ノート

ドライアイスを用いた食卓用保冷器による魚介類の腸炎ビブリオ増殖抑制

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丸山弓美[*1] 木村 凡[*1,†] 藤井建夫[*1]
徳永宜則[*2] 松林 潤[*2] 相川保史[*2]

Growth Inhibition of *Vibrio parahaemolyticus* in Seafood by Tabletop Dry Ice Cooler

Yumi MARUYAMA[*1], Bon KIMURA[*1,†], Tateo FUJII[*1], Yoshinori TOKUNAGA[*2], Megumi MATSUBAYASHI[*2] and Yasushi AIKAWA[*2]

([*1]Tokyo University of Marine Science and Technology: 4-5-7, Konan, Minato-ku, Tokyo 108-8477, Japan; [*2]Niitaka: 1-8-10, Niitaka, Yodogawa-ku, Osaka 532-8560, Japan; [†] Corresponding author)

Tabletop dry ice coolers (three types; dome model, cap model and tripod model), which are used in kitchens and hotel banquet halls to refrigerate fresh seafood, were investigated to determine whether growth of *Vibrio parahaemolyticus* was inhibited by their use. On TSA plates containing 1.8% NaCl and fresh seafood (fillets of squid, pink shrimp and yellowtail), *V. parahaemolyticus* (O3 : K6, TDH−) inoculated at 4 to 5 log CFU/sample and left at ambient temperature (25℃) grew by 1.0 to 2.8 orders in 4 hours. In contrast, with tabletop coolers no significant increase in viable count occurred in 3 to 4 hours, confirming that tabletop coolers inhibited the growth of *V. parahaemolyticus*. The temperature in each tabletop cooler was kept below 10℃ for 80 to 135 min, though the CO_2 gas concentration in them remained high for only a short time (0 to 75 min). It was presumed that the refrigeration function mainly contributed to growth inhibition. Our results indicate that tabletop dry ice coolers are helpful for prevention of food-borne disease due to *V. parahaemolyticus* in food-service locations, such as kitchens and banquet halls.

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Key words: 生鮮魚介類 fresh seafood; 腸炎ビブリオ *Vibrio parahaemolyticus*; 食卓用ドライアイス保冷器 tabletop dry ice cooler; 保冷器 tabletop cooler; ドライアイス dry ice; 冷却 refrigeration; 二酸化炭素ガス CO_2 gas

緒 言

旅館やホテルなどの外食産業において，魚介類を介した腸炎ビブリオ食中毒の発生事例が多くみられている[1]．腸炎ビブリオ (*Vibrio parahaemolyticus*) は中温性で，10℃以下の環境下ではほとんど増殖しないが，37℃の至適温度条件下での発育世代時間が約8分と，短時間で活発に増殖する[2]．そのため，原料汚染とともに温度管理の不備が食中毒発生要因として指摘されている．腸炎ビブリオ食中毒を防止するため，寿司および刺身などの魚介類調理品について冷蔵保存下を出てから可能な限り速やか（最大2時間以内）に消費するよう提言されている[3]．しかし，旅館やホテルでは調理から消費されるまでに要する時間が長く，室温放置されてしまうことがある．

本研究では，生鮮魚介類の衛生管理をより徹底する手段として食卓用ドライアイス装置（保冷器）の *V. parahaemolyticus* の増殖抑制効果について検討した．保冷器は，皿に盛られた生鮮魚介類をドライアイスを利用して冷やし，食感を保持する装置として，一部の旅館の調理場において大型冷蔵機器の代わりに，また宴会場においても喫食が遅れた場合に備えて使われている．本装置は，生鮮魚介類の保冷によって本菌の増殖を制御することが期待された．また，ガス置換包装による食品中の微生物制御に関する研究分野において，低温貯蔵に CO_2 置換包装を併用すると，多くの細菌に対して増殖抑制効果が高まることが認められていることから[4)-6)]，本装置のドライアイスから発生する CO_2 ガスが本菌に対して何らかの影響を与えている可能性も考えられた．そこで本研究では，食卓用ドライアイス装置の使用によって，生鮮魚介類に付着した *V. parahaemolyticus* の増殖抑制が可能であるか調査し，さ

† 連絡先
*1 東京海洋大学：〒108-8477 東京都港区港南4-5-7
*2 (株)ニイタカ：〒532-8560 大阪市淀川区新高1-8-10

らにそのメカニズムを解明するため，冷却機能と CO_2 ガス保持機能について調査した．

実験方法

1. 供試菌株

東京都立衛生研究所（現，東京都健康安全研究センター）から分与された食中毒患者由来である *V. parahaemolyticus* V02-36（O3：K6, TDH 陽性株）を使用した．

2. 魚介類試料

生食用のイカ（アカイカ），アマエビおよびハマチを東京都内の量販店で購入し，無菌的に約 10 g のフィレー状に切り，滅菌シャーレに入れて −20℃で凍結保存した．実験に供する際は，5℃下に約 2 時間貯蔵し，さらに室温 25℃下に約 20 分間放置して解凍した．これらの試料は，TCBS 寒天培地（栄研）にて緑色のコロニーを形成する *Vibrio* 属菌が検出されないことを確認した上で使用した．

3. 菌液の調整

NaCl 3.0%を含む Trypticase Soy Broth (TSBN) 培地 (BBL) にて 30℃，一晩培養した供試菌の前培養液（約 9 log CFU/mL）を新しい TSBN 培地を用いて段階希釈し，さらに 30℃，2 時間培養して得られた対数増殖器の培養液（5〜6 log CFU/mL）を実験に供した．

4. 保冷器内の温度と CO_2 ガス濃度の測定

室温 25℃下において，3 種類の保冷器［ドーム型，帽子型および三脚型保冷器（ニイタカ；Fig. 1）］を発泡スチロール製の板上に静置した後，ドライアイス充てん用容器にペレットドライアイスをドーム型および帽子型では 53±1 g，三脚型では 30±1 g 充てんし，各保冷器の上部に設置した．

その直後から経時的に保冷器内の中心（底部から約 3 cm）の温度（5 分ごと）および CO_2 ガス濃度（15 分ごと）を調べた．温度については，TM-150 型食品用デジタル温度計（アズワン）を用い，測定部位に温度センサーを固定して測定した．また CO_2 ガス濃度については，1.0 mL 容シリンジにより測定部位から捕集したガスに対して，G-5000A 型ガスクロマトグラフ（日立）および D-2500 型クロマトデータ処理装置（日立）を用いて測定した．

5. TSAN 平板培地における *V. parahaemolyticus* の挙動

保冷器内での本菌の挙動を調べるために，魚介類試料のモデル系として，NaCl 1.8% を含む Trypticase Soy Agar (TSAN) 平板培地 (BBL) を用いた．TSAN 平板培地に対数増殖期の供試菌液（5〜6 log CFU/mL）を 100 μL 接種し，塗抹した．4. と同様に室温 25℃で各保冷器にド

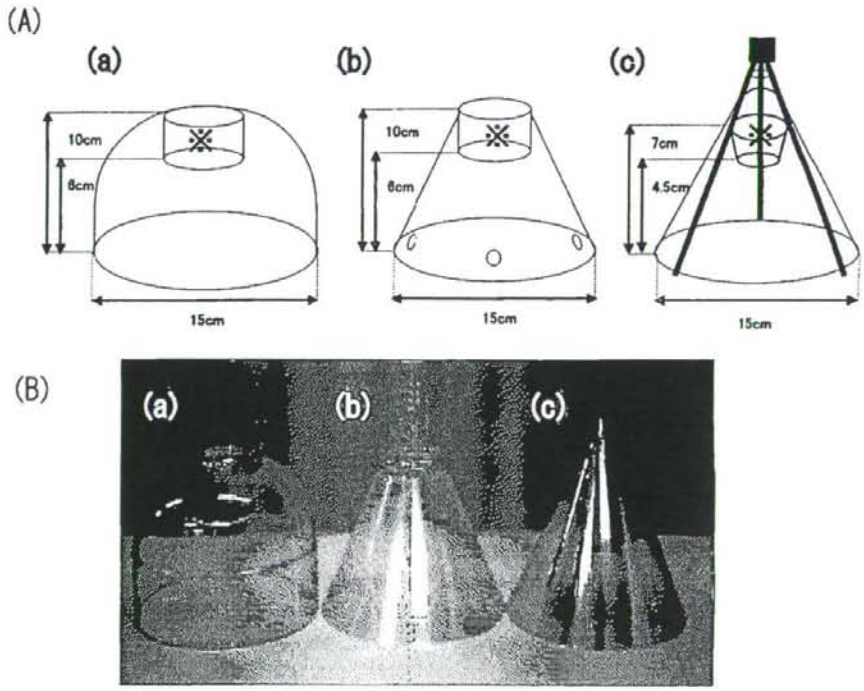

Fig. 1. Illustrations (A) and photographs (B) of tabletop dry ice coolers
(a), dome model; (b), cap model; (c), tripod model
The marks ✣ indicate the places where dry ice is placed.

