannosylerythritol lipid produced from Candidasp. SY16. Appl Microbiol Biotechnol, 1999, 52: 713-721.

41 Kim YB, Yun HS, Kim EK. Enhanced sophorolipid production by feeding-rate-controlled fed-batch culture. Bioresource Technology, 2009, 100: 6028-6032.

42 Kreger-vanRij NJW. The yeasts, a taxonomic study. Elsevier Science Publ Groningen, 1984.

43 Lee YJ, Choi JK, Kim EK, et al. Field experiments on mitigation of harmful algal blooms using a Sophorolipid-Yellow clay mixture and effects on marine plankton. HAB, 2008, 7: 154-162.

44 李建武. 生物化学实验原理和方法. 北京: 北京大学出版社, 2000, 131-132.

45 Lottermoser K, Schunck WH, Asperger O. Cytochromes P450 of the sophorose lipid-producing yeast Candida apicola: Heterogeneity and polymerase chain reaction-mediated cloning of two genes. Yeast, 1996, 12: 565-575.

46 鲁藜, 邓华瑜, 范维珂. MUC1的表达与乳腺癌细胞粘附的关系. 《癌症》, 2004, 23, 1294-1296.

47 Mulligan CN. Environmental applications for biosurfactants. Environmental Pollution, 2005, 133: 183-198.

48 Mulligan, CN, Yong, RN, Gibbs, BF. Removal of heavy metals from contaminated soil and sediments using the biosurfactant surfactin. Journal of

Soil Contamination, 1999, 8: 231-254.

49 Mulligan, CN, Yong, RN, Gibbs, BF. Remediation technologies for metal-contaminated soils and groundwater: an evaluation. Engineering Geology, 2001, 60: 193-207.

50 Napolitano LM. Sophorolipids in sepsis: Antiinflammatory or antibacterial? Critical Care Medicine, 2006, 34: 258-259.

51 Nunez A, Foglia TA, Ashby R. Enzymatic synthesis of a galactopyranose sophorolipid fatty acid-ester. Biotechnol Lett, 2003, 25: 1291-1297.

52 Nunez A, Ashby R, Foglia TA, Solaiman DKY. LC/MS analysis and lipase modification of the sophorolipids produced by *Rhodotorula bogoriensis*. Biotechnology Letters, 2004, 26: 1087-1093.

53 Oberbremer A, Muller-Hurtig R, Wagner F. Effect of the addition of microbial surfactants on hydrocarbon degradation in a soil population in a stirred reactor. Applied Microbiology and Biotechnology 1990, 32: 485-489.

54 Otto RT, Daniel HJ, Pekin G, Muller-Decker K, Furstenberger G, Reuss M, Syldatk C. Production of sophorolipids from whey. Appl Microbial Biotechnol 1999; 52: 495-501.

55 Passeri A, Schmidt M, Haffner T, et al. Marine biosurfactants. IV. Production, characterization and biosynthesis of an anionic glucose lipid from the marine bacterial strain MM1. Appl Microbiol Biotechnol, 1992, 37: 281-286.

56 Powalla M, Lang S, Wray V. Penta- and disaccharide lipid formation by Nocardia corynebacteroides grown on n-alkanes. Appl Microbiol Biotechnol, 1989, 31: 473-479.

57 Price p, Mcmillan TJ. Use of the tetrazolium assay in measuring the response of human tumor cells to ionizing radiation. Cancer Res 1990; 50: 1392-1396.

58 Rau U, Heckmann R, Victor W, et al. Enzymatic conversion of a sophorolipid

into a glucose lipid. Biotechnology Letters, 1999, 21: 973-977.

59 Rau.U, Hammen S, Heckmann R, Wray V, Lang S. Sophorolipids: a source for novel compounds. Industrial Crops and Products, 2001, 13: 85-92.

60 Rosenberg E, Ron EZ. High- and low-molecular-mass microbial surfactants. Appl Microbiol Biotechnol, 1999, 52: 154-162.

61 Schippers C, Gebner K, Muller T, Scheper T. Microbial degradation of phenanthrene by addition of a sophorolipid mixture. Journal of Biotechnology, 2000, 83: 189-198.

62 Scholz C, Mehta S, Bisht K, et al. Bioactivity of extracellular glycolipids-investigation of potentional anticancer activity of sophorolipids and sophorolipid-derivatives. Polymer preprints, 1998, 39: 168-169.

63 Shah S, Prabhune A. Purification by silica gel chromatography using dialysis tubing and characterization of sophorolipids produced from Candida bombicola grown on glucose and arachidonic acid. Biotechnol Lett, 2007b, 29: 267-272.

64 Shah V, Doncel GF, Seyoum T, et al. Sophorolipids, microbial glycolipids with anti-human immunodeficiency virus and sperm-immobilizing activities. Antimicrob Agents Chemother, 2005, 49: 4093-40100.

65 Shah V, Jurjevic M, Badia D. Utilization of Restaurant Waste Oil as a Precursor for Sophorolipid Production. Biotechnol. Prog., 2007a, 23: 512-515.

66 Shah V, Badia D, Ratsep P. Sophorolipids Having Enhanced Antibacterial Activity. Antimicrob Agents Chemother, 2007b, 51: 397-400.

67 Singh SK, Felse AP, Nunez A, Foglia TA, Gross RA. Regioselective enzyme-catalyzed synthesis of sophorolipid esters, amides, and multifunctional monomers. J Org Chem, 2003,68: 5466-5477.

68 Solaiman DKY, Ashby RD, Nunez A, Foglia TA. Production of sophorolipids by *Candida bombicola* grown on soy molasses as substrate. Biotechnology

Letters, 2004, 26: 1241-1245.

69 Sun XX, Choi JK, Kim EK. A preliminary study on the mechanism of harmful algal bloom mitigation by use of sophorolipid treatment. Journal of Experimental Marine Biology and Ecology, 2004a, 304: 35-49.

70 Sun XX, Lee YJ, Choi JK, Kim EK. Synergistic effect of sophorolipid and loess combination in harmful algal blooms mitigation. Marine Pollution Bulletin. 2004b, 48: 863 - 872.

71 Tonkova EV, Gesheva V. Glycolipids produced by Antarctic *Nocardioides* sp. During growth on n-paraffin. Process Biochemistry, 2005, 40: 2387-2391.

72 Van Bogaert INA, De Maeseneire SL, De Schamphelaire W, Develter D, Soetaert W, Vandamme EJ. Cloning, characterization and functionality of the orotidine-5'-phosphate decarboxylase gene (*URA3*) of the glycolipid-producing yeast *Candida bombicola*. Yeast, 2007, 24: 201-208.

73 Van Bogaert INA, De Maeseneire SL, Develter D, Soetaert W, Vandamme EJ. Development of a transformation and selection system for the glycolipid-producing yeast *Candida bombicola*. Yeast, 2008a, 25: 273-8.

74 Van Bogaert INA, De Maeseneire SL, Develter D, Soetaert W, Vandamme EJ. Cloning and characterisation of the glyceraldehyde 3-phosphate dehydrogenase gene of *Candida bombicola* and use of its promoter. J Ind Microbiol Biotechnol, 2008b, 35: 1085-1092.

75 Van Bogaert INA, Sabirova J, Develter D, Soetaert W, Vandamme EJ. Knocking out the MFE-2 gene of Candida bombicola leads to improvedmedium-chain sophorolipid production . FEMS Yeast Res, 2009, 9: 610-617.

76 Wang YB, Lou Y, Luo ZF, Zhang DF, Wang YZ. Induction of apoptosis and cell cycle arrest by polyvinylpyrrolidone K-30 and protective effect of α-tocopheroi. Biochemical and Biophysical Research Communications, 2003, 308: 878-884.

77 薛庆善. 体外培养的原理与技术. 北京: 科学出版社, 2001.

78 Yoo DS, Lee BS, Kim EK. Characteristics of Microbial Biosurfactant as an Antifungal Agent Against Plant Pathogenic Fungus. J. Microbiol. Biotechnol, 2005, 15: 1164–1169.

79 张天胜等. 生物表面活性剂及其应用. 北京: 化学工业出版社, 2005.

80 Zakeri Z, Bursch W, Tenniswood M, lockshin RA. Cell death: Programmed, apoptosis, necrosis, or other. Cell death diff, 1995, 2: 87-96.

81 Zhou QH, Klekner V, Kosaric N. Production of sophorose lipids by Torulopsis bombicola from safflower oil and glucose. JAOCS, 1992, 69: 89-91.

攻读学位期间已发表和录用的论文

Lingjian Shao, Xin Song, Xiaojing Ma, Hui Li, Yinbo Qu. Bioactivties of Sophorolipid with Different Structures against Human Esophageal Cancer Cells. Journal of Surgical Research. doi:10.1016/j.jss.2010.09.013

宋欣，曲音波，邵凌健，马晓静，李慧。槐糖脂在制备抗皮肤癣菌药物中的应用。专利号：ZL200710114796

The Inhibition of Sophorolipids to Dermatophytes. Journal of Surgical Research. 投稿中

邵凌健，宋欣，曲音波（2007）。生物表面活性剂槐糖脂的补料发酵和抑菌作用。第二届中国资源生物技术与糖工程学术研讨会论文集。山东威海。（第一作者）

致 谢

本论文是在导师曲音波教授和宋欣教授的悉心指导下完成的。曲老师渊博的知识，分析问题的深度和宽度，严谨的科研风范，是我一生学习的榜样。宋老师求真务实、精益求精的科研作风，无时无刻不在给与我熏陶和教诲，在本论文的写作过程中，给与了我非常严格细致的指导，使我顺利完成学业。宋老师不仅在学习上对我有很大帮助，在生活上对我也无微不至的关心，教会了我很多做人的道理，是我人生路上的指路明灯。在此我衷心的感谢两位导师五年来的关怀、指导和培养。

感谢实验室的赵建老师、方诩老师、林建强老师、李雪芝老师、秦玉琪老师给予我的无私帮助和指导。

感谢孙彩云老师、巩海涛老师在仪器分析方面给予的大力支持。

感谢实验室的陈静师姐、孙宪昀师兄、张志华师姐、刘艳师姐和李莉师姐对我的帮助，感谢林彦良、程艳飞、刘凯、韦小敏、马晓静、刘国栋、李慧、付娟、陈梅、申静、赵丹，你们的真诚鼓励和热情帮助让我深刻体会到友情的温馨与珍贵。

