การโคลนยีนไคติเนสจาก *Bacillus circulans* เข้าสู่แบคทีเรียที่ผลิตกรดแลคติก

Cloning of Chitinase-Encoding Gene from *Bacillus circulans* into Lactic Acid Bacteria

มณฑล เลิศคณาวนิชกุล (Monthon Lertcanawanichakul)[1]

บทคัดย่อ

ทำการโคลนชิ้นยีนไคติเนสจากแบคทีเรียแกรมบวกชนิด *Bacillus circulans* No. 4.1 เข้าใน *Enterococcus faecalis* JCM8726 และ *Lactococcus lactis* L14 ที่แยกได้จากหมูหมัก ปรากฏว่าไม่มีการแสดงออกของยีนไคติเนสในเชื้อทั้งสองชนิด และยังเกิดปรากฏการณ์ที่ พลาสมิดหลายชนิดไม่สามารถอยู่ร่วมกันได้ในเซลล์เดียวกันที่เรียกว่า plasmid incompatibility ใน *L. lactis* อย่างไรก็ตามการไม่แสดงออกของยีนไคติเนสใน Enterococci ไม่เกี่ยวกับการเกิดตัดขาดของชิ้นยีนเพราะเมื่อได้นำพลาสมิดดังกล่าวที่สกัดจาก Enterococci ไปถ่ายกลับเข้าสู่ *Escherichia coli* DH5α ปรากฏว่ายังคงมีการแสดงออกของยีนไคติเนส ดังเดิม ดังนั้นการแสดงออกของยีนอาจจะขึ้นอยู่กับกลไกควบคุมภายในเซลล์ต่างชนิดกัน เช่น โปรโมเตอร์ ตำแหน่งที่ให้ไรโบโซมมาเกาะ หรือ การเลือกใช้รหัสพันธุกรรมที่ไม่เหมือนกันในแต่ละเชื้อ เป็นต้น

Abstract

A Chitinase gene from the Gram-positive bacterium *Bacillus circulans* No. 4.1 was cloned in *Enterococcus faecalis* JCM8726 and in *Lactococcus lactis* L14, isolated from fermented pork. The chitinase gene was not expressed as an active enzyme in either strain. Moreover, *L. lactis*, containing several native plasmids in their cells, showed incompatibility to the plasmid. The chitinase gene expression in the Enterococci strain was not due to plasmid deletion, because the plasmid isolated from them showed normal chitinase activity when retransformed into *Escherichia coli* DH5α. It might be caused by regulatory processing in different cells, such as promoter, Shine-Dalgano (SD) sequence, codon usage, etc.

คำสำคัญ: ยีนไคติเนส แบคทีเรียที่ผลิตกรดแลคติก

Keywords: chitinase gene, lactic acid bacteria

[1] *ผู้ช่วยศาสตราจารย์ สำนักวิชาสหเวชศาสตร์และสาธารณสุขศาสตร์ มหาวิทยาลัยวลัยลักษณ์, e-mail: lmonthon@wu.ac.th*

Introduction

The chitinases are classified as endochitinase, exochitinase (EC.3.2.1.14), β-*N*-acetylglucosaminase, and chitobiase (EC.3.2.1.30). Endochitinase splits the chitin polymer internally, exochitinase releases chitobiose from one end, β-*N*-acetylglucosaminase releases *N*-acetyl-D glucosamine (NAG) monomers, and chitobiase hydrolyzes chitobiose to NAG (Blaak et al., 1993; Flach et al., 1992). The roles of these chitinases vary depending on their sources. A major role in fungi, cructaceans, and insects is modification of their structural constituent chitin by a binary chitinase system (Fugamizo et al., 1986; Roberts and Selitrennikoff, 1988; Gooday, 1990; Leah et al., 1991). Bacteria produce chitinase to digest chitin, primarily to utilize it as a carbon and energy source. In plants, chitinases are induced in response to attack by phytopathogenic fungi, and they are reported to combat fungal infection in concert with other antifungal polypeptides (Schlumbaum et al., 1986; Kombrink et al., 1988; Gooday, 1990; Roby et al., 1990; Oppenheim and Chet, 1992. Such, chitinases are enzymes with anti-fungal activity (Gooday 1990). Also, during recent years, some lactic acid bacteria (LAB) were reported concerning their anti-fungal activity (Corsetti et al., 1988; Lavermicocca et al., 2000; Strom et al., 2002; Lavermicocca et al., 2003; Lertcanawanichakul, 2005a). Due to the "generally regarded as safe" (GRAS) status of LAB, the interest in using them for biopreservative, disease treatment and prevention as well as health restoration and maintenance will be further elucidated.