ライアイスを充てん後，保冷器内の中心に TSAN 平板培地をシャーレのふたを開けた状態で一定時間（0, 2, 3, 4 時間）放置した．また接種後の TSAN 平板培地を室温 25℃ 下に保冷器を用いずに同時間放置したものを対照区とした．処理後は TSAN 培地を回収し，5 倍量の希釈水を加えホモジナイズした後，段階希釈し TCBS 寒天平板に塗抹して，25℃ で 18～24 時間培養後，生菌数を計測した．以上の実験を 3 回行い，生菌数の平均値を表した．

6. 生鮮魚介類における *V. parahaemolyticus* の挙動

TSAN 培地平板と同様に，魚介類試料に対し本菌を接種し，各保冷器内に一定時間（0, 2, 3, 4 時間）放置後，TCBS 寒天平板を用いて生菌数を計測した．予備実験では，ハマチフィレーに接種した本菌が 25℃ 保存下，4 時間後に約 0.5 オーダーのみの増加にとどまったため (Fig. 3(D))，ハマチを試料として用いた場合については，増殖が促進された 4～8 時間後の間において保冷器の効果を調査した．菌液接種後，25℃ に 4 時間放置したハマチに対して，各保冷器内に一定時間（0, 2, 3, 4 時間）放置して，TCBS 寒天平板を用いて生菌数を計測した．以上の実験を 3 回行い，生菌数の平均値を表した．

結果および考察

1. 保冷器内の温度と CO_2 ガス濃度の測定

本装置の冷却機能と CO_2 ガス保持機能を調べるために，保冷器内部の中心温度および CO_2 ガス濃度を経時的に測定した．その結果，ドーム型および帽子型保冷器では，三脚型保冷器と比較して，ドライアイスの昇華が緩慢であり，10℃以下および 40%以上の高 CO_2 ガス濃度の保持時間が長かった．すなわちドーム型および帽子型保冷器内において，使用開始から 115～135 分間，10℃以下に保持され，160～175 分後にすべてのドライアイスが昇華し，その後 30 分以内に室温 25℃に戻った．一方，三脚型保冷器内において，10℃以下の保持時間は約 80 分間にとどまり，約 90 分後にすべて昇華し，その後 30 分以内に室温に戻った (Fig. 2(A))．また CO_2 ガス濃度についても，ドーム型および帽子型保冷器では 60～75 分間ほど 40% 以上に保持されたが，三脚型保冷器では使用開始後に約 33%にいったん上昇した後，速やかに室内と同じ濃度に戻った (Fig. 2(B))．このように，低温や CO_2 ガスの保持しやすさに差がみられたのは，ドーム型および帽子型では，ドライアイス充てん量が 53±1 g であり，装置の構造上密閉性が比較的高く，また三脚型では充てん量 30±1 g であり，密閉性が低かったことが要因として考えられた．

使用開始後 0℃以下の低温に保持された時間については，ドーム型および帽子型では 25 分間であったが，三脚型では使用開始後－22℃以下の大幅な温度低下が起きたため，60 分間と比較的長かった．しかし，三脚型では，CO_2 ガス濃度が他の保冷器よりも常に低かった．これは装置下部が，他の保冷器よりも大きく開いており，装置内での対流が起こりにくいため，CO_2 ガスが装置外に排出され，ドライアイスから発生した新しい冷気が常に供給されていることが考えられた．このような理由から，温度の低下と CO_2 ガス濃度の上昇は一致しないと考えられた．

また保冷器使用による生鮮魚介類（ハマチフィレー）の内部温度を測定したところ，0℃以下に達することはなく，生鮮魚介類が凍結することはなかった（非提示）．

2. TSAN 平板培地における *V. parahaemolyticus* の挙動

魚介類試料のモデルとして用いた TSAN 平板培地に対して，対数増殖期の *V. parahaemolyticus* V02-36 を 4～5 log CFU/平板接種後，室温 25℃下に放置した場合，本菌は活発に増殖し，4 時間後に約 2.8 オーダー増加した．しかし，ドーム型および帽子型保冷器を使用した場合では，本菌は 3 時間後に約 1.4, 0.5 オーダーそれぞれ減少し，4 時間後まで著しい菌数変化はみられなかった．また

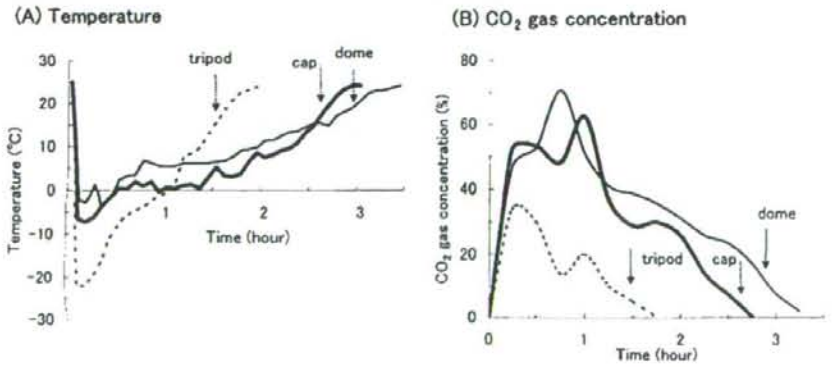

Fig. 2. Temperature (A) and CO_2 gas concentration (B) in tabletop dry ice coolers in locations at room temperature (25℃)
Thin line, dome model tabletop cooler; thick line, cap model tabletop cooler; thin dotted line, tripod model tabletop cooler

Arrows indicate the time when all the dry ice had completely sublimed.

三脚型保冷器では，2時間後にいったん約1.0オーダー減少し，4時間後に約0.3オーダー増加したが，初期の菌数と差がみられなかった(Fig. 3(A))．これより，いずれの保冷器においても，4時間の増殖抑制効果が確認された．

3. 生鮮魚介類における *V. parahaemolyticus* の挙動

TSAN平板培地において本菌の増殖抑制効果が確認されたため，TSAN平板培地を用いた実験と同様に，実際に魚介類試料（生食用のイカ，アマエビおよびハマチの各フィレー）に本菌を接種し，保冷器による増殖抑制効果を調べた．イカフィレーに接種して，室温25℃下に放置した場合，本菌は4時間後に約1.0オーダー増加したが，3種類の保冷器を用いた場合，著しい菌数増加はみられず，4時間の増殖抑制効果が確認された(Fig. 3(B))．

アマエビフィレーにおいて，開放下の場合，本菌は4時間後に約2.1オーダー増加した．ドーム型，帽子型および三脚型保冷器を使用した場合，3時間後まで著しい菌数増加はみられなかったが，4時間後にそれぞれ約0.4, 0.4, 0.9オーダー増加したことから，3時間までの増殖抑制効果が確認された(Fig. 3(C))．予備実験では，ハマチフィレーに接種した本菌の増殖が25℃保存下で4時間後まで緩慢であり，4～8時間後の間において促進されたことから，本試験では接種後4時間放置したものに対して保冷器の効果を調査した．その結果ハマチフィレーにおいて室温25℃下の場合は，接種4時間後と比較して，6～8時間後に0.8～1.0オーダー増加したが，3種類の保冷器を使用した場合，6～8時間後に－0.8～0.3オーダーと著しい菌数増加がみられなかったことから，保冷器未使用の25℃，4時間保存下を含めて，8時間の増殖抑制効果が確認された(Fig. 3(D))．

魚介類試料のうち，本菌の増殖が25℃下で活発であったアマエビフィレーにおいては，保冷器の増殖抑制効果が3時間と比較的短かった．このように本菌が増殖しやすい魚介類では，保冷器内のドライアイスの昇華後，速やかに菌数増加するので注意が必要である．

本研究において，保冷器内における本菌の減少がTSAN培地上およびハマチフィレー上でみられたが，イカおよびアマエビフィレー上では増殖抑制にとどまった(Fig. 3)．これはTSAN培地平板では本菌が平板表面上に残り，本装置の低温の影響を受けやすかったことに対して，魚介類試料では表面構造が複雑で，組織が脆弱であることから，本菌がその組織内に入り込み，低温にさらされにくかったことが推測された．またハマチフィレーのpH

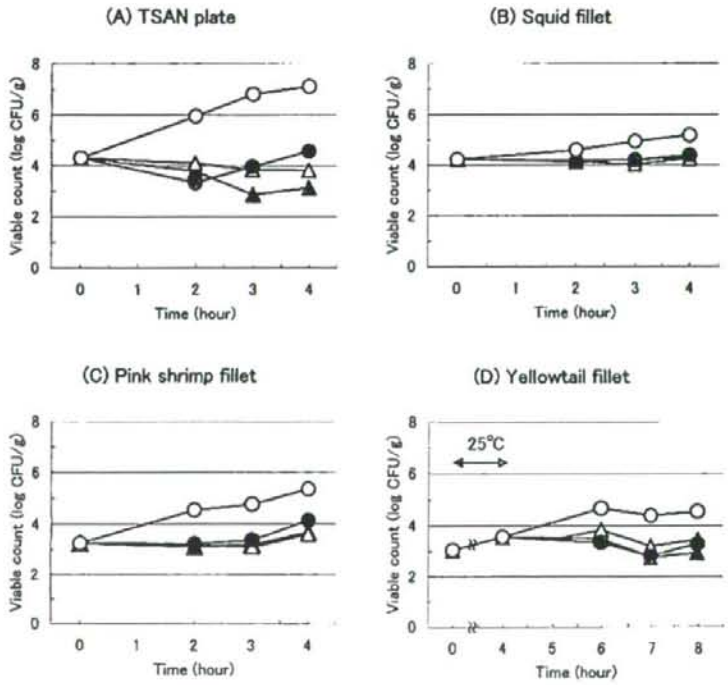

Fig. 3. Inhibition of growth of *V. parahaemolyticus* V02-36 inoculated on TSAN plate (A), squid fillet (B), pink shrimp fillet (C) and yellowtail fillet (D) in tabletop dry ice coolers in locations at room temperature (25℃)

▲, dome model tabletop cooler; △, cap model tabletop cooler; ●, tripod model tabletop cooler; ○, opened (25℃)
The arrow in (D) indicates a period at room temperature (25℃) when the dry ice coolers were not used.
These data are presented as mean of three experiments.