感谢我的父母，你们无私的爱是我最大的精神支柱。感谢丁昊一直以来默默的支持、包容和鼓励，亲人们的关爱是支持我顺利完成学业巨大的动力，并将永远激励着我不停的前进和奋斗。

感谢所有的朋友，感谢你们的关心、支持和帮助。

2010年10月于济南

学位论文评阅及答辩情况表

<table>
<tr><td rowspan="6">论文评阅人</td><td colspan="2">姓　名</td><td>专业技术职务</td><td>所 在 单 位</td><td>对论文总体评价※</td></tr>
<tr><td colspan="2"></td><td></td><td></td><td></td></tr>
<tr><td colspan="2"></td><td></td><td></td><td></td></tr>
<tr><td colspan="2"></td><td></td><td></td><td></td></tr>
<tr><td colspan="2"></td><td></td><td></td><td></td></tr>
<tr><td colspan="2"></td><td></td><td></td><td></td></tr>
<tr><td rowspan="10">答辩委员会成员</td><td colspan="2">姓　名</td><td>专业技术职务</td><td>所 在 单 位</td><td>备　注</td></tr>
<tr><td>主席</td><td></td><td></td><td></td><td></td></tr>
<tr><td rowspan="8">委
员</td><td></td><td></td><td></td><td></td></tr>
<tr><td></td><td></td><td></td><td></td></tr>
<tr><td></td><td></td><td></td><td></td></tr>
<tr><td></td><td></td><td></td><td></td></tr>
<tr><td></td><td></td><td></td><td></td></tr>
<tr><td></td><td></td><td></td><td></td></tr>
<tr><td></td><td></td><td></td><td></td></tr>
<tr><td colspan="2">答辩委员会对论文的总体评价※</td><td></td><td>答辩秘书</td><td></td><td>答辩日期</td><td></td></tr>
<tr><td>备注</td><td colspan="6"></td></tr>
</table>

※ 优秀为“A”；良好为“B”；合格为“C”；不合格为“D”。

Journal of Surgical Research ■, 1–6 (2010)
doi:10.1016/j.jss.2010.09.013

Bioactivties of Sophorolipid with Different Structures Against Human Esophageal Cancer Cells

EQ1

Lingjian Shao,* Xin Song, Ph.D.,*,†,[1] Xiaojing Ma,* Hui Li,* and Yinbo Qu, Ph.D.*,†,[1]

*State Key Laboratory of Microbial Technology; and †National Glycoengineering Research Center, Shandong University, Jinan, China

Submitted for publication July 1, 2010

Background. **Sophorolipids (SLs) obtained from yeast broth are mixtures of many sophorolipid molecules with different structures, and have attracted more attention since they were found to have good antimicrobial, anticancer, anti-inflammatory, and anti-HIV activities. We investigated the effects of sophorolipid molecules with different structures on human esophageal cancer cells in the present work.**

Materials and Methods. **Ten sophorolipid (SL) molecules were separated and purified from the sophorolipids mixture using preparative HPLC, and their structures were identified by MS analyses. The effects of sophorolipid molecules with different structures on two human esophageal cancer cell lines, KYSE 109 and KYSE 450, were investigated by MTT assay.**

Results. **The structures of the 10 sophorolipid molecules differ in acetylation degree of sophorose, unsaturation degree of hydroxyl fatty acid, and lactonization or ring opening. The results demonstrated that the inhibition of diacetylated lactonic sophorolipid on two esophageal cancer cells (total inhibition at 30 μg/mL concentration) was stronger than that of monoacetylated lactonic sophorolipid (totally inhibition at 60 μg/mL concentration). Difference of unsaturation degree of hydroxyl fatty acid in SL molecules also had obvious influence on their cytotoxicity to esophageal cancer cells. The sophorolipid with one double bond in fatty acid part had the strongest cytotoxic effect on two esophageal cancer cells (total inhibition at 30 μg/mL concentration). Acidic sophorolipid showed hardly any anticancer activity against esophageal cancer cells.**

Conclusions. **In this study, the relationship of anticancer activities of natural sophorolipid molecules and the differences in their structures was revealed, which probably further reveals the mechanism of SL bioactivities and will be helpful in the modification of SL structures to obtain more novel SLs with excellent bioactivities.**

Key Words: **sophorolipid; esophageal cancer; acetylation; unsaturation degree; lactonization.**

INTRODUCTION

Sophorolipids (SLs) are glycolipid biosurfactants produced by yeasts and composed of a sophorose moiety (hydrophilic part) linked by a glycosidic bond to a long chain hydroxyl fatty acid (lipophilic part). They are mixtures of many sophorolipid molecules differing in acetylation degree of sophorose, acetyl group position in the sophorose moiety, chain length and unsaturation degree of hydroxyl fatty acid, hydroxyl group position in the fatty acid moiety, and lactonization or not (Fig. 1) [1–4]. Due to the properties of low toxicity, high biodegradability, biocompatibility, and produced in the largest amount in all the biosurfactants [5], sophorolipids have great application prospects in cosmetics, food, detergent industries such as emulsifiers, in environmental industry as bioremediation agent, and in petroleum industry as enhanced oil recovery agent [6–9]. Recently, sophorolipids have attracted more attention since they were found to have good antimicrobial, anticancer, anti-inflammatory, and even anti-HIV activities [10–19]. This will broaden the applications of sophorolipids in the pharmaceutical sector.

Natural sophorolipid molecules and their specific derivatives (such as sophorolipid methyl ester, sophorolipid ethyl ester, and so on) have recently been reported to have anticancer effects against pancreatic cancer cells [19]. In our previous studies, the inhibition on four human cancer cell lines H7402 (liver cancer line),

[1] To whom correspondence and reprint requests should be addressed at State Key Laboratory of Microbial Technology, Shandong University, Shan Da Nan Road 27, Jinan, 250100 China. E-mail: songx@sdu.edu.cn or quyinbo@sdu.edu.cn.

0022-4804/$36.00

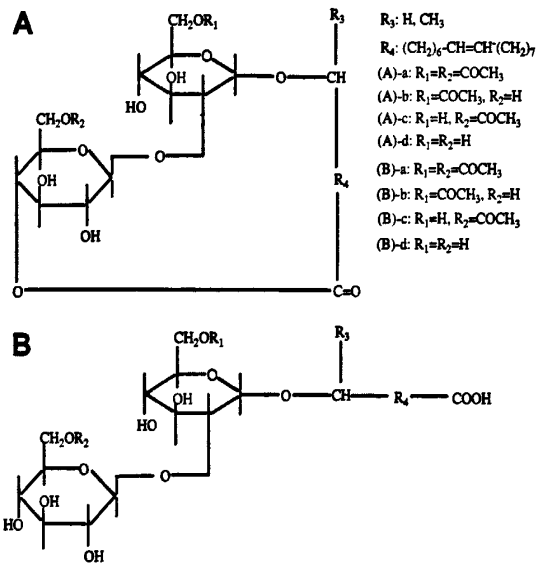


FIG. 1. Structure of sophorolipids: (A) lactonic type; (B) acidic type.

A549 (lung cancer line), HL60 and K562 (leukemia line) by a purified diacetylated lactonic sophorolipid with a C18 monounsaturated fatty acid was also investigated, and the inhibition on the human liver cancer cells H7402 was attributed to cell apoptosis induced by this diacetylated lactonic sophorolipid [10]. However, the relationship of the bioactivities of natural sophorolipid molecules and the differences in their structures have not been revealed so far. Considering that the differences in SL structures are mainly the differences in acetylation degree of sophorose, unsaturation degree of hydroxyl fatty acid, and lactonization or not, 10 sophorolipid molecules of different structures were purified, and the effects of them on human esophageal cancer cell KYSE 109 and KYSE 450 were investigated in the present work.

MATERIALS AND METHODS

Production of Crude Sophorolipids

The yeast *Wickerhamiella domercqiae* var. sophorolipid was maintained on YEPD agar slants at 4°C and was transferred at regular intervals. A loopful of inoculum was inoculated to 100 mL medium in 500 mL conical flasks. The composition of the medium for batch cultivation was as follows (g/L): glucose 80.0, yeast extract 3.0, KH_2PO_4 1.0, $Na_2HPO_4 \cdot 12H_2O$ 1.0, $MgSO_4 \cdot 7H_2O$ 0.5, and rapeseed oil 60.0. After the strain was cultivated for 7 d in 5-L fermentor at 30°C with 1.0 vvm aeration rate, the products of sophorolipids were extracted with ethyl acetate, and then ethyl acetate was removed by vacuum evaporation at 50°C. The residue was washed with hexane to remove the remaining rapeseed oil, followed by the removal of hexane by vacuum evaporation, the crude sophorolipids were obtained.

Purification and Structure Elucidation of Sophorolipid

The crude sophorolipids were applied to Kromasil C18, 5 μm columns (250 mm × 4.6 mm, Agela Technologies Inc.) for HPLC separation. The fluid phase consisted of acetonitrile-water and was programmed from 40% to 90% in 55 min, which was pumped at a flow rate of 1.0 mL/min. The injection volume was 5 μL and the eluent was monitored with UV detector at 207 nm. Ten fractions were collected, respectively. Preparative HPLC was performed using a PrepHT XDB C_{18} column (250 mm × 21.2 mm, Agela Technologies Inc). The injection volume was 500 μL and the flow rate was 15 mL/min. The fluid phase was programmed as the same of analytical HPLC.

All 10 purified SL molecules were applied to MS analysis for structure elucidation on an API 4000 mass spectrometer (Applied Biosystems, Foster City, CA).