Previous study demonstrated that *Bacillus circulans* No. 4.1 could degrade colloidal chitin (insoluble chitin) over a wide range of pHs (Wiwat et al., 1996). Genetic engineering techniques could be used to introduce the chitinase-encoding gene into LAB, in order to inhibit fungal growth. Considerable efforts have been made to improve important characteristics of LAB or to introduce new traits into these organisms. Chitinase LAB producing seemed to be a possible candidate for a biological system to control fungal growth in production or prophylaxis to fungal infection in immunocompromised patients.

Material and Methods

Bacterial strains and plasmids: *Enterococcus faecalis* JCM8726 (bacteriocin non-producing, kindly provided by Professor S. Shioya, Osaka University, Japan) (Losteinkit et al., 2001), *Lactococcus lactis* L14, fermented pork (Nham) isolate with anti-fungal activity (Lertcanawanichakul, 2005a) were grown at 30°C in Mann Rogosa Sharpe (MRS) broth (Oxoid). *Escherichia coli* DH5α was grown at 37°C in Luria-Bertani (LB: 10 g NaCl, 10 g peptone, 5 g yeast extract) broth. All of them were used as cloning hosts for expression of chitinase-encoding gene (*chi*) from *B. circulans*. Whenever appropriate, antibiotics were added to the media as follows: erythromycin (Er) at 5 μg/ml for LAB and at 150 μg/ml for *E. coli*. The solid medium were prepared by addition of 1.5% agar (Difco) to the liquid medium.

All pure cultures of bacteria were kept as stock cultures in 15% glycerol at -70°C.

The *chi* gene (GenBank accession No. AF54827) from *B. circulans* No. 4.1 (pCHIB43) was used in this study. The recombinant plasmid pCHIB43 contained a 2.6-kb insert of the *chi* gene from *B. circulans* No. 4.1. The gene was cloned into *E. coli* by using pBluescript II KS and SK. Both

plasmids could express chitinolytic activity, demonstrating that the chitinase portion contained in pCHIB43 has its own promoter that is active in *E. coli*. The synthesized chitinase has a molecular mass of 65 kDa (Wiwat et al., 2002).

The plasmid vector, pOri253, was constructed from the plasmid pIL253 (5.2 kb) and the 0.89-kb fragment of *oriColEI* from pBluescript II KS. The bifunctional plasmid pOri253 conferred erythromycin resistance in both *E. coli* and *E. faecalis*. (Lertcanawanichakul, 2005b). It was used as cloning vector for expression of chi gene from *B. circulans* in cloning hosts.

Subcloning of the chitinase-encoding gene from recombinant plasmid pCHIB43

A single colony of *E. coli* transformant harboring recombinant plasmid pCHIB43 was inoculated into 5 ml of LB broth, and incubated at 37°C for 24 h on a rotary shaker (150 rpm). Cells were harvested, and recombinant chitinase gene was prepared with a Qiaprep Spin Miniprep Kit (Qiagen, Germany) according to the manufacturer's directions. The recombinant plasmid pCHIB43 was completely double digested with *Bam*HI-*Eco*RI and electrophoresed on 0.7% agarose. The *chi* fragment, 2.5-kb in size, was cut from the gel and extracted with a Qiagen Extraction Kit (Qiagen, Germany) before ligation with pOri253 and transformation into *E. coli* DH5α by means of the heat shock method (Sambrook et al., 1989). The recombinant chitinase gene was extracted as previously described, and subsequently transformed into LAB by means of electroporation (Holo and Nes, 1989). The transformed *E. coli* and LAB cells were spread on LB agar containing 150 μg/ml of Em and MRS agar containing 5 μg/ml of Em, respectively. Erythromycin-resistant colonies were replicated on tryptone-glucose-yeast extract (TGY) agar (Biswas et al., 1991) supplemented with Em as previously described and with and without colloidal chitin (0.3%). The transformants growing on TGY agar were screened by the rapid method with 4-methylumbelliferyl -β-D-N,N'-diacetylchitobioside [4-MU$(GlcNAc)_2$] (Sigma-Aldrich, USA) used as a substrate. Transformants that produced light blue fluorescence around colonies were confirmed for clear zones on colloidal chitin agar plates.