はpH 6付近と低く，本菌の増殖環境として不適であり，その条件下で本装置の低温の影響を受けたため死滅したと推測された．

3種類の保冷器の増殖抑制効果の強さにはわずかに差が見られるものの，効果の持続時間はほぼ同じであった．最初低温およびCO_2ガスが増殖抑制作用を示すと期待されたが，保冷器内における40%以上の高CO_2濃度の保持時間は0～75分間と短かった．これより，本菌に対する保冷器内のCO_2ガスの影響はごく小さく，保冷器の増殖抑制効果は主に低温によるものと示唆された．

ま と め

ドライアイスを用いた保冷器を使用することにより，魚介類に付着した*V. parahaemolyticus*に対して室温(25℃)下でも3～4時間の増殖抑制効果があることが示された．各保冷器内の温度は10℃以下に80～135分間保持されていたが，高CO_2濃度の保持時間が短かったことから(0～75分間)，増殖抑制効果は主に冷却機能によるものであったと思われた．旅館，ホテルや飲食店などの調理場や宴会場において，刺身やたたきなどの生鮮魚介類の調理から消費までの時間，長時間の室温放置を防止する手段として利用することは，食品の品質保持のみならず，腸炎ビブリオ食中毒防止に役立つであろう．

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調査・資料

各種温度条件下における微生物増殖予測プログラムの開発

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藤川 浩*1,† 矢野一好*1 諸角 聖*1
木村 凡*2 藤井建夫*2

Development of a Predictive Program for Microbial Growth under Various Temperature Conditions

Hiroshi FUJIKAWA*1, Kazuyoshi YANO*1, Satoshi MOROZUMI*1, Bon KIMURA*2 and Tateo FUJII*2

(*1Tokyo Metropolitan Institute of Public Health: 3-24-1, Hyakunin-cho, Shinjuku-ku, Tokyo 169-0073, Jpan; *2Tokyo University of Marine Science and Technology: 4-5-7, Konan, Minato-ku, Tokyo 108-8477, Jpan; †Corresponding author)

A predictive program for microbial growth under various temperature conditions was developed with a mathematical model. The model was a new logistic model recently developed by us. The program predicts *Escherichia coli* growth in broth, *Staphylococcus aureus* growth and its enterotoxin production in milk, and *Vibrio parahaemolyticus* growth in broth at various temperature patterns. The program, which was built with Microsoft Excel (Visual Basic Application), is user-friendly; users can easily input the temperature history of a test food and obtain the prediction instantly on the computer screen. The predicted growth and toxin production can be important indices to determine whether a food is microbiologically safe or not. This program should be a useful tool to confirm the microbial safety of commercial foods.

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Key words: コンピュータプログラム computer program; 予測微生物学 predictive microbiology; 増殖モデル growth model; ロジスティックモデル logistic model; 変動温度 varying temperature

緒 言

微生物による食中毒事件および食品腐敗の発生を抑制するための手段は数多くあり，その代表的なものとしては温度，酸素濃度，水素イオン濃度，水分活性およびそれに関与する塩分・糖濃度，保存料濃度などがある．しかし，その大部分の要因はその食品自体に固有な値であり，製造・包装後の変動は少ないと考えられる．製造後変動が可能であり，しかも最も微生物増殖に大きな影響を与える環境要因として温度が挙げられる．しかも，食品への各種添加物の使用が安全性の面から控えられている現在，微生物制御を行う最も有効な手段の1つは温度管理である．したがって，食品をいかに適切な温度で製造し，流通させるかは食品の安全性を確保する上で非常に重要である．

一方，製造および流通過程で対象食品が受けた温度履歴から，それを汚染する有害微生物の増殖を予測できれば，その予測結果から対象食品の微生物学的安全性に対する客観的な判断ができる．これまで国際的にいくつかの増殖モデルが発表され，なかでもゴンペルツモデルとバラニーモデルはよく知られている[1,2]．特にバラニーモデルは最近多くの研究者によって使われているが，変動温度下での増殖にはうまく対応できない場合がある[3]．一方，ゴンペルツモデルは数学的に変動温度には適用が困難である．

国際的にはコンピュータ上の微生物増殖プログラムとして，アメリカ農務省のPathogen Modeling Program (http://ars.usda.gov/service/docs.htm?docid=6786) がよく知られ，入力したデータに対して瞬時に増殖予測結果を得ることができる．しかし，このプログラムはゴンペルツモデルなどを増殖モデルとして使っているため，変動温度には適用できない．

最近，私たちはロジスティックモデルを基本にしたモデルを新たに開発し，このモデルを新ロジスティックモデルと名づけた[3]．このモデルは大腸菌，サルモネラ，黄色ブド

† 連絡先
*1 東京都健康安全研究センター：〒169-0073 東京都新宿区百人町3-24-1
*2 東京海洋大学：〒108-8477 東京都港区港南4-5-7

ウ球菌など各種の微生物の増殖挙動を高い精度で表すことができた[1~5]．しかも，本モデルは変動温度下の増殖を高い精度で予測することが示された[4,6,8]．

今回，この新ロジスティックモデルを用いて，これまで発表された微生物増殖データを基に，各種の温度条件下での微生物増殖を予測するコンピュータプログラムの開発を行った．このプログラムは食品の生産・加工・流通および食品衛生行政の関係者，研究者あるいは一般消費者を対象として作られている．そのため，ユーザーに対してできる限り易しく，使いやすいように設計を心がけた．すなわち，増殖モデルに関する知識を必要とせずに簡単に必要なデータを入力でき，瞬時に対象微生物の増殖予測がコンピュータ画面上に表示されるようにした．

2. 開発方法

2.1 増殖予測モデル

増殖予測プログラムに用いる増殖モデルは私たちが最近開発したモデルを用いた．このモデルは生物個体数あるいは人口を表すためにこれまでよく用いられてきたロジスティックモデルを基本にし，それを拡張したものであるため，新ロジスティックモデルと呼ぶ[1~5]．その式を下に示す．

$$\frac{dN}{dt} = rN\left\{1-\left(\frac{N}{N_{max}}\right)^m\right\}\left\{1-\left(\frac{N_{min}}{N}\right)^n\right\}$$

ここで N：菌数，t：時間，r：速度定数，N_{max}：最大値（定常期菌数），N_{min}：最小値（接種菌数），m, n：調節パラメータである．

2.2 増殖データ

微生物増殖データはこれまでに報告された液体培地中の大腸菌増殖[5]，牛乳中の黄色ブドウ球菌増殖とエンテロトキシン生成量[8]，液体培地中の腸炎ビブリオ増殖*1を用いた．

2.3 プログラム開発方法

上記の新ロジスティックモデルは数値解法（ルンゲークッタ法）を用いて解いた[1~5]．速度定数 r は各時間での温度からアレニウスモデルあるいは平方根モデルを用いて求めた[1~5]．ブドウ球菌エンテロトキシン生成量は牛乳の受けた温度履歴データから予測した[8]．

予測プログラムは表計算ソフトウェア Microsoft Excel を用いて作られた[1~5]．また，ユーザーに使いやすくするため，Excel 中の Visual Basic Application および操作ボタンを使って自動化した．

3. 増殖予測プログラムの開発結果：内容と使用手順

3.1 概要

本プログラムは新ロジスティックモデルを使った一般ユーザー向けの初めての増殖予測プログラムである．ユーザーは対象とする菌種および初期菌数，温度履歴を入力すると，その条件下での増殖予測を瞬時に行う．なお，牛乳中の黄色ブドウ球菌についてはエンテロトキシン生成量も予測できる．

温度履歴は2つの入力方法がある．1つは手動でキーボードから温度が変化するステップごとにその温度と時間を入力する方法である．もう1つはデジタル温度記録計で得られた時間-温度データを貼り付ける方法である．

3.2 使用手順

本プログラムの使い方を実際のコンピュータ画面で表示しながら解説する．ここでは温度履歴を手動入力する方法での操作手順を示す．

①対象とする菌種をリストボックス中の「大腸菌・黄色ブドウ球菌・腸炎ビブリオ」から選択し，次にその初期菌数を入力する (Fig. 1)．この例では，黄色ブドウ球菌（牛乳中）を選択し，その初期菌数を 1,000 CFU/mL とした．

②温度履歴を入力する (Fig. 2)．最初に開始温度を入力し，次に各温度ステップでの時間と温度を入力する．一定温度のステップではその時間と温度を入力し，経時的な温度勾配のある場合はその温度と経過時間を入れ，「勾配を付ける」というボックスにチェックを入れる．ただし，予測適用温度の範囲は 15℃から 35℃とした．