Cell Culture

Human esophageal cancer cell KYSE 109 and KYSE 450 were obtained from Qilu Hospital of Shandong Province, China. The two cell lines were cultured in flat flasks with RPMI 1640 medium (Gibco,) supplemented with 10% calf serum (Hangzhou Sijiqing Co., China), 100 units/mL penicillin and 100 μg/mL streptomycin sulfate (AMRESCO,) in an incubator with an atmosphere containing 5% CO_2 at 37°C. Each purified sophorolipid molecule dissolved in ethanol was added to the cells grown in the RPMI 1640 medium and the final concentration of ethanol was below 0.05% (vol/vol). The RPMI 1640 medium without the addition of SL was used as the blank.

MTT Assay

Fifty microliters of the cell suspension (1.0×10^5 cells/mL) was seeded into the wells of a 96-well plate and incubated for 24 h, and then sophorolipid solution diluted in different concentrations was added to the wells. After incubation of the treated cells for 24 h, cell viability was determined by MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenytetrazolium, Sigma) assay [20]. Ten microliters of MTT was added to the wells and the cells were cultured for 4 h at 37°C in an incubator with an atmosphere containing 5% CO_2, then 100 μL of 10% SDS was added to the wells and left overnight. The formed formazan was measured by spectrophotometry (Spectra Max 190 Spectrophotometer; USA) at 570 nm.

Data Analysis

The results are expressed as the mean ± SD, and accompanied by the number of experiments performed independently. Statistical analysis was done by *t*-test.

RESULTS

Separation and Purification of Sophorolipids

As shown in Fig. 2, the crude sophorolipids produced by *Wickerhamiella domercqiae* var. sophorolipid are a mixture composed of many molecules. Ten sophorolipid molecules were separately collected.

Structure Elucidation of the Purified Sophorolipids

In this study, the structures of the 10 sophorolipid molecules were elucidated by MS analysis. Their structure details were listed in Table 1. It was found that all the sophorolipid molecules are sophorolipids with C_{18} fatty acid. Their structures differ in acetylation degree of sophorose, unsaturation degree of hydroxyl fatty acid, and lactonization or ring opening. Compound 10 with m/z peak of 313 does not correspond to any of

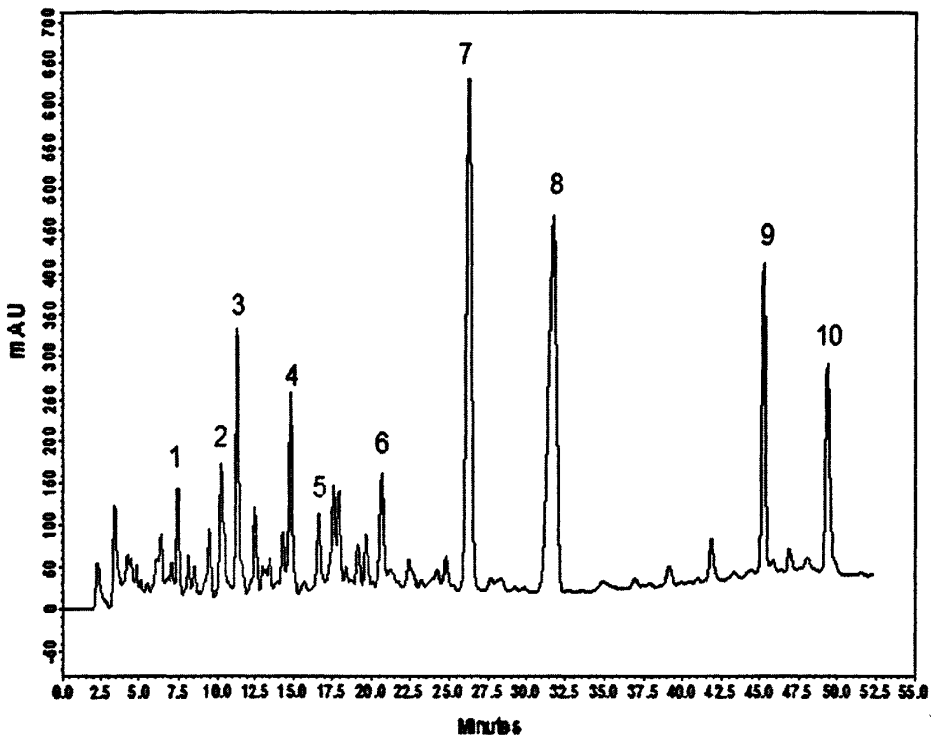


FIG. 2. HPLC chromatogram of crude sophorolipids produced by *Wickerhamiella domercqiae* var. sophorolipid. Ten sophorolipid molecules were separately collected.

the SL analogs or its precursors. This peak was probably obtained during fragmentation of sophorolipids.

Effects of SL on Cell Viability

The cytotoxic effects of purified lactonic sophorolipid with one or two acetylated groups of sophorose on esophageal cancer cells KYSE109 and KYSE450 are shown in Fig. 3. The cells were treated with sophorolipid of different concentrations, ranging from 0 to 60 μg/mL, respectively. C18:1 DLSL showed the strongest cytotoxic effect on KYSE109 and KYSE450, almost no viable cells of the two cell lines were observed after the treatment by SL at concentrations higher than

TABLE 1

Structure Elucidation of Sophorolipid Molecules Based on *m/z* Peaks of Protonated Molecular Ion [M + H^+]

No. as assigned in Fig. 2	Retention time (min)	*m/z* Peak of protonated molecular ions	Sophorolipid structure	Abbreviation
1	7.42	621	Unacetylated acidic SL with a C18 diunsaturated fatty acid	C18:2 NASL
2	10.21	663	Monoacetylated acidic SL with a C18 diunsaturated fatty acid	C18:2 MASL
3	11.35	665	Monoacetylated acidic SL with a C18 monounsaturated fatty acid	C18:1 MASL
4	14.81	705	Diacetylated acidic SL with a C18 diunsaturated fatty acid	C18:2 DASL
5	16.64	707	Diacetylated acidic SL with a C18 monounsaturated fatty acid	C18:1 DASL
6	20.68	647	Monoacetylated lactonic SL with a C18 monounsaturated fatty acid	C18:1 MLSL
7	26.31	687	Diacetylated lactonic SL with a C18 diunsaturated fatty acid	C18:2 DLSL
8	31.71	689	Diacetylated lactonic SL with a C18 monounsaturated fatty acid	C18:1 DLSL
9	45.21	691	Diacetylated lactonic SL with a C18 saturated fatty acid	C18:0 DLSL
10	49.35	313	Unknown	

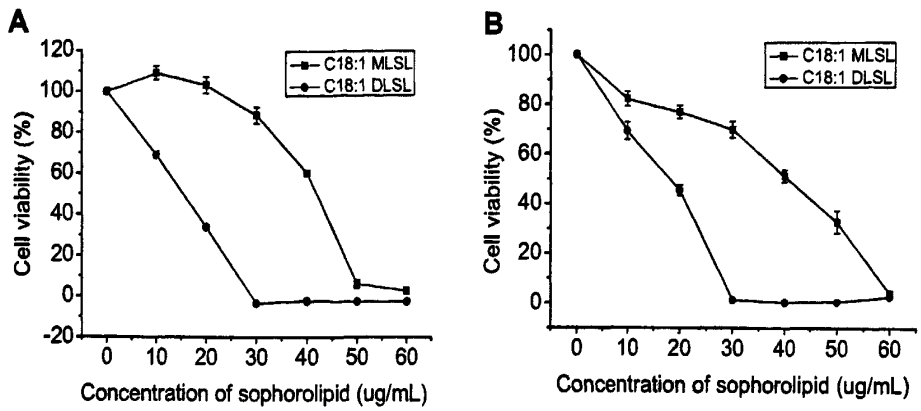


FIG. 3. Effects of lactonic sophorolipid with different acetylation degree of sophorose on cell viabilities of KYSE109 (A) and KYSE450 (B). Cell viability was determined by MTT assay. C18:1 MLSL = monoacetylated lactonic SL with a C18 monounsaturated fatty acid; C18:1 DLSL = diacetylated lactonic SL with a C18 monounsaturated fatty acid. The data represent mean ± SD;. $n = 6$ in all groups; $P < 0.01$ compared with the control.

30 μg/mL, while C18:1 MLSL showed almost complete inhibition when SL concentration was elevated to 60 μg/mL, which indicated a weaker cytotoxic effect of C18:1 MLSL than C18:1 DLSL on KYSE109 and KYSE450 cells.

As shown in Fig. 4, after the cells of KYSE109 and KYSE450 were treated with sophorolipid of different concentrations, ranging from 0 to 60 μg/mL, lactonic SL with different unsaturation degree in their fatty acid moieties showed different effects. C18:1 DLSL presented the strongest cytotoxic effects on KYSE109 and KYSE450 cells, cell viabilities of the two cell lines were almost 0, at the SL concentration of 30 μg/mL, while C18:2 DLSL, complete inhibition of the cells of KYSE109 and KYSE450 required higher SL concentration (50 and 60 μg/mL, respectively). C18:0 DLSL showed the weakest cytotoxic effect, after the treatment by 60 μg/mL SL concentration, 77% and 76% cell viability of KYSE109 and KYSE450 cells were retained, respectively.

Three acidic sophorolipids, which differ in acetylation degree of sophorose or unsaturation degree in fatty acid moieties were chosen to act on the cells of KYSE109 and KYSE450 esophageal cancer cell lines. As shown in Fig. 5, C18:2 MASL, C18:1 DASL, and C18:2 DASL have little inhibition to KYSE109 cells and can even increase the cell viability of KYSE450 cells. Even when the SL concentration was lifted to 60 μg/mL, the cells of the two cell lines retained almost 100% of cell viabilities.