Determination of chitinolytic activity

Rapid method. Chitinolytic activity of transformants was determined by the rapid method of Tronsmo and Harman (1993). Bacterial colonies were grown on TGY agar at 37°C for 2 d. Then the colonies were lysed with chloroform vapor for 15 min before overlaying with 5 ml of 0.7% Bacto agar containing 20 μg/ml of 4-MU$(GlcNAc)_2$ and incubation at 37°C for 15 min. A light blue fluorescence around colonies, as observed by UV transilluminator, indicated chitinolytic activity.

Detection of chitinase activity in colloidal chitin agar plate. Positive chitinase transformants were also confirmed by using colloidal chitin as substrate. Cloning hosts and colonies suspected of carrying the recombinant plasmid were spotted on TGY agar plates containing 0.3% (wt/vol) colloidal chitin (colloidal chitin agar plate) prepared from crab shell chitin (Sigma-Aldrich, USA) as previously described (Wiwat et al., 1996), and were incubated at 37°C for at least 7 d in a sealed petri dish. Chitinolytic activity was indicated by the ability of bacterial colonies to produce hydrolytic clear zones.

Results

The chitinase gene of *B. circulans* was cloned as a 2.6-kb *Hind*III fragment in the multiple cloning site of the high copy number *E. coli* vector pBluescript II KS (Promega), and the resulting plasmid was designated as pCHIB43. The 2.6-kb fragment comprises the coding region of the chitinase (1794 bp) and expressed chitinase by itself promoter. As promoters have elements that so-called −35 and −10 boxes of *E. coli* transcription initiation signals (Guchte et al., 1992), the chitinase with its native upstream region was subcloned directly into the plasmid vector pOri253 (This study). The 2.5-kb chitinase fragment was inserted as *Bam*HI-*Eco*RI fragment in pOri253 and initially transformed to *E. coli* giving rise to pOrichi. Its restriction enzyme map is shown in Figures 1 & 2. Transformed *E. coli* cells were found to be positive for chitinase activity with both the 4-MU $(GlcNAc)_2$ and colloidal chitin agar assay (Figure 3).

The recombinant plasmid pOrichi was extracted from transformed *E. coli* cells and then electroporated into LAB. Screening of LAB erythromycin-resistant transformants were done by restriction enzyme analysis of plasmid DNA. The recombinant pOrichi constructs were only transformed to *E. faecalis*, but were not able to transform any of the *L. lactis* fermented pork isolates. However, no chitinase activity was detected in transformed *E. faecalis* cells with both methods as described in Materials and Methods (Figure 3). To examine whether this was due to a mutation introduced in pOrichi after the plasmid was transferred to *E. faecalis*, pOrichi isolated from *E. faecalis* was restransformed to *E. coli*. Extracts of *E. coli* harboring pOrichi, retrived from *E. faecalis* showed normal chitinase activity, indicating that no mutation had occurred in the construct (Figure 2).

Discussion

In this work, the LAB harboring the chitinase-encoding gene from the *B. circulans* could not produce chitinase. The recombinant plasmid pOrichi, comprising pIL253 and chitinase gene from *B. circulans*, was not able to transform any of the *L. lactis* fermented pork isolates, although the broad-host range vector pIL253 has been used in an expression study with other *L. plantarum* strains (Jones and Warner, 1990). Lelie et al. (1988) showed that incompatibility of an introduced vector and a resident plasmid generally correlated with a low transformation efficiency. As the tested fermented pork strain (*L. lactis*) may be contained several plasmids (Brurberg et al. 1994), lack of transformability by recombinant chitinase gene pOrichi could be caused by plasmid incompatibility. In contrast, the *E. faecalis* JCM8726 did not contain resident plasmids (Losteinkit et al., 2001), so they could be transformed by pOrichi. However, transformant *E. faecalis* did not express the chitinase gene.