③入力した温度履歴を確認する (Fig. 2)．「温度履歴確認」ボタンを押し，入力した温度履歴が正しいかをグラフで確認する．温度履歴曲線は赤線で示される．もし，間違っていれば②に戻って数値を入れ直し，再度「温度履歴確認」ボタンを押して確認する．

④「増殖予測」ボタンを押し，温度履歴に対応した増殖予測曲線を得る (Fig. 3)．増殖予測曲線は青い線で示される．黄色ブドウ球菌の場合はエンテロトキシンの産生予測量を得ることもできる．予測毒素量は紫色の線で示される．

⑤指定した時間での予測菌数を知る (Fig. 3)．指定した時間を入力し，「表示」ボタンを押すとその時刻における予測菌数が得られる．黄色ブドウ球菌の場合はその時刻におけるエンテロトキシンの予測産生量を知ることもできる．この図では 20 時間後の値を求めた．

⑥出力した増殖予測曲線を印刷する．「グラフ印刷」ボタンを押した後，Excel の通常の操作指示に従って印刷することができる．

デジタル温度記録計などによる連続した温度データも同様に使うことができる (Fig. 4)．対象菌種とその初期菌数を入力した後，時間-温度データは右側の表に貼り付け，「増殖予測」ボタンを押す．Fig. 4 では前述した手動による場合と同じ温度データを入力した場合の予測結果を示す．

4. 増殖予測プログラムの活用

対象食品の微生物学的安全性は最終的には実際に微生物

*1 関根暢子，木村　凡，丸山弓美，藤井建夫：投稿中

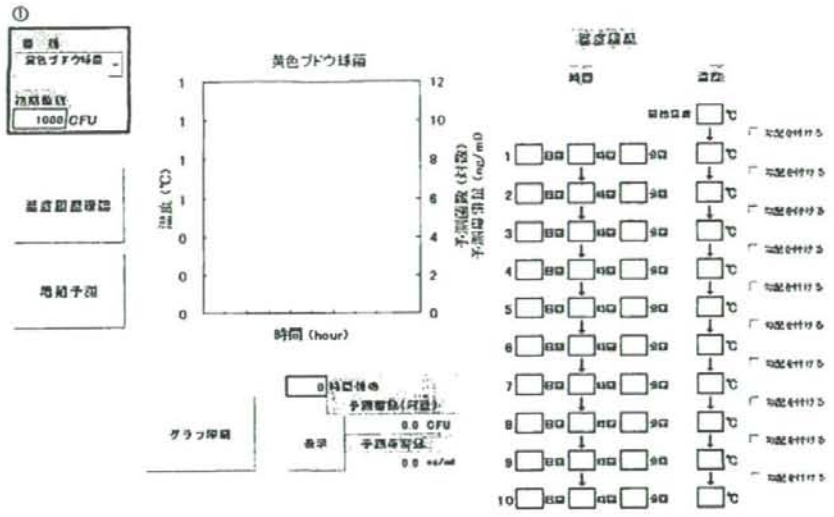

Fig. 1. Procedure-1: Input of microbial species and initial cell count
Circled number corresponds to the number of the procedure.

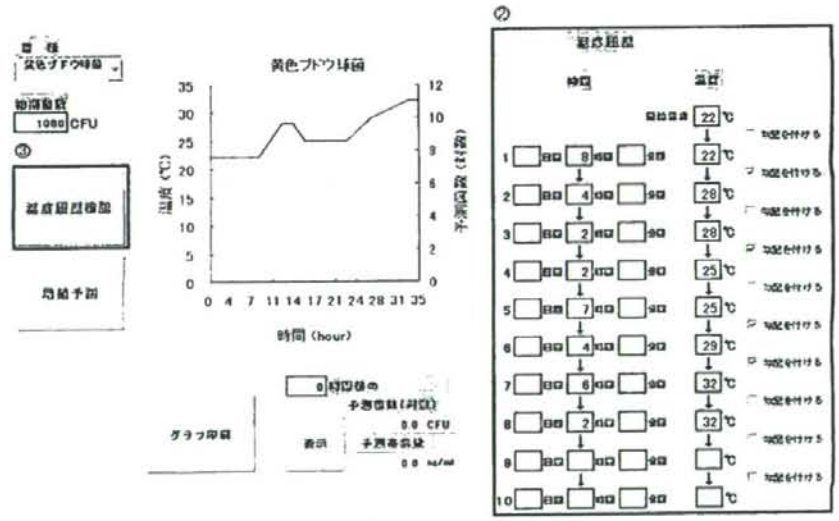

Fig. 2. Procedures-2 and 3: Input of the temperature history and its confirmation
Circled number correspond to the numbers of the procedures.

検査をしなければ知ることはできないが，それには相当の時間と費用が必要となる．それに対して，本プログラムは微生物学的安全性を瞬時に推測する手段となりうる．それで得られた予測結果が微生物学的な許容限度を超えたかどうかでその食品あるいは工程が微生物学的に安全であったか判断できるであろう．

この許容限度は食品の変敗を対象とした一般細菌数と食中毒を起こす病原菌数とでは当然その値が異なる．惣菜など一般的な市販食品の細菌数は，食品の種類にもよるが概ね 10^1～10^6 CFU/g の範囲に入り，微生物による変敗は一般に細菌数が $10^{7\sim8}$ CFU/g 以上で認められる[9~11]．実際の食品行政上の指導基準は細菌数に関して 10^5 あるいは 10^6 CFU/g としている自治体が多い[4]．したがって，対象食品の細菌数に関する許容限度を，例えば 10^6 CFU/g とし，本プログラムによる最終予測菌数がこの値以下であれば，その工程には温度管理上の問題がなく，食品の微

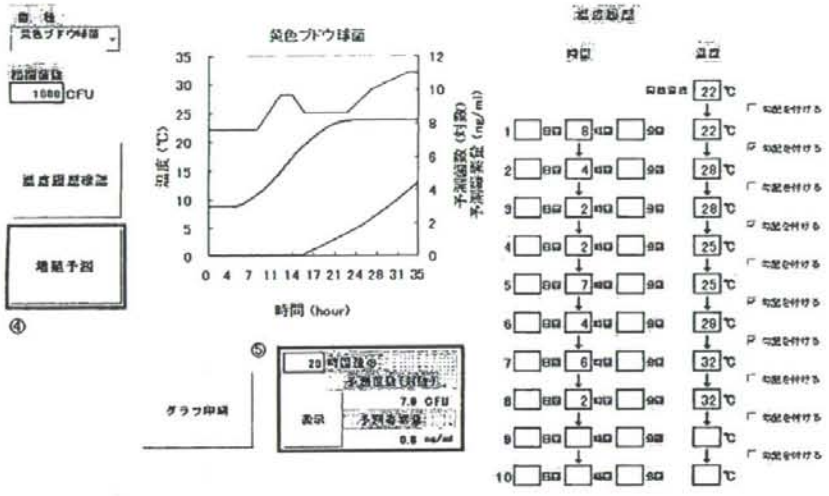

Fig. 3. Procedures-4 and 5: Implementation of growth prediction
Circled number correspond to the numbers of the procedures.

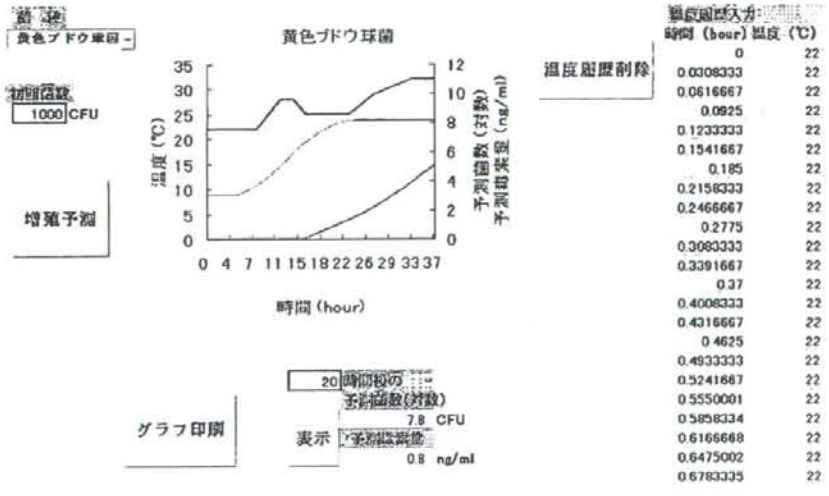

Fig. 4. Growth prediction using continuous temperature data

生物学的安全性および品質も確保されていると考えてよいだろう．

食中毒起因細菌に関しては，対象食品の初期汚染量は一般にゼロあるいは非常に低く，同時に正確な値が得られないことが多い．そのため，対象食品に関して増殖予測する際に病原微生物の初期汚染量を設定することは難しい．初期汚染量が不明であれば，その工程の温度履歴が得られても，当然最終増殖予測はできない．このような場合，筆者らの考えでは病原菌についての予測結果に明らかな増殖が認められなければ，その工程における食品の微生物学的安全性はほぼ確保されているとしてよいだろう．例えば，ある病原菌の初期汚染量を 10 CFU/g であったと仮定し，その工程での最終増殖予測結果が 100 CFU/g 以下，すなわち 1 桁以内の増殖であればその工程の安全性は一応確保されていると考えてよいかもしれない．しかし，もっと厳しい基準であれば，増殖予測結果も 10 CFU/g であること，すなわち増殖を認めないことを要求される場合もある．この判断基準は対象微生物によっても異なるであろう．