DISCUSSION

Nature sophorolipids are mixtures of SLs with different structures. In our study, 10 sophorolipid molecules were separated and purified from sophorolipids mixture produced by *Wickerhamiella domercqiae* var. sophorolipid using preparative HPLC. Their structures were elucidated by MS analysis. The structures of the 10 sophorolipid molecules differ in acetylation degree

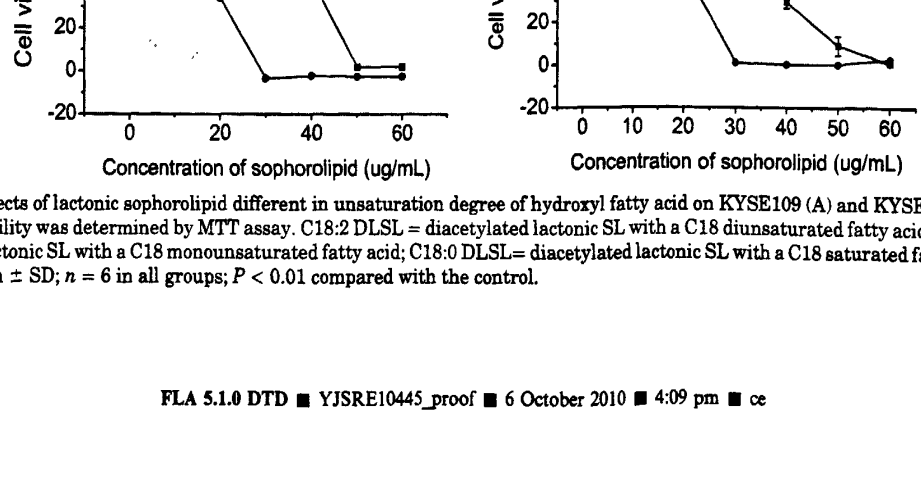


FIG. 4. Effects of lactonic sophorolipid different in unsaturation degree of hydroxyl fatty acid on KYSE109 (A) and KYSE450 (B) cell viability. Cell viability was determined by MTT assay. C18:2 DLSL = diacetylated lactonic SL with a C18 diunsaturated fatty acid; C18:1 DLSL = diacetylated lactonic SL with a C18 monounsaturated fatty acid; C18:0 DLSL= diacetylated lactonic SL with a C18 saturated fatty acid. Values represent mean ± SD; $n = 6$ in all groups; $P < 0.01$ compared with the control.

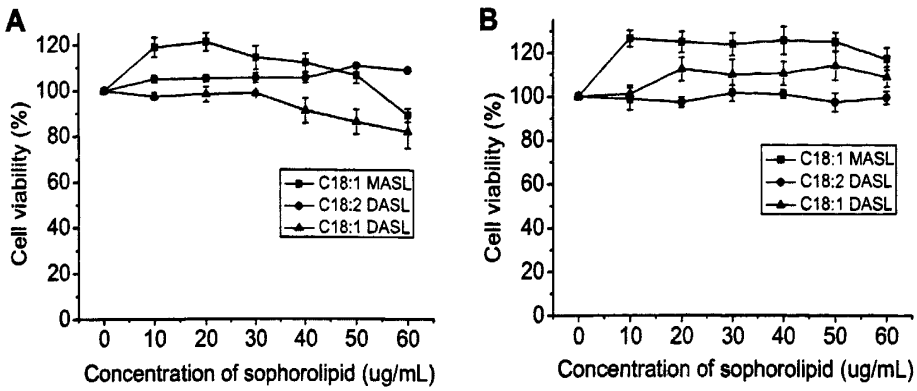


FIG. 5. Effects of acidic sophorolipid on KYSE109 (A) and KYSE450 (B) cell viability. Cell viability was determined by MTT assay. C18:1 MASL = monoacetylated acidic SL with a C18 monounsaturated fatty acid; C18:2 DASL = diacetylated acidic SL with a C18 diunsaturated fatty acid; C18:1 DASL = diacetylated acidic SL with a C18 monounsaturated fatty acid. Values represent mean ± SD; $n = 6$ in all groups; $P < 0.01$ by comparison with the control.

of sophorose, unsaturation degree of hydroxyl fatty acid, and lactonization or ring opening. The anticancer effects of sophorolipids with different structures on human esophageal cancer cell KYSE 109 and KYSE 450 were researched.

In 1998, Scholz *et al.* have reported that SL derivatives, acetylated sophorolipid ethyl ester and acetylated sophorolipid butyl ester, possessed strong cytotoxicity to suspension cell leukemic Jurkat and attached cell head and neck cancer Tu 138. However, unacetylated sophorolipid ethyl ester and unacetylated sophorolipid butyl ester showed little cytotoxicity to the two tumor cells. The author determined that the anticancer activity of SL was related to the acetylation of SL molecules [21]. The results in the present study indicated that the inhibition of diacetylated lactonic sophorolipid to esophageal cancer cells was stronger than mom acetylated lactonic sophorolipid, which confirmed that anticancer activity of SLs was affected by their acetylation degree in sophorose moiety.

Our results showed that sophorolipid with different unsaturation degree of hydroxyl fatty acid also had different cytotoxic effects on esophageal cancer cells. Sophorolipid having one double bond in fatty acid part had the strongest cytotoxic effect (totally inhibition at 30 μg/mL concentration) on esophageal cancer cells, sophorolipid with two double bonds had a little weaker anticancer effect (totally inhibition at 60 μg/mL concentration), while sophorolipid with no double bond had the weakest cytotoxic effect (only 20% of cells were inhibited at 60 μg/mL concentration) among the three sophorolipid molecules. This was the first study to reveal the relationship of bioactivities of natural sophorolipid molecules with different unsaturation degree in hydroxyl fatty acid and their structures.

Interestingly, acidic SL with one or two double bonds in fatty acid part, with monoacetylated group or diacetylated groups in sophorose part, have little anticancer effect against esophageal cancer cells. This result agrees with the previous report that acidic SL possessed weaker bioactivity than lactonic SL and has little cytotoxicity [13]. However, recently, Fu *et al.* have demonstrated that acidic sophorolipid has stronger cytotoxicity (35.5% cytotoxicity at 100 μg/mL concentration) than lactonic sophorolipid (26.2% cytotoxicity at 100 μg/mL concentration) against pancreatic cancer cells [19]. These different results are probably attributed to the inhibition mechanism of SL on pancreatic cancer cells, which are different from other cancer cells.

In our previous studies [10], the inhibition mechanism of diacetylated lactonic sophorolipid with a C18 monounsaturated fatty acid on the human liver cancer cells H7402 has been proved to induce cell apoptosis. We also have studied the inhibition mechanism of two sophorolipid molecules on human esophageal cancer cell KYSE 450 and it turned out to be cell apoptosis. The results will be presented in another paper.

In conclusion, in this study, the relationship of anticancer activities of natural sophorolipid molecules having different structures in acetylation degree of sophorose, unsaturation degree of hydroxyl fatty acid, and lactonization or not and their structures was revealed, which probably further reveals the mechanism of SL bioactivities and will be helpful in the modification of SL structures to obtain more novel SLs with excellent bioactivities.

ACKNOWLEDGMENTS

The authors acknowledge support for this work by Encouragement Foundation of Shandong Province for Young Scientists (2006BS02010), Key Scientific and Technological Project of Shandong Province (2007GG10002002), National Natural Science Foundation of China (30870048, 30970052), and National Key Technology R and D Program in the 11th Five-Year Plan of China (2008BAI63B08). The

authors thank Qilu Hospital of Shandong province in China for supplying human esophageal cancer cells KYSE 109 and KYSE 450.

REFERENCES

1. Cooper DG, Paddock DA. Production of a biosurfactant from *Torulopsis bombicola*. Appl Environ Microbiol 1984;47:173.
2. Asmer HJ, Lang S, Wagner F, et al. Microbial-production, structure elucidation and bioconversion of sophorose lipids. J Am Oil Chem Soc 1988;65:1460.
3. Cavalero DA, Cooper DG. The effect of medium composition on the structure and physical state of sophorolipids produced by *Candida bombicola* ATCC 22214. J Biotechnol 2003; 103:31.
4. Hu YM, Ju LK. Purification of lactonic sophorolipids by crystallization. J Biotechnol 2001;87:263.
5. Haba E, Espuny MJ, Busquets M, et al. Screening and production of rhamnolipids by *Pseudomonas aeruginosa* 47T2 NCIB 40044 from waste frying oils. J App Microbiol 2000; 88:379.
6. Klekner V, Kosaric N. Biosurfactant for cosmetic. In: Kosaric N, Ed. Biosurfactant: Production, properties, applications. New York: Marcel Dekker, 1993:6599.
7. Mulligan CN. Environmental applications for biosurfactants. Environ Pollution 2005;133:183.
8. Ron E, Rosenberg E. Natural roles of biosurfactants. Environ Microbiol 2001;3:229.
9. Banat IM. Biosurfactants production and possible uses in microbial enhanced oil recovery and oil pollution remediation. Bioresour Technol 1995;51:1.
10. Chen J, Song X, Zhang H, et al. Sophorolipid produced from the new yeast strain *Wickerhamiella domercqiae* induces apoptosis in H7402 human liver cancer cells. Appl Microbiol Biotechnol 2006;72:52.
11. Ito S, Kinta M, Inoue S. Growth of yeasts on alkanes: Inhibition by a lactonic sophorolipid produced by *Torulopsis bombicola*. Agric Biol Chem 1980;44:2221.
12. Yoo DS, Lee BS, Kim EK. Characteristics of microbial biosurfactant as an antifungal agent against plant pathogenic fungus. J Microbiol Biotechnol 2005;15:1164.
13. Shah V, Doncel GF, Seyoum T, et al. Sophorolipids, microbial glycolipids with anti-human immunodeficiency virus and sperm-immobilizing activities. Antimicrob Agents Chemother 2005;49:4093.
14. Shah V, Badia D, Ratsep P. Sophorolipids having enhanced antibacterial activity. Antimicrob Agents Chemother 2007; 51:397.
15. Sun XX, Lee YJ, Choi JK, et al. Synergistic effect of sophorolipid and loess combination in harmful algal blooms mitigation. Mar Pollut Bull 2004;48:863.
16. Hardin R, Pierre J, Schulze R, et al. Sophorolipids improve sepsis survival: Effects of dosing and derivatives. J Surg Res 2007; 142:314.
17. Bluth MH, Kandil E, Mueller CM, et al. Sophorolipids block lethal effects of septic shock in rats in a cecal ligation and puncture model of experimental sepsis. Crit Care Med 2006;34:188.
18. Napolitano LM. Sophorolipids in sepsis: Anti-inflammatory or antibacterial? Crit Care Med 2006;34:258.
19. Fu SL, Wallner SR, Bowne WB, et al. Sophorolipids and their derivatives are lethal against human pancreatic cancer cells. J Surg Res 2008;148:77.
20. Price P, McMillan TJ. Use of the tetrazolium assay in measuring the response of human tumor cells to ionizing radiation. Cancer Res 1990;50:1392.
21. Scholz C, Mehta S, Bisht K, et al. Bioactivity of extracellular glycolipids-investigation of potential anticancer activity of sophorolipids and sophorolipid derivatives. Polymer preprints 1998; 39:169.