The 2.5-kb *Bam*HI-*Eco*RI chitinase fragment is a deletion derivative of the chitinase gene (2.6-kb) from *B. circulans* No. 4.1 (Wiwat et al., 2002). As there is no apparent transcriptional terminator present behind the 65 kDa replication protein gene, the chitinase activity in *E. faecalis* could be explained by a transcriptional read-through from the replication protein gene. A read-through transcript could affect chitinase expression negatively in pOrichi, by inhibition of the chitinase transcript (Brurberg et al., 1994). However, transformant *E. coli* harboring the same plasmid still expressed the chitinase gene. We did not study in detail the factors that limit expression in the LAB as compared to *E. coli*. Alternatively, one might expect that the use of an SD sequence

from heterologous gene can limit initiation of translation in LAB, but not in *E. coli.* Moreover, sequences located outside the SD region may also have an effect on the efficiency of translation intitiation. Another limiting factor for expression factor for expression of the chitinase in LAB may be the codon usage (Guchte et al., 1992). Interestingly, the genetic manipulation of LAB has many potential applications in food safety and/or health maintenance, but still requires the development of host/vector systems which allow homologous and heterologous genes to be transferred and expressed.

Conclusions

The present results illustrated the power of the recently developed genetic tools for manipulation of LAB, although in this work it cannot express the heterologous gene. At least the plasmid vector, pOri253, was stably maintained in *Enterococci*. However, the differences of LAB, with respect to the functional expression of a heterologous gene, indicate that establishment of a specific genetic systems for LAB may be necessary in some cases.

Acknowledgements

This work was Partly supported by the National Research Council of Thailand Grant No. B-07-1/2547.

References

Biswas, S.R., Ray, P., Johnson, M.C. and Ray, B. 1991. Influence of growth conditions on the production of a bacteriocin, pediocin AcH, by *Pediococcus acidilactici* H. **Appl. Environ. Microbiol**. 57: 1265-1267.

Blaak, H., Schnellmann, J., Walter, S., Henrissat, S. and Schrempf, H. 1993. Characteristics of an exochitinase from *Streptomyces olivaceoviridis*, its corresponding gene, putative protein domains and relationship to other chitinases. **Eur. J. Biochem.** 214: 659-669.

Brurberg, M.B., Haandrikman, A.J., Leenhouts, K.J., Venema, G. and Nes, I.F. 1994. Expression of a chitinase gene from *Serratia marcescens* in *Lactococcus lactis* and *Lactobacillus plantarum*. **Appl. Microbiol. Biotechnol**. 42: 108-115.

Corsetti, A., Gobbetti, M., Rossi, J. and Damiani, P. 1998. Antimould activity of sourdough lactic acid bacteria: identification of a mixture of organic acids produced by *Lactobacillus sanfrancisco* CB1. **Appl. Microbiol. Biotechnol.** 50: 253-256.

Flach, J., Pilet, P.E. and Jolles, P. 1992. What's new in chitinase research?. **Experimentia** 48: 701-716.

Fugamizo, Y., Kramer, K.L., Mueller, D.D., Schaefer, J., Garbow, J. and Jacob, G.S. 1986. Analysis of chitin structure by nuclear magnetic resonance spectroscopy and chitinolytic enzyme digestion. **Arch. Biochem. Biophys.** 249: 15-26.

Gooday, G.W. 1990. The ecology of chitin degradation. **Adv. Microbiol. Ecol.** 11: 387-430.

Guchte, M. van de, Kok, J. and Venema, G. 1992. Gene expression in *Lactococcus lactis*. **FEMS Microbiol. Rev.** 88: 73-92.

Holo, H. and Nes, I.F. 1989. High efficiency transformation by electroporation of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. **Appl. Environ. Microbiol.** 55: 3119-3123.

Jones, S. and Warner, P.J. 1990. Cloning and expression of alpha amylase from *Bacillus amyloliquefaciens* in a stable plasmid vector in *Lactococcus plantarum*. **Lett. Appl. Microbiol.** 11: 214-219.

Kombrink, E., Schroder, M. and Hahlbrock, K. 1988. Several "pathogenesis-related" proteins in potato are 1,3-β-glucanases and chitinases. **Proc. Natl. Acad. Sci. USA.** 85: 782-786.

Lavermicocca, P., Valerio, F., Evidente, A., Lazzaroni, S., Corsetti, A. and Cobbetti, M. 2000. Purification and characterization of novel antifungal compounds from the sourdough *Lactobacillus plantarum* strain 21B. **Appl. Environ. Microbiol.** 66: 4084-4090.

Lavermicocca, P., Valerio, F. and Visconti, A. 2003. Antifungal activity of phenyllactic acid against molds isolated from bakery products. **Appl. Environ. Microbiol.** 69: 634-640.

Leah, R., Tommerup, H., Svendsen, I. and Mundy, J. 1991. Biochemical and molecular characterization of three barley seed proteins with antifungal properties. **J. Biol. Chem.** 266: 1564-1573.