以上のような予測結果に基づいて，その製品をそのまま

次の工程に送るか，あるいは破棄・再加工などを行うか判断できる．このようにして，本増殖予測プログラムは食品の微生物学的安全性確保のための道具として活用できると考えられる．

5. 今後の課題

本プログラムは前述したように一般ユーザー向けに初めて開発したものである．まだ対象菌種も3種類であり，用いた基質（食品）も液体ブロスおよび牛乳である．ただし，この3菌種はそれぞれ黄色ブドウ球菌が乳肉原材料由来の食中毒起因細菌，腸炎ビブリオが海産物由来の食中毒起因細菌，大腸菌が一般的な通性嫌気性細菌に対応している．また，著者らの大腸菌を用いた最近の実験では，栄養が十分ある環境において液体，固体表面，固体内部（あるいは包装容器内）での増殖挙動はほぼ等しかった[4),6)]．したがって，今回示した増殖予測は一般の食品の各部位でほぼ当てはまるのではないかと考えられる．ただし，今後さらに多くの菌種および食品におけるデータは必要である．

また，微生物は同じ温度でも食品固有のpH，水分活性，保存料濃度などの要因によって増殖は変化する．これらの要因のとりうる範囲の値をすべて使って増殖実測をすることは不可能に近い．しかも食品のpHまたは水分活性が同一であっても，含まれる酸，塩あるいは糖の種類によって微生物の挙動は異なる．そのため，対象とする食品についてできる限り精度の高い増殖予測をするためには，実際にその食品を使った定常温度での増殖実測値が必要となる．しかし，これらの要因の濃度はその食品群ごとにそれほど変わらないことが多いので，実測データを一度得ておけば，そのデータは多方面で活用できると考えられる．

今回用いた増殖モデルはその予測可能な温度条件，すなわち温度帯，温度変化速度などについてさらに確認しておく必要があろう．今回はモデル構築時に行った実験条件から10～35度の範囲を予測可能温度帯とした．一方，コンピュータプログラムは今後さらに使いやすくし，新機能を加えるなどユーザーの要望に沿った改良を行いたい．

なお，本プログラムは(財)食品産業センター(http://www.shokusan.or.jp/haccp/)から公開されている．

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Rapid Separation and Counting of Viable Microbial Cells in Food by Nonculture Method with Bioplorer, a Focusing-Free Microscopic Apparatus with a Novel Cell Separation Unit

TOMONORI SHIMAKITA,[1] YOSHIKAZU TASHIRO,[1] AKIRA KATSUYA,[1] MIKAKO SAITO,[2,3] AND HIDEAKI MATSUOKA[2,3]*

[1]*Biosensing Business Project, Matsushita Ecology Systems Co., Ltd., 4017 Shimonakata, Takaki-Cho, Kasugai, Aichi 486-8522, Japan;* [2]*Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology, Koganei, Tokyo 184-8588, Japan; and* [3]*CREST, Japan Science and Technology Agency, Honcho 4-1-8, Kawaguchi, Saitama 332-0012, Japan*

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ABSTRACT

A nonculture method utilizing a novel apparatus, the bioplorer, was developed. The bioplorer is composed of an efficient cell separation unit, a focusing-free microscopic device, and an image analysis program. A meat or vegetable suspension is poured into the cell separation funnel, and insoluble matter in the sample suspension is trapped by prefilters. Microbial cells passing through the two prefilters are then trapped by the membrane filter (pore size, 0.4 μm). Trapped cells are double-stained with 4′,6′-diamidino-2-phenylindole and propidium iodide, and the membrane filter is removed and set on the focusing-free microscope. A fluorescent image is then recorded. Total numbers of viable and dead cells on the membrane filter can thus be determined automatically. One assay can be performed within 10 min, which is much faster than the culture method. The results obtained with both the nonculture method and the culture method for meat and vegetable samples were highly correlated (r = 0.953 to 0.998). This method is feasible for the practical purpose of food safety control.

To protect food from contamination, it is important to detect viable microbial cells as rapidly as possible. A rapid method implies the direct counting of cells without culture, either in liquid or on solid media, and may be referred to as a nonculture method (NCM) as opposed to a conventional culture method (CM) based on counting colonies on an agar plate. The NCM is based on various indicators of living cells such as cell membrane permeability *(5, 8, 11)*, intracellular esterase activity *(4)*, microscopic changes in cell shape *(9)*, respiratory activity *(5, 9)*, and nutrient uptake *(1, 7, 15)*. Dyes that allow visualization of these indicators are now commercially available.

This study was focused on the permeability of the cell membrane to ionic dyes, which is not equivalent to cell viability but can be used as a practical indicator of it. Propidium iodide (PI) is an ionic dye used for this purpose because this dye can permeate the damaged cell membrane and combine with DNA to form a fluorescent compound. Therefore, PI can detect only dead cells. 4′,6′-diamidino-2-phenylindole (DAPI) is a nonionic dye used to stain DNA. Because DAPI can permeate intact and damaged cell membranes, it can make both viable and dead cells fluorescent. Therefore, the combined use of PI and DAPI enables the enumeration of both viable and dead cells.

When dealing with clean samples containing only microbial cells, an NCM based on fluorescent staining is useful and reliable; successful applications already have been reported *(6, 13)*. However, a NCM cannot be regarded as reliable for food samples because these samples contain various unidentified compounds that may cause pseudopositive or pseudonegative signals.

Another problem to consider when using a NCM is the need for a fluorescent microscope. Microscopic observation requires careful focusing and image scanning, and such time-consuming and labor-intensive operations are unsuitable for frequent use. Therefore, more rapid and less labor-intensive techniques are essential for practical applications.

To overcome these problems, we devised a smart apparatus, the bioplorer, which is composed of an efficient cell separation unit (CSU), a focusing-free microscopic device (FFM), and image analysis software. Here, we describe the specifications of this novel apparatus and its successful application for the rapid counting of viable cells in food samples.

MATERIALS AND METHODS

Materials. The feasibility of the bioplorer NCM was evaluated in two stages. First, we determined whether the bioplorer NCM could produce the same cell count as the conventional agar plate CM independent of species. For this purpose, we selected typical examples of gram-positive and gram-negative bacteria and yeasts from among those often encountered in food safety screenings. Species tested were *Escherichia coli* NBRC 3301, *Staphylococcus aureus* NBRC 12732, *Enterobacter intermedius* ATCC 33110, *Citrobacter freundii* ATCC 8090, *Klebsiella pneumoniae* NBRC 14940, *Bacillus subtilis* NBRC 3023, *Pseudomonas aeruginosa* NBRC 13275, *Salmonella* Enteritidis IFO 3315, *Candida*

* Author for correspondence. Tel: +81-42-388-7029; Fax: +81-42-387-1503; E-mail: bio-func@cc.tuat.ac.jp.

TABLE 1. *Applicability of the bioplorer NCM to analysis of various food samples*

Sample	SC[a]	FP[b]	RFC[c]	BNCM[d]	SR[e]
Refreshing drinks					
Water, mineral water	L	○	○	○	GF
Cola, soda	L	○	○	○	GF
Carbonated drinks	L	×	×	×	GF
Juice	L	×	×	×	
Tea	L	△	×	×	
Coffee	L	×	×	×	
Milk-based drinks					
Milk	L	×	×	×	
Lactic acid drink	L	○	○	○	
Alcoholic drinks					
Japanese sake	L	○	○	×	GF
Beer	L	△	○	△	GF
Meats					
Beef	S	○	○	○	
Pork	S	○	○	○	
Chicken	S	○	○	○	
Gyoza	S	○	○	○	
Vegetables					
Cabbage	S	○	○	○	
Lettuce	S	○	○	○	
Cut vegetables	S	○	○	○	
Flour	S	○	○	○	
Mushrooms	S	○	○	○	
Radish sprouts	S	○	○	○	
Bean sprouts	S	○	○	○	
Seafoods					
Fish	S	○	○	○	
Oysters	S	×	×	×	
Dairy products					
Butter	S	×	○	×	
Margarine	S	○	×	×	
Yogurt	L	○	○	○	
Grains					
Rice	S	○	○	○	
Boiled rice	S	×	○	×	
Noodles	S	○	○	○	
Buckwheat noodles	S	○	○	○	
Seasonings					
Salt	S	○	○	○	
Powdered soy sauce	S	○	×	×	
Syrup	L	○	○	○	

[a] Sample condition: L, liquid; S, solid.
[b] Filtration possibility: ○, possible; ×, impossible; △, depending upon sample conditions.
[c] Removal of fluorescent contamination: ○, can be removed; ×, cannot be removed.
[d] Bioplorer NCM: ○, possible; ×, impossible; △, depends upon sample conditions.
[e] Special requirement: GF, germ-free condition is required.