The Inhibition of Sophorolipids to Dermatophytes*

Lingjian Shao, Xin Song Ph.D.,** Xiaojing Ma, Hui Li,

Yinbo Qu, Ph.D.**

State Key Laboratory of Microbial Technology,Shandong University, Jinan 250100, China

Introduction. Sophorolipids, a class of glycolipid biosurfactants produced by yeasts, have been attracting great attentions for their various bioactivities, such as antimicrobial, anticancer activities and even the anti-HIV activity, which will broaden their applications in pharmaceutical sector besides food, detergent and cosmetics industries. In the present study, we investigated the inhibition of sophorolipids to three common clinical dermatophytes, *Trichophyton rubrum*, *Trichophyton gypseum*, and *Microsporum canis* and probable inhibition mechanism.

Materials and methods. Three dermatophytes were treated with increasing concentrations of lactonic sophorolipids and acidic sophorolipids. Minimum Inhibitory Concentration (MIC), Minimal Fungicidal Concentration (MFC) and inhibition ratio to hypha extension of sophorolipids to dermatophytes were measured. TEM observation of three dermatophytes before and after being treated with sophorolipids was done to research inhibition mechanism of sophorolipids to dermatophytes.

Results. Lactonic or acidic sophorolipids showed different inhibition on the growth of three dermatophytes. When lactonic sophorolipids concentration was 0.5 mg/mL, the inhibition ratio on hypha extension of *Trichophyton rubrum*, *Trichophyton gypseum*

*Supported by Encouragement Foundation of Shandong Province for Young Scientists (2006BS02010), Key Scientific and Technological project of Shandong Province (2007GG10002002) and National Natural Science Foundation of China (No. 30870048 and No. 30970052)

** Corresponding author. Tel: 0531-88362883; Fax::0531-88565610; E-mail: songx@sdu.edu.cn
Tel: 0531-88369495; Fax: 0531-88565610; E-mail: quyinbo@sdu.edu.cn

and *Microsporum canis* was 53.8%, 62.5% and 68.2%, respectively. The MIC_{50} of lactonic sophorolipids to the three dermatophytes was 0.0625, 0.125, 0.0625 mg/mL, respectively. The TEM analysis indicated that, after treated by sophorolipids, three dermatophytes have some obvious changes in their microstructures. The cell wall became thicker and loose, the cytoplasm agglomerated, the membranes of organelles were disappearing, and no integrated organelles and clear nuclear zone were found in the cytoplasm.

Conclusions. These results suggest that sophorolipids may have great potential in treating skin diseases. Lactonic or acidic sophorolipids can be used to treat skin diseases induced by different dermatophytes.

Keywords: inhibition, lactonic sophorolipids, acidic sophorolipids, dermatophyte

Introduction

Sophorolipids are glycolipid biosurfactants produced by yeasts and composed of a sophorose moiety (hydrophilic part) linked by a glycosidic bond to a long chain hydroxyl fatty acid (lipophilic part). They are mixtures of up to 14 sophorolipids differing in acetylated degree of sophorose, chain length and unsaturation degree of hydroxyl fatty acid, and hydroxyl group position in the fatty acid moiety [1-4]. As shown in Fig.1, there are two main types of sophorolipids-acidic sophorolipids and lactonic sophorolipids. Due to their properties of low toxicity, high biodegradability and biocompatibility [5], sophorolipids have great application prospects in cosmetics, food, detergent industries as emulsifiers, in environmental industry as bioremediation agent, in petroleum industry as enhanced oil recovery agent [6-9]. Recently, sophorolipids have been proved to have good antimicrobial, anticancer activities and even the anti-HIV activity, which will broaden the applications of sophorolipids in pharmaceutical sector [10-13].

As is reported, sophorolipids possess antibacterial properties and are more effective against gram-positive bacteria than gram-negative bacteria [14]. Besides the inhibition to bacteria, sophorolipids are also good antifungal agents against plant pathogenic fungi such as *Phytophthora* sp. and *Pythium* sp. [12], antialgal reagents for algal bloom inhibition [15], and even have strong activities of anti-human immunodeficiency virus and of sperm-immobilization [13]. Rosemarie Hardin et al. [16] reported that sophorolipids tend to decrease sepsis-related mortality in a rat model. However, little information regarding the inhibition mechanism of sophorolipids to microorganism is available, it stays however a matter of debate whether the effect is caused by modulation of immune and inflammatory responses or by the direct destroy of sophorolipid molecules to cell structures of microorganisms [17,18]. So far, the inhibition of sophorolipids to skin disease-causing fungi has not been reported. *Trichophyton rubrum*, *Trichophyton gypseum*, and *Microsporum canis* are three common clinical dermatophytes which can cause serious skin tinea on hand, foot, nail, head and so on. In the present study, we investigated the inhibition of

sophorolipids to the three dermatophytes and probable inhibition mechanism.

Materials and methods

Production of sophorolipids

The yeast *Wickerhamiella domercqiae* var. sophorolipid was maintained on YEPD agar slants and was transferred at regular intervals. A loopful of inoculum was inoculated to 100 mL medium in 500 mL shake flasks. The medium for batch cultivation contained (per liter deionized water): glucose 80.0 g, yeast extract 3.0 g, KH_2PO_4 1.0 g, $Na_2HPO_4 \cdot 12H_2O$ 1.0 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, and rapeseed oil 60.0 g. After the strain was cultivated for 7 days in 5 L fermentor at 30 °C with 1 vvm aeration rate, the products of sophorolipids were extracted by ethyl acetate, and then ethyl acetate was removed by vacuum distillation at 50 °C. The residue was washed with hexane to remove the remaining rapeseed oil. After the residual hexane was evaporated by vacuum distillation, the crude lactonic sophorolipids were obtained. The medium for acidic sophorolipids cultivation contained (per liter deionized water): glucose 80.0 g, yeast extract 3.0 g, $(NH_4)_2SO_4$ 4.0 g, KH_2PO_4 1.0 g, $Na_2HPO_4 \cdot 12H_2O$ 1.0 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, and rapeseed oil 60.0 g. After the strain was cultivated for 7 days at 30 °C, hexane was first added to the fermentation broth to remove residual lipophilic substrate, followed by centrifugation for 15min at 6000 rpm to remove the yeast cells, the supernatant was concentrated at 80 °C by vacuum distillation to a brown, sticky semi-solid product ,that is, the acidic sophorolipids.

Dermatophytes

Three dermatophytes, *Trichophyton rubrum*, *Trichophyton gypseum*, and *Microsporum canis* were obtained from Institute of Dermatology in Chinese Academy of Medical Sciences. Dermatophytes were maintained on Sabouraud's Agar Medium consisting of glucose 40.0 g/L, peptone 10.0 g/L, agar 20.0 g/L.

Effect of sophorolipids on dermatophyte growth

Lactonic sophorolipids were dissolved with dimethyl sulfoxide to the final

concentrations of 0, 8, 20, 40 g/L respectively. Two hundred and fifty microlitres of lactonic sophorolipid solutions were poured into sterilized plates and 20 mL medium was then added. The medium and the lactonic sophorolipids solution were fully mixed. The final concentrations of lactonic sophorolipids were 0, 0.1, 0.25, 0.5 g/L respectively. The medium without sophorolipids solutions were used as blank. Acidic sophorolipids were dissolved with deionized water to the final concentrations of 8, 20, 40 g/L respectively. Two hundred and fifty microlitres of acidic sophorolipid solutions were added into sterilized plates followed by the addition of 20 mL medium, and then they were fully mixed. The final concentration of acidic sophorolipids was 0.1, 0.25, 0.5 g/L respectively. The medium without sophorolipids solutions was used as control. One hundred microlitres of spore suspension (1×10^3 spores/mL) of each dermatophyte was spread on the prepared agar plates with and without sophorolipids and cultured for 5 days at 30 °C. The growth of each dermatophyte was observed every 24 hours.

Inhibition ratio to hypha extension of sophorolipids to dermatophytes

Sophorolipids in different concentrations were mixed with Sabouraud's Agar Medium and then added into sterilized plates. The medium and each concentration of sophorolipids solution were fully mixed. The final concentrations were 0, 0.065, 0.125, 0.25, 0.5 g/L, respectively. Medium without sophorolipids solutions were used as control. An agar cake of fungal inoculum (6 mm in diameter) was removed from a 5-day-old previous culture of all the dermatophytes tested and placed upside down on the Sabouraud's Agar Medium plates with sophorolipids of different concentrations. The plates were cultured for 5 days at 30 °C. The diameter of the hypha was measured and the inhibition ratio to hypha extension was calculated using the following formula:

inhibition ratio (%)=$\{$1-radial growth of treatment (mm)/radial growth of control (mm)$\} \times 100$

Minimum Inhibitory Concentration (MIC) and Minimal Fungicidal Concentration (MFC)

Fifty microlitres of lactonic sophorolipids with different concentrations was added into 5 mL medium in different tube, and the final concentrations of sophorolipids were 0, 0.0625, 0.125, 0.25, 0.5 g/L respectively. The medium with 50 μl dimethyl sulfoxide was used as control. One hundred microlitres of spore suspension (1×10^6 spores/mL) of dermatophyte was added into the tubes with and without sophorolipids and cultured for 3 days at 30 °C. The minimum concentration of sophorolipids in which tube no dermatophyte was grown was the MIC. One hundred microlitres of medium in which tubes no dermatophyte was grown was spread onto the Sabouraud's Agar Medium plates and cultured for 5 days at 30 °C. The minimum concentration of sophorolipids in which plate no dermatophyte was grown was the MFC.