Lelie, D. van der, Vossen, J.M.B.M. van der and Venema, G. 1988. Effect of plasmid incompatibility on DNA transfer to *Streptococcus cremoris*. **Appl. Env. Microbiol.** 54: 865-871.

Lertcanawanichakul, M. 2005a. Isolation and selection of anti-*Candida albicans* producing lactic acid bacteria. **Walailak J. Sci & Tech.** 2: 179-187.

Lertcanawanichakul, M. 2005b. Construction of plasmid vector for expression of Bacteriocin N15-encoding gene and effect of engineered bacteria on *Enterococcus faecalis*. **Walailak J. Sci & Tech.** 2: 189-199.

Losteinkit, C., Uchiyama, K., Ochi, S., Takakoya, T., Nagahisa, K. and Shioya, S. 2001. Characterization of Bacteriocin N15 produced by *Enterococcus faecium* N15 and cloning of the related genes. **J. Biosci. Bioeng.** 91: 390-395.

Oppenheim, A.B. and Chet, I. 1992. Cloned chitinase in fungal plant pathogen control strategies. **Trends Biotechnol.** 10: 392-394.

Roberts, W.K. and Selitrennikoff, C.P. 1988. Plant and bacterial chitinases differ in antifungal activity. **J. Gen. Microbiol.** 134: 169-176.

Roby, D., Broglie, K., Cressman, R., Biddle, P., Chet, I. and Broglie, R. 1990. Activation of bean chitinase promoter in transgenic tobacco plants by phytopathogenic fungi. **Plant Cell** 2: 999-1007.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. **Molecular cloning. A laboratory manual.** 2nd ed., New York: Cold Spring Harbor Laboratory Press.

Schlumbaum, A., Mauch, F., Vogeli, U. and Baller, T. 1986. Plant chitinases are potent inhibitors of fungal growth. **Nature** 324: 365-367.

Strom, K., Sjogren, J., Broberg, A. and Schnurer, J. 2002. *Lactobacillus plantarum* MiLAB 393 produces the antifungal cyclic dipeptides cyclo (L-phe-L-pro) and cyclo (L-phe-trans-4-OH-L-pro) and 3-phenyllatic acid. **Appl. Environ. Microbiol.** 68: 4322-4327.

Tronsmo, A. and Harman, G.E. 1993. Detection and quantitation of *N*-acetyl- -D-glucosaminidase, chitobiosidase, and endochitinase in solutions and on gels. **Anal. Biochem.** 208: 74-79.

Wiwat, C., Lertcanawanichakul, M., Siwayaprahm, P., Pantuwatana, S. and Bhumiratana, A. 1996. Expression of chitinase-encoding genes from Aeromonas hydrophila and *Pseudomonas maltophilia* in *Bacillus thuringiensis* subsp. *Israelensis*. **Gene** 197: 119-126.

Wiwat, C., Thepouyporn, A., Siwayaprahm, P. and Bhumiratana, A. 2002. Cloning, sequencing, and expression of a chitinase-encoding gene from *Bacillus circulans* No. 4.1. **Curr. Microbiol.** 44: 167-172.