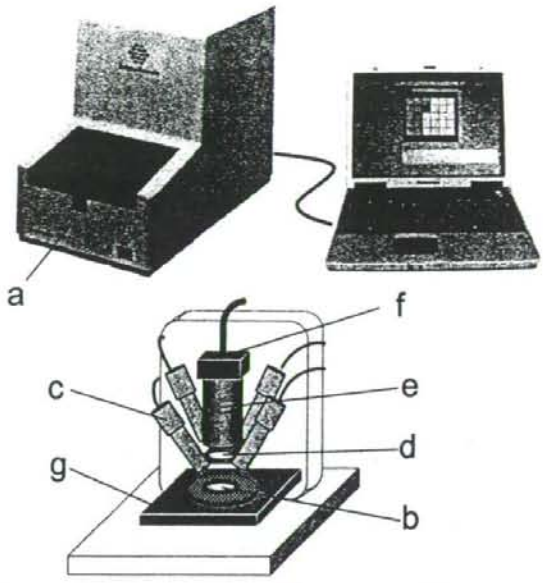


FIGURE 1. *Schematic diagram of the bioplorer. (a) Appearance; (b) cell trap membrane filter; (c) UV and green light–emitting diodes; (d) absorption filter; (e) objective lens; (f) charge-coupled device; and (g) XY-automatic stage.*

albicans NBRC 1594, and *Saccharomyces cerevisiae* NBRC 0555. Standard method agar (Nissui Seiyaku, Tokyo, Japan) was used for the colony count CM. In this first stage, water samples spiked with one of the above species were used.

The second stage focused on whether the bioplorer NCM is applicable to naturally occurring bacteria in actual food samples. Among the materials that were applicable to the bioplorer NCM without pretreatment for cell separation (Table 1), we selected meats (beef, pork, chicken, and fish), vegetables (cabbage, lettuce, and radish sprouts), and grain (rice) for further quantitative comparison with the agar plate CM. At this stage, no spike test was performed. Meats, vegetables, and rice were purchased fresh from the market and were not cooked or processed. Based on preliminary experiments, we determined the proper dilution conditions such that the range of cell concentration was 10^2 to 10^5 cells per ml.

DAPI and PI were obtained from Wako Pure Chemicals Co., Ltd. (Osaka, Japan). Other reagents were of commercially available analytical grade.

Spike test. Each species was cultured on standard agar medium at 36°C for 24 h, and a single colony was selected and suspended in physiological saline. Cells were then diluted in distilled water to give the prescribed concentrations. Spiking tests were performed only with these water samples.

Optical system. A schematic diagram of the bioplorer is given in Figure 1. The optical system is composed of two sets of light-emitting diodes and filters. One set is for DAPI and comprises four UV light–emitting diodes, a 375-nm excitation filter, and a 435- to 580-nm absorption filter. The other set is for PI and comprises four green light–emitting diodes, a 525-nm excitation filter, and a 580- to 650-nm absorption filter. The excitation light source is illuminated for 6 s through the excitation filter on the cell trap membrane filter of the CSU. The fluorescent light emitted

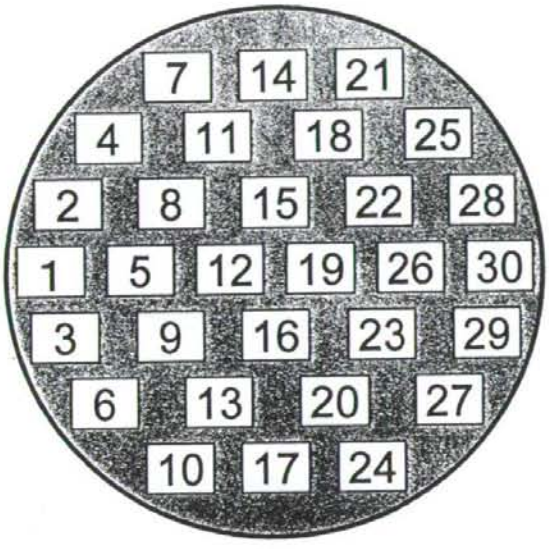


FIGURE 2. *Cell counting area of a cell trap membrane filter.*

from 1 of 30 sections (Fig. 2) of the cell trap membrane filter passes through an objective lens and an absorption filter and is projected onto a charge-coupled device. Under these conditions, one pixel of the charge-coupled device corresponds to 1.8 by 1.6 μm^2 on the cell trap membrane filter. All 30 sections were scanned automatically, and fluorescent spots in each section were counted.

CSU. The CSU is composed of three parts (Fig. 3). The top part is a funnel with two prefilters (unwoven nylon cloth and cellulose membrane). The unwoven nylon cloth is 9 mm in diameter, and the cellulose membrane is 9 mm in diameter and has an 8-μm mesh size. These prefilters are vital for the efficient trapping of fluorescent contaminants and other large particles. The middle part is a cell trap membrane filter 9 mm in diameter with a 0.45-μm pore size. This membrane is coated with gold by ion spattering to ensure a clean surface, free from fluorescent background. The prefilters facilitate uniform cell distribution for the cell trap membrane filter. The bottom part is a needle that allows for convenient one-touch thrust into the rubber plate of a suction unit.

Image analysis program. The image analysis program was developed by Matsushita Ecology Systems Co., Ltd. The cell counting area is 30 sections (Fig. 2). Total area is 50% of the whole membrane area. Each fluorescent spot is registered as a microbial cell when its intensity exceeds the lowest intensity limit and its size is smaller than the largest spot size limit. The lowest light intensity limit was adjusted to ensure that the bioplorer NCM results agreed with those of direct microscopic observation. For this adjustment, we used water samples spiked with each of the test strains at various concentrations. The size limit of the largest spot was determined based on a database produced from comparative CM studies of various practical samples. Spots larger than 30 pixels were excluded from the cell count.

The number of DAPI-fluorescent microbial cells (N_{DAPI}) corresponds to the total number of living and dead cells, whereas the number of PI-fluorescent microbial cells (N_{PI}) corresponds to the number of dead cells. Therefore, the number of living cells is given by $N_{DAPI} - N_{PI}$. N_{DAPI} and N_{PI} were determined for all 30 sections per membrane. Therefore, the total numbers of viable and dead cells per membrane are respectively given by the following formulas:

$$2 \times \sum_{i=1}^{30} (N_{DAPI} - N_{PI})_i \quad \text{and}$$

$$2 \times \sum_{i=1}^{30} (N_{PI})_i.$$

Numbers of both viable and dead cells on the respective membranes are displayed and saved for later use.

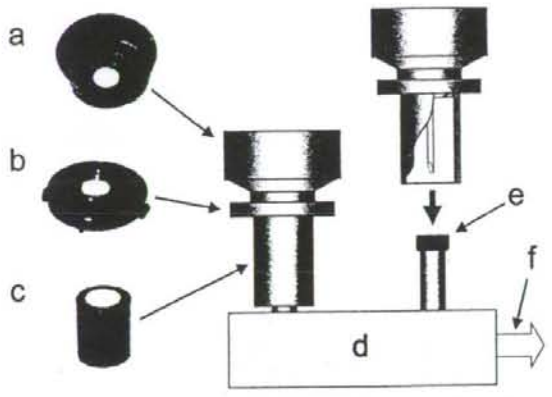


FIGURE 3. *Cell separation unit (CSU). (a) Funnel with prefilters; (b) cell trap membrane filter; (c) base with a needle; (d) suction unit; (e) silicon rubber cap; and (f) to a suction line.*

Preparation of test cell suspensions. Raw meat or raw vegetable (10 g of solid matter) was suspended in 90 ml of physiological saline and transferred into a stomacher bag (a plastic bag with a mesh filter at the nozzle). The bag was agitated at 8 strokes per s for 30 s. The contents of the bag were then poured out through the mesh filter. The resultant suspension was diluted with physiological saline from 100 to 100,000 times to prepare test cell suspensions. These test cell suspensions were assayed by both the bioplorer NCM and the standard agar colony count CM.

For the bioplorer NCM assay, the cell suspension (1 ml) was directly transferred into the CSU funnel. For the CM, the cell suspension (0.1 ml) was spread onto a standard agar plate and incubated at 36°C for 24 or 48 h.

Cell trapping and staining. The CSU was placed on a suction unit such that the needle of the CSU pierced the rubber plate (Fig. 3). One hundred microliters of Tween 80 (0.1%, vol/vol) was added onto the prefilter to improve filterability. One milliliter of cell suspension was added to the CSU and filtrated by suction, and the prefilter was then washed with 3 ml of physiological saline. The funnel was removed, and 100 μl of solution containing DAPI (1.0 μg/ml) and PI (2.5 μg/ml) was placed on the membrane filter. After incubation for 2 min, the dye solution was removed by suction from below. Another 100 μl of DAPI (1.0 μg/ml) was added onto the membrane filter to compensate for the relatively weak staining of DAPI compared with PI. The DAPI solution was removed immediately by suction from below. Finally cells were rinsed with 0.1 ml of saline.