Morphological observation of dermatophytes by TEM

For transmission electron microscopy (TEM) observation, hyphae treated with or without lactonic sophorolipid (0.5 g/L for 48 h) were collected and washed once with PBS (0.85% NaCl, 0.02% KCl, 0.285% $Na_2HPO_4 \cdot 12H_2O$, 0.027% KH_2PO_4, pH 7.2, w/v) and centrifuged for 10 min at 1,000 rpm. Pellets were then stood overnight at 4 °C in 2.5% glutaraldehyde, treated with 1% osmium tetroxide for 2 h at 4 °C, dehydrated in different acetone/water concentration gradient (25~100% acetone), and embedded in epoxy resin. Ultrathin sections (70~90 nm thick) were prepared with a diamond knife using a Richart Ultracut II microtome and examined by a JEM-1200EX electron microscope (Japan) [19].

Result

Inhibition of lactonic sophorolipids to *Trichophyton rubrum*, *Trichophyton gypseum* and *Microsporum canis*

As shown in Fig.2, after being cultured 5 days, *Trichophyton rubrum* on medium without sophorolipids solutions and medium only with 0.25 mL dimethyl sulfoxide

were grown well. The colonies of *Trichophyton rubrum* on medium with 0.1 g/L and 0.25 g/L lactonic sophorolipids were smaller and less than the two controls. 0.5 g/L lactonic sophrolipids could fully inhibit the growth of *Trichophyton rubrum*. As to *Trichophyton gypseum* and *Microsporum canis*, the hyphae also grew well on the two controls. There were no colonies on medium with 0.1 g/L, 0.25 g/L and 0.5 g/L lactonic sophorolipids, which indicated the stronger inhibition of lactonic sophorolipids to *Trichophyton gypseum* and *Microsporum canis* than *Trichophyton rubrum*. (The results regarding the inhibition of lactonic sophorolipids to *Trichophyton gypseum* and *Microsporum canis* were omitted.)

The inhibition of acidic sophorolipids to *Trichophyton rubrum*, *Trichophyton gypseum* and *Microsporum canis*

As shown in Fig.3, after being cultured 5 days, the colonies of *Trichophyton gypseum* on medium with 0.1 g/L acidic sophorolipids were smaller and less than the ones on medium without sophorolipids solutions. There were no colonies on medium with 0.25 g/L and 0.5 g/L acidic sophorolipids, which indicated strong inhibition of acidic sophorolipids to *Trichophyton gypseum*. The colonies of *Trichophyton rubrum* and *Microsporum canis* were less and less with the increase of the concentrations of acidic sophorolipids. There were no colonies on medium with 0.5 g/L acidic sophorolipids. (The results regarding the inhibition of acidic sophorolipids to *Trichophyton rubrum* and *Microsporum canis* were omitted.)

Comparison of the inhibition of acidic sophorolipids with that of lactonic sophorolipids to *Trichophyton rubrum*, *Trichophyton gypseum* and *Microsporum canis*

We investigated the inhibition of acidic sophorolipids and lactonic sophorolipids to *Trichophyton rubrum* and *Trichophyton gypseum* from the fifth day to the seventh day, and to *Microsporum canis* from the fifth day to the tenth day. Only the results after 7 days' cultivation were shown in Table 1. The inhibition of acidic sophorolipids to *Trichophyton gypseum* was much better than to *Trichophyton rubrum* and

Microsporum canis. At the seventh day, there were no colonies of *Trichophyton gypseum* grown on the medium with 0.25 g/L acidic sophorolipids. However, some colonies of *Trichophyton rubrum* and *Microsporum canis* were grown on the medium with 0.5 g/L acidic sophorolipids. Lactonic sophorolipids has the strongest inhibition to *Microsporum canis*, the growth of the dermatophyte was fully inhibited by low concentration of lactonic sophorolipids (0.1 g/L), they also showed good inhibition to *Trichophyton gypseum*, no colonies appeared on the medium with 0.25 g/L and 0.5 g/L sophorolipids, in comparison, lactonic sophorolipids presented inhibition for *Trichophyton rubrum* growth, some colonies could still be observed on the medium with 0.5 g/L sophorolipids.

To *Trichophyton rubrum*, the inhibition of lactonic sophorolipids was a little better than acidic ones. As the increase of the concentrations of lactonic and acidic sophorolipids, the colonies of *Trichophyton rubrum* were less and less. When the concentration of sophorolipids was 0.5 g/L, there were no colonies grown on the plates at the fifth day. With the prolongation of culture time, the colonies were grown out gradually and they were a little less on the medium with lactonic sophorolipids than on the medium with acidic ones at the same concentrations.

Both acidic and lactonic sophorolipids have strong inhibition to *Trichophyton gypseum*. They could not only inhibit the size and quantity of the colonies, but also inhibit *Trichophyton gypseum* for a long time. At low concentrations, the inhibition of lactonic sophorolipids was much better than acidic ones. After being cultured for 5 days, there were no colonies on the medium with 0.05 g/L lactonic sophorolipids, but some colonies on the medium with 0.1 g/L acidic sophorolipids were observed. When at higher concentrations, the inhibition of acidic sophorolipids was much better than that of lactonic ones. After being cultured for 10 days, there were no colonies on the medium with 0.5 g/L acidic sophorolipids, but on the medium with 0.5 g/L lactonic sophorolipids, *Trichophyton gypseum* was growing slowly and some colonies were observed.

To *Microsporum canis*, the inhibition of lactonic sophorolipids was much better

than acidic ones. At lower concentrations, acidic sophorolipids showed a little inhibition to *Microsporum canis,* at higher concentration (0.5 g/L), acidic sophorolipids showed good inhibition to *Microsporum canis*, they could retard the growth of *Microsporum canis* until the fifth day. After being cultured for up to 7 days, there were no colonies on the medium with 0.1 g/L lactonic sophorolipids, which indicated strong lasting inhibition of lactonic sophorolipids to *Microsporum canis*.

Inhibition ratio to hypha extension

It could be seen from the table 2 that, lactonic sophorolipids could inhibit the extension of hypha much better than the acidic ones. When acidic sophorolipids concentration was 0.5g/L, the inhibition ratio to hypha extension of *Trichophyton rubrum*, *Trichophyton gypseum* and *Microsporum canis* was only 19.2%, 12.5% and 9.09% respectively. When treated by higher concentrations of lactonic sophorolipids, the colony diameters of three dermatophytes were smaller, and the inhibition ratio is higher. When lactonic sophorolipids concentration was 0.5 g/L, the inhibition ratio to hypha extension of *Trichophyton rubrum*, *Trichophyton gypseum* and *Microsporum canis* was 53.8%, 62.5% and 68.2% respectively. Because lactonic sophorolipids were hard to dissolve in water, when the concentration was 0.5 g/L, the medium was opaque. After hypha cake was cultured on the medium, there was transparent circle around the hypha cake, which indicated that sophorolipids were absorbed into the hypha, and acted on the inside of cell.

Minimum Inhibited Consistency (MIC) and Minimal Fungicidal Consistency (MFC)

In liquid culture, acidic sophorolipids have little inhibition to three dermatophytes, so only MIC and MFC of lactonic sophorolipids to three dermatophytes were investigated. MIC range of lactonic sophorolipids to three dermatophytes was 0.0625-0.5 mg/mL. MIC_{50} of lactonic sophorolipids to *Trichophyton rubrum*, *Trichophyton gypseum* and *Microsporum canis* was 0.0625, 0.125, 0.0625 mg/mL

respectively. MIC_{90} of lactonic sophorolipids to *Trichophyton rubrum*, *Trichophyton gypseum* and *Microsporum canis* was 0.125, 0.25, 0.125 mg/mL respectively. MFC of lactonic sophorolipids to *Trichophyton rubrum*, *Trichophyton gypseum* and *Microsporum canis* was 0.5, 0.5, 0.25 mg/mL respectively.

TEM observation

Before being treated by sophorolipids, the cell wall was intact and the thickness of cell wall was even. The cytoplasm was homogeneous, and organelles and vacuoles could be seen clearly in the cytoplasm. The membranes of organelles and vacuoles were intact. The nuclear zone and the boundary were clear. After being treated by lactonic sophorolipids, *Trichophyton gypseum*, *Trichophyton rubrum* and *Microsporum canis* have some obvious morphorlogical changes (Fig. 4). The cell wall became thicker and loose, the cytoplasm became agglomerate and formed grain structure with high electron density, the membranes of organelles were disappearing and the organelles were destroyed. There were no integrated organelles and clear nuclear zone in the cytoplasm.

Discussion

As a kind of glycolipid biosurfactant, sophorolipids have been attracting more and more interests for their various activities in pharmaceutical area such as anti-cancer, anti-bacterial, anti-algal and anti-HIV activities [10-13]. Sophorolipids have also been reported to present inhibition to plant pathogenic fungi such as *Phytophthora* sp. and *Pythium* sp. [12], however, there have not been any studies on the inhibition of sophorolipids to pathogenic fungi which can cause human skin diseases. In this paper, sophorolipids showed strong inhibition to three common clinical dermatophytes, *Trichophyton rubrum*, *Trichophyton gypseum*, and *Microsporum canis* which can cause skin diseases, and acidic and lactonic sophorolipids showed some different effects, which have not been reported before.

In our previous study, one diacetylated lactonic sophorolipid was purified to act on the human liver cancer cells H7402 and the inhibition of this sophorolipid was

attributed to its induction of cell apoptosis of liver cell [10]. In clinical therapy of skin diseases, purified component of sophorolipid was not necessary, so crude lactonic and acidic sophorolipids were used to inhibit dermatophytes in this study. We compared the inhibition of acidic sophorolipids with that of lactonic ones to the three dermatophytes. Lactonic or acidic sophorolipids showed inhibition on the growth of all the three dermatophytes and showed different inhibition towards each of them. To *Trichophyton rubrum*, the inhibition of lactonic sophorolipids was a little better than acidic ones. Toward *Trichophyton gypseum*, when at low concentrations, the inhibition of lactonic sophorolipids was much better than that of acidic ones. However, when at high concentrations, the inhibition of acidic sophorolipids was much better than lactonic ones. We speculate that, for acidic sophorolipids, a critical concentration may account for the above results. When the concentration of acidic sophorolipids was higher than the critical concentration, they could fully inhibit the growth of *Trichophyton gypseum*. Toward *Microsporum canis*, the inhibition of lactonic sophorolipids was much better than acidic ones. The inhibition of acidic sophorolipids to *Microsporum canis* was not obvious. For the inhibition of hypha extension, lactonic sophorolipids could inhibit the extension of hypha much better than the acidic ones. And in liquid culture, acidic sophorolipids have little inhibition to three dermatophytes. Therefore, lactonic sophorolipids may have more potential in clinical therapy of skin diseases.