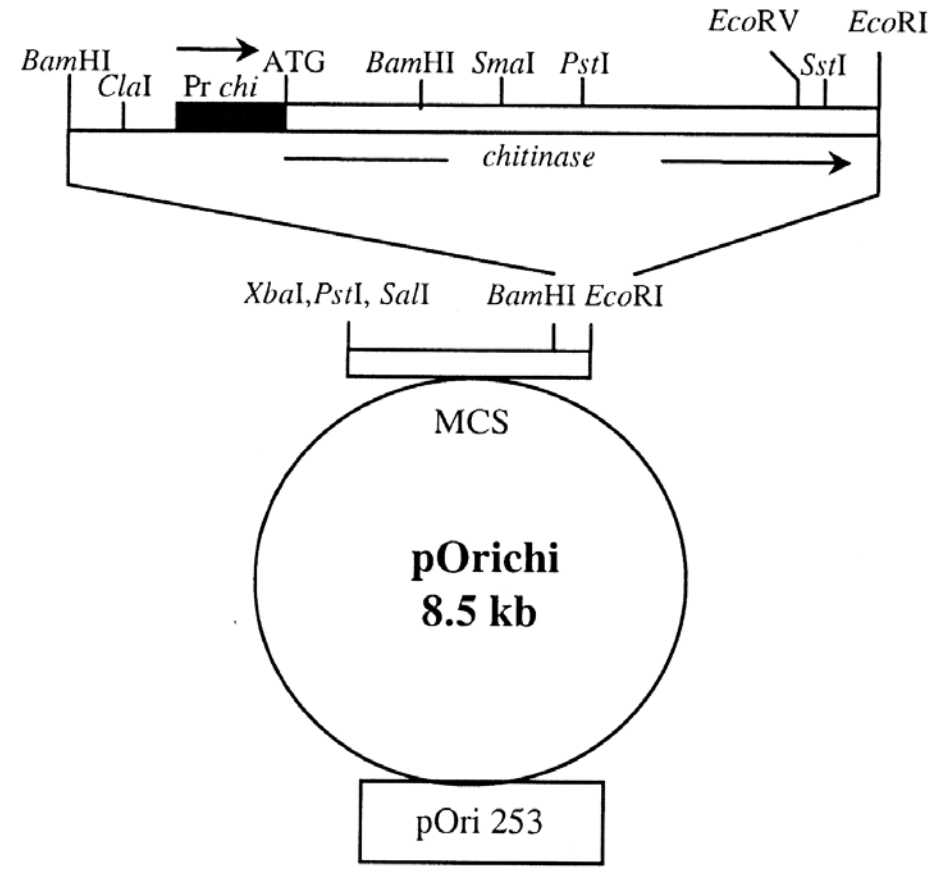

Figure 1. Physical map of plasmid pOrichi and expanded restriction map of the 2.5 kb *Bam*HI/*Eco*RI DNA fragment (chitinase gene) from recombinant plasmid pCHIB43. Pr *chi*, promoter of chitinase gene. Only restriction sites relevant for this work are shown.

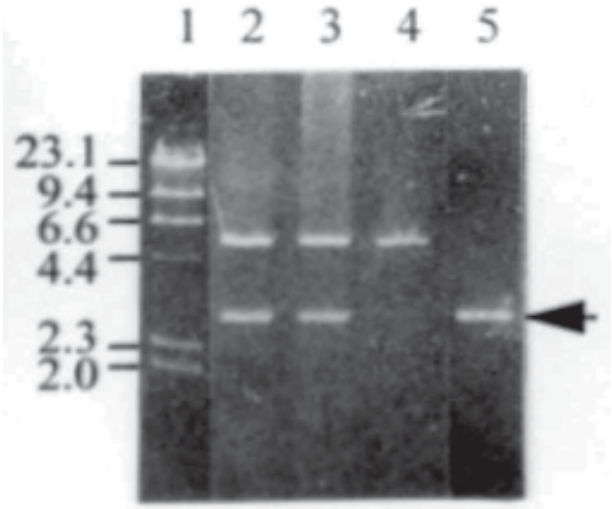

Figure 2. Electrophoretic pattern of recombinant chitinase gene pOrichi from *E. coli* DH5α and *E. faecalis* JCM 8726. Lane 1, λDNA was digested with *Hind*III; lane 2, pOrichi from *E. coli* digested with *Bam*HI+*Eco*RI; lane 3, pOrichi from retransformed *E. coli* digested with *Bam*HI+*Eco*RI; lane 4, pOri253 digested with *Bam*HI+*Eco*RI; lane 5, *Bam*HI/*Eco*RI-digested chitinase gene fragment from pCHIB43. The arrow head indicates the chitinase gene. The standard marker is indicated at the left hand as kilobase (kb)- insize in lane 1.

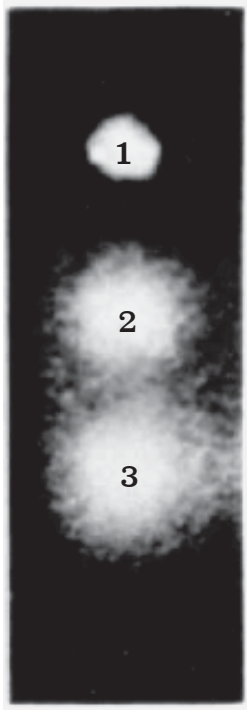

Figure 3. Chitinase activity from transformed *E. coli* and *E. faecalis* cells, using 4-MU$(GlcNAc)_2$ as substrate. Light blue fluorescence could be seen around the colony of *E. coli* harboring pOrichi (2) and retransformed *E. coli* harboring the same plasmid (3), but could not be seen around the colony of transformats *E. faecalis* (1).