RESULTS

Applicability of the bioplorer NCM for various foods. When a sample can be filtered and fluorescent contamination can efficiently be removed, the sample can be

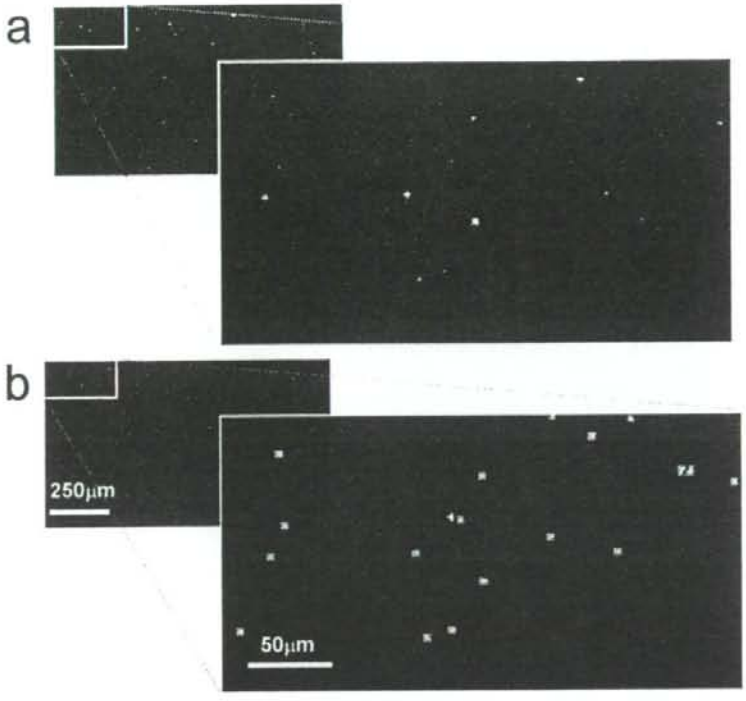


FIGURE 4. *Fluorescent images of DAPI-stained* E. coli *cells from a water sample spiked with* E. coli. *When a fluorescent spot satisfies the criteria of light intensity limit and spot size, the spot is intensified by the image processing program. (a) Before image processing; (b) after image processing.*

assayed by the bioplorer NCM. Table 1 summarizes the results for 33 types of food and drink. For each type, we tested 5 to 50 brands, and raw samples were used throughout. A total of 21 types of food and drink were applicable to the bioplorer NCM, including meats and vegetables. For some items, some brands could be assayed with the bioplorer NCM but others could not, e.g., beer. In such cases, evaluation depended on sample conditions.

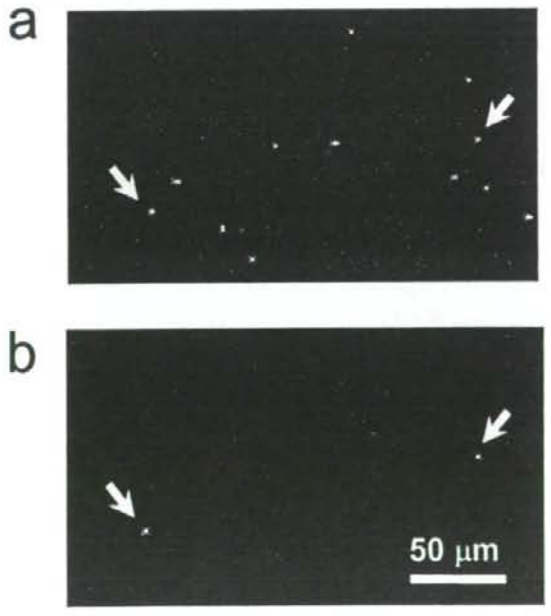


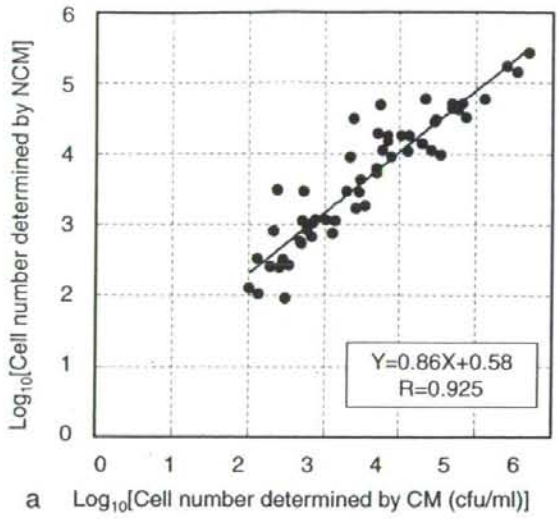
FIGURE 5. *Fluorescent images of DAPI-stained (a) and PI-stained (b) cells separated from beef. Arrows indicate dead cells, which were stained with both dyes.*

Fluorescent image of microbial cells. Spiked water samples cause no particular problems with fluorescent background, but food samples often contain many fluorescent contaminants that cannot completely be removed by CSU. This problem was solved with the image analysis program.

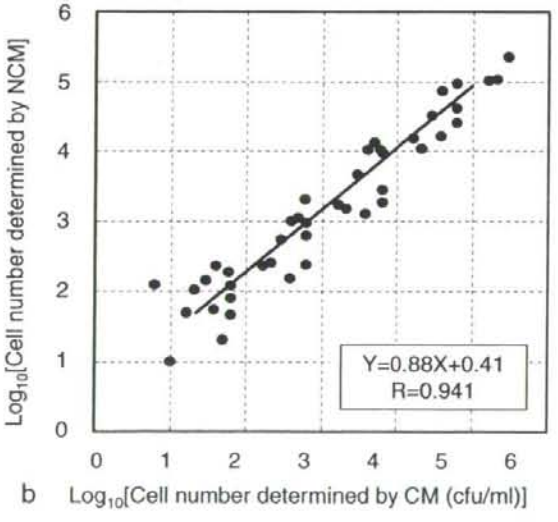
An aliquot of meat sample was applied to the bioplorer, and the fluorescent image of the trapped cells was displayed on a monitor. Figure 4a shows an image of bacteria before image processing; many fluorescent spots with irregular shapes are visible. After image processing, only the spots that satisfy the criteria of light intensity and spot size are designated as discrete spots (Fig. 4b).

Figure 5 shows a pair of DAPI-stained (Fig. 5a) and PI-stained (Fig. 5b) images of the same meat sample. Those cells that emitted fluorescence in both images (indicated by arrows) were considered dead cells, whereas those that emitted fluorescence only in Figure 5a were regarded as viable cells. These results indicate that the numbers of viable and dead cells can be determined simultaneously.

Number of viable microbial cells in water spiked with test species. The bioplorer NCM was applied to test sample suspensions containing only one species. Aliquots of distilled water were spiked with the prescribed number of test species. The number of viable cells determined by the bioplorer NCM was plotted against the number of colonies determined by CM (Fig. 6a). The results of these two methods were highly correlated. In the same manner, sam-



a



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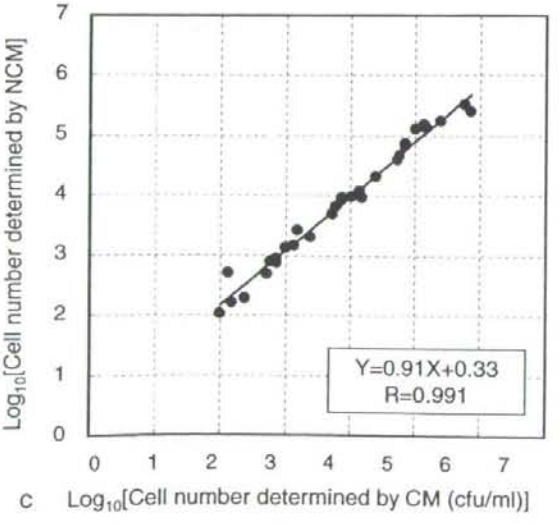

c

TABLE 2. *Correlations of numbers of microbial cells as determined with the bioplorer NCM (direct count) and CM (plate count) for water samples spiked with various microbial strains*[a]

Strains	Correlation coefficients
Gram-negative species	
Escherichia coli	$Y = 0.86X + 0.58$, $r = 0.925$
Salmonella Enteritidis	$Y = 0.88X + 0.39$, $r = 0.941$
Klebsiella pneumoniae	$Y = 0.97X + 0.25$, $r = 0.999$
Enterobacter intermedius	$Y = 0.85X + 0.15$, $r = 0.994$
Citrobacter freundii	$Y = 0.96X + 0.35$, $r = 0.989$
Proteus vulgaris	$Y = 0.89X + 0.06$, $r = 0.998$
Pseudomonas aeruginosa	$Y = 1.14X - 0.34$, $r = 0.967$
Gram-positive species	
Bifidobacterium species	$Y = 1.07X + 0.10$, $r = 0.996$
Lactobacillus species	$Y = 0.89X + 0.54$, $r = 0.993$
Staphylococcus aureus	$Y = 0.91X + 0.33$, $r = 0.991$
Bacillus subtilis	$Y = 0.98X$, $r = 0.994$
Yeasts	
Candida albicans	$Y = 0.40X$, $r = 0.995$
Saccharomyces cerevisiae	$Y = 1.60X$, $r = 0.950$

[a] For all strains, number of cells per membrane ranged from 10^2 to 10^5.

ples containing other species were assayed using both methods (Fig. 6b and 6c and Table 2), and the results were highly correlated.

Therefore, the bioplorer NCM and agar plate colony count CM were designated as giving equivalent results with clean samples containing only microbial cells.