In order to understand the mechanism of the inhibition of sophorolipids to the three dermatophytes, morphological changes of the three dermatophytes after the treatment with lactonic sophorolipids was fulfilled through TEM observation. The TEM observation results indicated that, after being treated by lactonic sophorolipids, three dermatophytes have some obvious changes in their microstructures. The cell wall became thicker and loose, the cytoplasm agglomerated, the membranes of organelles were disappearing, and no integrated organelles and clear nuclear zone were found in the cytoplasm. One probable reason of these changes attributes to surfactant trait of sophorolipids, and another reason probably lie in that sophorolipids can induce cell

apoptosis based on the effect of sophorolipid to human liver cancer cell line H7402. Newly-discovered dermatophyte inhibition bioactivity of sophorolipids will make sophorolipids as a clinical candidate drug for the treatment of skin tinea, and will enrich the applications of sophorolipids in pharmaceutical area.

Acknowledgements

Our work was supported by Encouragement Fundation of Shandong Province for Young Scientists (2006BS02010), Key Scientific and Technological project of Shandong Province (2007GG10002002) and National Natural Science Foundation of China (No. 30870048). We wish to thank Institute of Dermatology in Chinese Academy of Medical Sciences to supply the three skin disease-causing fungi *Trichophyton rubrum*, *Trichophyton gypseum*, and *Microsporum canis*.

References

1. Cooper DG, Paddock DA. Production of a biosurfactant from Torulopsis bombicola. Appl Environ Microbiol 1984;47:173 - 176

2. Asmer HJ, Lang S, Wagner F, Wray V. Microbial-production, structure elucidation and bioconversion of sophorose lipids. J Am Oil Chem Soc 1988;65:1460 - 1466

3. Cavalero DA, Cooper DG. The effect of medium composition on the structure and physical state of sophorolipids produced by Candida bombicola ATCC 22214. J Biotechnol 2003;103:31 - 41

4. Hu YM, Ju LK. Purification of lactonic sophorolipids by crystallization. J Biotechnol 2001;87:263 - 272

5. Haba E, Espuny MJ, Busquets M, Manresa A. Screening and production of rhamnolipids by Pseudomonas aeruginosa 47T2 NCIB 40044 from waste frying oils. J App Microbiol 2000;88:379– 387

6. Klekner V, Kosaric N. Biosurfactant for cosmetic. In: Kosaric N (ed) Biosurfactant: production, properties, applications. Marcel Dekker, New York, 1993: 65 - 99

7. Mulligan CN. Environmental applications for biosurfactants. Environmental Pollution 2005;133: 183-198

8. Ron E, Rosenberg E. Natural roles of biosurfactants. Environ Microbiol 2001;3:229 - 236

9. Banat IM. Biosurfactants production and possible uses in microbial enhanced oil recovery and oil pollution remediation. Bioresour Technol 1995;51:1 - 12

10. Chen J, Song X, Zhang H, Qu YB, Miao JY. Sophorolipid produced from the new yeast strain Wickerhamiella domercqiae induces apoptosis in H7402 human liver cancer cells. Appl Microbiol Biotechnol 2006;72:52–59

11. Ito S, Kinta M, Inoue S. Growth of yeasts on alkanes: inhibition by a lactonic sophorolipid produced by Torulopsis bombicola. Agric Biol Chem 1980;44:2221

12. Yoo DS, Lee BS, Kim EK. Characteristics of microbial biosurfactant as an antifungal agent against plant pathogenic fungus. J Microbiol Biotechnol 2005;15:1164 - 1169

13. Shah V, Doncel GF, Seyoum T, Eaton KM, Zalenskaya I, Hagver R, Azim A, Gross R. Sophorolipids, microbial glycolipids with anti-human immunodeficiency virus and sperm - immobilizing activities. Antimicrob Agents Chemother 2005;49:4093 - 4100

14. Shah V, Badia D, Ratsep P. Sophorolipids Having Enhanced Antibacterial Activity. Antimicrob Agents Chemother 2007; 51:397–400

15. Sun XX, Lee YJ, Choi JK, et al. Synergistic effect of sophorolipid and loess combination in harmful algal blooms mitigation. Mar Pollut Bull 2004; 48:863

16. Hardin R, Pierre J, Schulze R, Mueller CM, Fu SL, Wallner SR, Stanek A, Shah V, Gross RA, Weedon J, Nowakowski M, Zenilman ME, Bluth MH. Sophorolipids improve sepsis survival: effects of dosing and derivatives. Journal of Surgical Research 2007;142:314–319

17. Bluth MH, Kandil E, Mueller CM, Shah V, Lin YY, Zhang H, Dresner L, Lempert L, Nowakowski M, Gross R, Schulze R, Zenilman ME. Sophorolipids block lethal effects of septic shock in rats in a cecal ligation and puncture model of experimental sepsis. Crit Care Med 2006;34:188 - 195

18. Napolitano LM. Sophorolipids in sepsis: Antiinflammatory or antibacterial? Crit Care Med 2006;34:258 - 259

19. Catelas I, Petit A, Vali H, Fragiskatos C, Meilleur R, Zukor DJ, Antoniou J, Huk OL. Quantitative analysis of macrophage apoptosis vs necrosis induced by cobalt and chromium ions in vitro. Biomaterials 2005;26:2441 - 2453

Figure captions

Fig.1 Structure of sophorolipids (A) lactonic type; (B) acidic type.

Fig.2 Inhibition of lactonic sophorolipids to *Trichophyton rubrum.*

A: medium without sophorolipids solutions; B: medium only with 0.25mL dimethyl sulfoxide; C: medium with 0.1g/L lactonic sophorolipids; D: medium with 0.25 g/L lactonic sophorolipids; E: medium with 0.5 g/L lactonic sophorolipids.

Fig.3 Inhibition of acidic sophorolipids to *Trichophyton gypseum.*

A: medium without sophorolipids solutions; B: medium with 0.1 g/L acidic sophorolipids; C: medium with 0.25 g/L acidic sophorolipids; D: medium with 0.5 g/L acidic sophorolipids.

Fig. 4 TEM observation of *Trichophyton gypseum*, *Trichophyton rubrum* and *Microsporum canis* with sophorolipids treatment

A: *Trichophyton gypseum* without being treated by sophorolipids; B: *Trichophyton gypseum* treated by 0.5 g/L sophorolipids for 48h; C: *Trichophyton rubrum* without being treated by sophorolipids; D: *Trichophyton rubrum* treated by 0.5 g/L sophorolipids for 48 h; E: *Microsporum canis* without being treated by sophorolipids; F: *Microsporum canis* treated by 0.5 g/L sophorolipids for 48h.

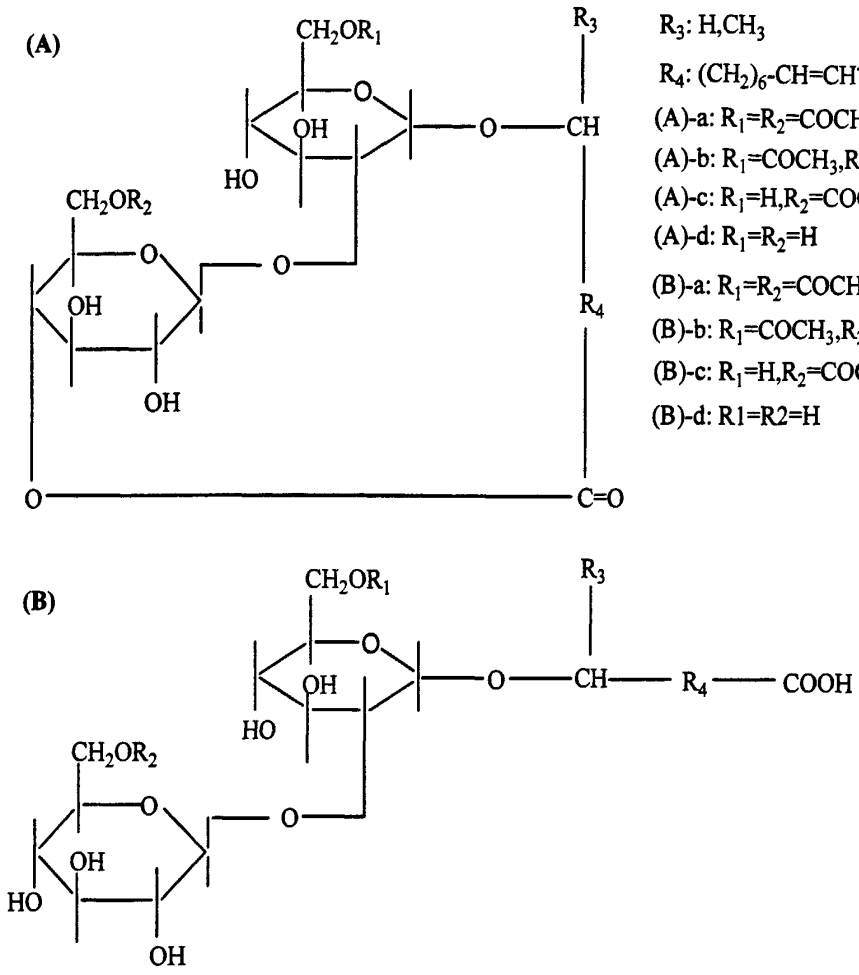

(A)
CH_2OR_1
R_3
O
OH
HO
O
CH
CH_2OR_2
O
O
OH
OH
R_4
O
C=O
R_3: H,CH_3
R_4: $(CH_2)_6$-CH=CH-$(CH_2)_7$
(A)-a: R_1=R_2=$COCH_3$
(A)-b: R_1=$COCH_3$,R_2=H
(A)-c: R_1=H,R_2=$COCH_3$
(A)-d: R_1=R_2=H
(B)-a: R_1=R_2=$COCH_3$
(B)-b: R_1=$COCH_3$,R_2=H
(B)-c: R_1=H,R_2=$COCH_3$
(B)-d: R1=R2=H
(B)
CH_2OR_1
R_3
O
OH
HO
O
CH
R_4
COOH
CH_2OR_2
O
O
OH
HO
OH

Fig.1

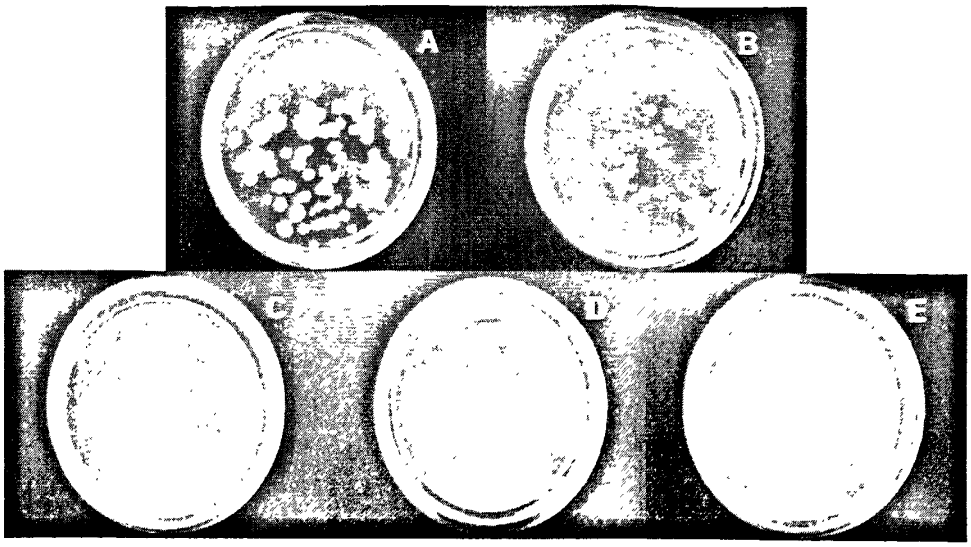


Fig.2

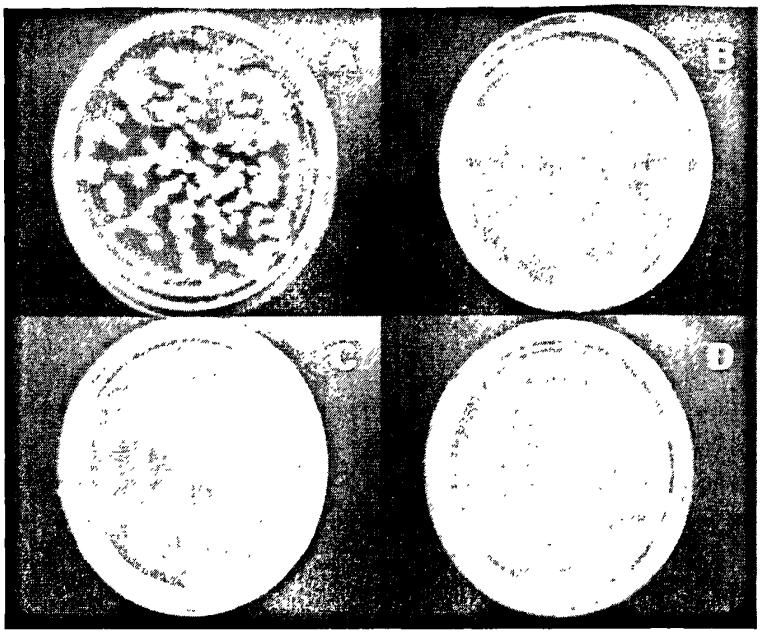


Fig.3

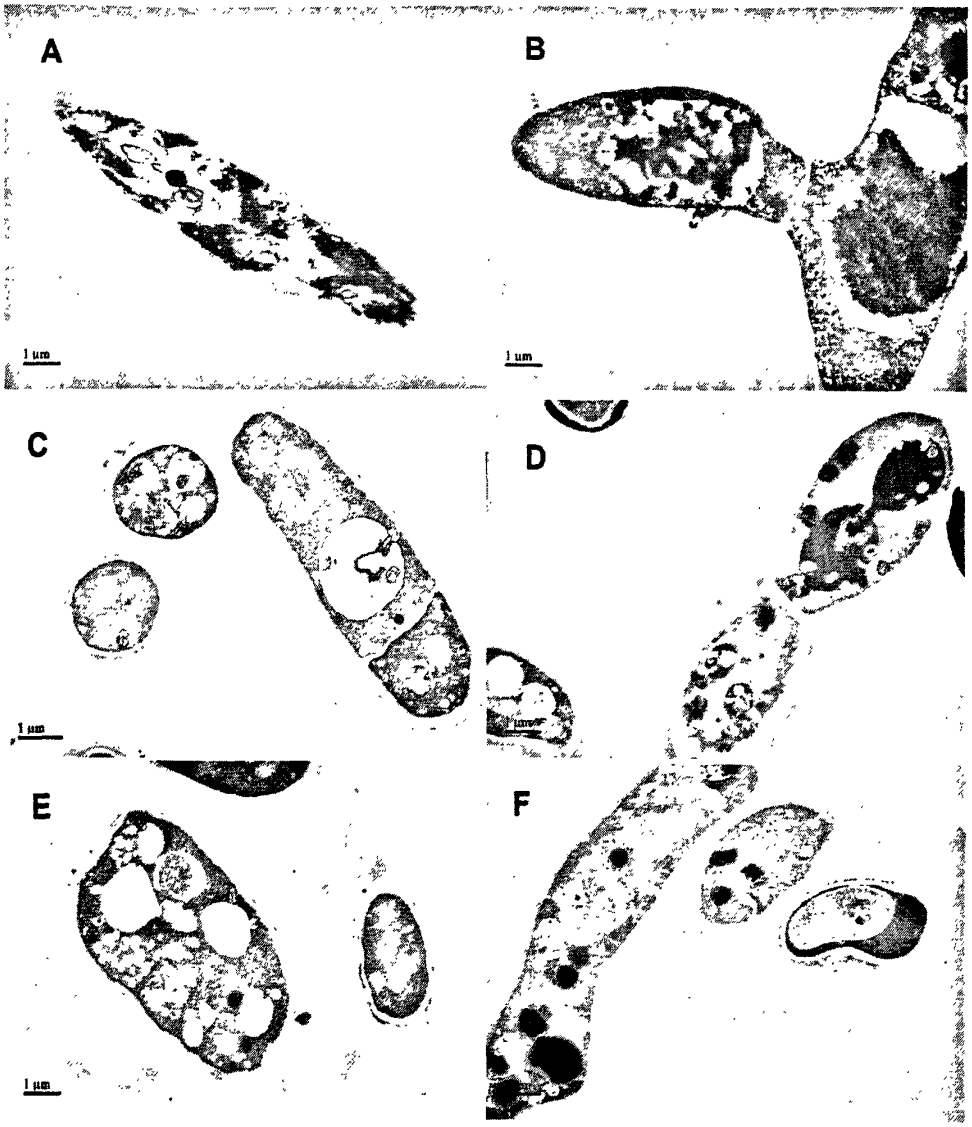


Fig. 4

Table 1 Comparison of the inhibition of acidic sophorolipids with that of lactonic sophorolipids to *Trichophyton rubrum*, *Trichophyton gypseum* and *Microsporum canis* at the seventh day of cultivation

Sophorolipids concentration	*Trichophyton rubrum*	*Trichophyton gypseum*	*Microsporum canis*
Control*	+++++	+++++	+++++
0.1 g/L ASLs	+++++	++++	+++++
0.25 g/L ASLs	+++++	−	+++++
0.5 g/L ASLs	+++	−	+++
DSMO*	+++++	++++	+++++
0.05 g/L LSLs	+++++	++	+
0.1 g/L LSLs	++++	+	−
0.25 g/L LSLs	+++	−	−
0.5 g/L LSLs	++	−	−

Control* was medium without sophorolipids; DSMO* was medium only with 0.25 mL dimethyl sulfoxide; + showed the amount of the colonies; − indicated that no colonies was grown; ASLs was acidic sophorolipids; LSLs was lactonic sophorolipids

Table 2 Inhibition ratio to hypha extension

Sophorolipids concentration	*Trichophyton rubrum*		*Trichophyton gypseum*		*Microsporum canis*	
	Diameter (mm)	Inhibition ratio (%)	Diameter (mm)	Inhibition ratio (%)	Diameter (mm)	Inhibition ratio (%)
Control	26	0.0	24	0.00	22	0.00
0.0625 g/L ASLs	25	3.84	23	4.17	22	0.00
0.125 g/L ASLs	24	7.69	22	8.33	21	4.55
0.25 g/L ASLs	24	7.69	21	12.5	20	9.09
0.50 g/L ASLs	21	19.2	21	12.5	20	9.09
DMSO*	23	11.5	22	8.33	20	9.09
0.0625 g/L LSLs	16	38.5	16	33.3	15	31.8
0.125 g/L LSLs	15	42.3	12	50.0	13	40.9
0.25 g/L LSLs	14	46.2	11	54.1	9	59.1
0.50 g/L LSLs	12	53.8	9	62.5	7	68.2

Control* was medium without sophorolipids; DSMO* was medium only with 0.2 mL dimethyl sulfoxide; ASLs was acidic sophorolipids; LSLs was lactonic sophorolipids

Table 3 Minimum Inhibitive Consistency (MIC) and Minimal Fungicidal Consistency (MFC) of lactonic sophorolipids to three dermatophytes

Dermatophytes	MIC (mg/mL)			MFC(mg/mL)
	MIC range	MIC_{50}	MIC_{90}	
Trichophyton rubrum	0.0625-0.5	0.0625	0.125	0.5
Trichophyton gypseum	0.0625-0.5	0.125	0.25	0.5
Microsporum canis	0.0625-0.5	0.0625	0.125	0.25