Number of viable microbial cells collected from meats and vegetables. The performance of the bioplorer NCM was investigated with food samples that contained unidentified contaminants. The numbers of living cells determined with the bioplorer NCM and the CM were closely correlated (Fig. 7a). Similar results were obtained with lettuce samples (Fig. 7b).

The number of viable cells determined with the bioplorer NCM was correlated with the results for the CM, which indicates that insoluble contaminants were removed effectively by the CSU prefilters and that potential fluorescent contaminants had no effect on counts for the bioplorer NCM. Linear relationships and high correlation coefficients were also obtained for several other meat and vegetable samples (Table 3).

DISCUSSION

The use of a membrane filter and prefilter for rapid collection of microbial cells from food samples has been described previously *(3, 10, 14)*. Pettipher and Rodrigues

←

FIGURE 6. Correlations of microbial cell numbers determined with the bioplorer NCM (direct count) and the CM (plate count). Water samples were spiked with E. coli *(a),* Salmonella *Enteritidis (b), and* S. aureus *(c). For the CM, cultures were incubated for 24 h at 36°C.*

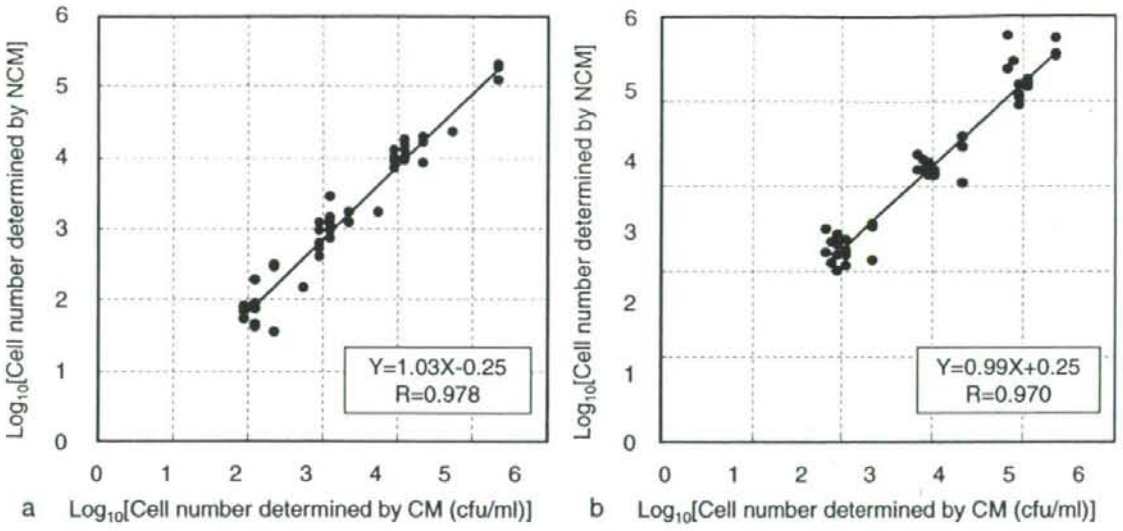

FIGURE 7. *Correlations of microbial cell numbers determined with the bioplorer NCM (direct count) and the CM (plate count). Food samples contained naturally occurring microbial cells. (a) Beef; (b) lettuce. For the CM, cultures were incubated for 48 h at 36°C.*

(10) reported the rapid count of microorganisms in foods by the NCM based on fluorescent dye staining and microscopic observation. Another pretreatment method was the use of alkaline protease in a meat sample to facilitate membrane permeation *(14)*. As a result, a close correlation was obtained between the NCM and the CM with certain food samples. Despite these pioneering studies, a more convenient method or device that can be applied to various other food and drink samples was required. Nonspecific fluorescent background has been a particularly difficult problem to overcome. However, the apparatus developed in the present study has solved this problem and satisfies the requirement of wide applicability.

A comparison of the bioplorer NCM and CM results for meat revealed that the number of cells determined by the bioplorer NCM tended to be higher than that determined by the CM. One of the reasons for this difference might be the influence of viable but nonculturable cells (VNC) *(2, 4, 8)*. VNC may be counted as viable cells by the NCM because the cell membrane properties of VNC more closely resemble those of viable cells than of dead cells. In contrast, VNC do not form colonies under typical culture conditions and thus are not counted as viable cells by the CM. *(6)*

Considering the principle of the NCM, it is not surprising that differences were seen in the number of cells determined with the two methods. Different NCM techniques will not necessarily coincide because the indicators used for different NCM techniques reflect different aspects of the live state, i.e., a highly vital state or a seriously wounded state.

For practical application of the NCM, conditions under which the NCM results are highly correlated with those of CM were identified. Very close correlation between results for both methods was observed for counts of microbial cells from meats and vegetables. Insoluble matter inevitably contaminates homogenates of food samples, but such contaminants were efficiently eliminated by the CSU. The number of VNC in these samples is likely to be small. Therefore, the bioplorer is useful for the rapid detection of microbial contamination in meat and vegetable samples.

TABLE 3. *Correlations of numbers of microbial cells as determined with the bioplorer NCM (direct count) and CM (plate count) for naturally contaminated meats and vegetables*[a]

Samples[b]	Correlation coefficients
Beef	$Y = 1.03X - 0.25$, $r = 0.978$
Pork	$Y = 1.15X - 0.28$, $r = 0.995$
Chicken	$Y = 1.18X + 0.60$, $r = 0.998$
Cabbage	$Y = 0.89X + 0.10$, $r = 0.982$
Lettuce	$Y = 0.99X + 0.25$, $r = 0.970$
Radish sprouts	$Y = 0.97X - 1.33$, $r = 0.997$
Rice	$Y = 0.81X + 0.56$, $r = 0.954$
Fish	$Y = 1.21X + 0.01$, $r = 0.902$

[a] For all sample types, the number of microbial cells per membrane ranged from 10^2 to 10^5.

[b] Stomacher-treated filtrates were diluted 100-fold and then assayed.

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ORIGINAL PAPER

Kohtaro Fujioka · Ikuko Kozone · Mikako Saito
Hideaki Matsuoka

Rapid evaluation of the efficacy of microbial cell removal from fabrics

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Abstract The efficacy of microbial cell removal (EMR) from fabrics is a practically important indicator for the evaluation of cleansers and detergents. EMR is expressed quantitatively by the relative number of viable cells remaining on a fabric swatch after the treatment with these reagents. In order to count the viable cells on the swatch directly and rapidly, we have developed a unique microscopic imaging system with an ultra-deep focusing range. Standard swatches of cotton fabric were inoculated with microorganisms such as *Pseudomonas fluorescence*, *Staphylococcus aureus*, or *Candida albicans*. After the incubation on an agar medium, each swatch was treated with a fluorescent glucose, 2-[*N*-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino]-2-deoxyglucose, to stain only viable cells. The images of every cell distributed within the surface layer with no greater than 130 μm thickness could be integrated into one image. Thus visualized cells could be counted automatically by a novel imaging program. Using a pair of cotton swatches ($0.5\times1.0\,cm^2$) inoculated with *C. albicans*, EMR was evaluated quantitatively. Before washing, the total number of viable cells found on the observation area ($3.8\times10^{-4}\,cm^2$) was 288 cells. After washing with a test detergent, no cell (<1) was detected. For this case, EMR was given by the formula: $\log(288/<1)=$greater than 2.5. The imaging and cell count of a test fabric could be performed within 1 h.

K. Fujioka
Kobe Technical Center, Procter & Gamble Far East, Inc., 17, Koyo-cho Naka 1-Chome, Higashinada-ku, Kobe, 658-0032, Japan

I. Kozone · M. Saito · H. Matsuoka (✉)
Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology, Koganei, Tokyo, 184-8588, Japan
E-mail: bio-func@cc.tuat.ac.jp
Tel.: +81-42-3887029
Fax: +81-42-3871503

I. Kozone · M. Saito · H. Matsuoka
CREST, Japan Science and Technology Agency, Honcho 4-1-8, Kawaguchi, Saitama, 332-0012, Japan

Keywords Ultra-deep focusing range (UDF) fluorescent microscope · Efficacy of microbial cell removal (EMR) · A fluorescent glucose · Viable cell imaging

Introduction

In recent years, there are many household products on the global market claiming the efficacy of microbial cell removal (EMR) from fabrics and other solid products [2, 6, 10, 12, 14, 23, 27]. The evaluation of EMR is especially important from the viewpoint of laundry treatment evaluation. To evaluate EMR, several guidelines have been issued [9, 24, 25, 26]. These guidelines recommend traditional agar plating and incubation procedures. Practically, however, a more rapid and more direct method is required. To meet this requirement, non-culture rapid methods using fluorescent staining dyes have been proposed and recognized to be potentially applicable to liquid samples. However, they could hardly be applied to solid samples with rough surface.

Recent advances in fluorescent bio-imaging [4, 5] have enabled the visualization of particles with 10 μm in diameter existing at a depth of 1 mm [5]. However, to detect microbial cells with no greater than 0.5 μm, the magnification of the objective lens should be 60× or 100×. The working distance and the in-focus depth of these lenses (VC60×oil, VC100×oil) are 130 μm and a few micrometers, respectively. It is only those cells being located within the in-focus depth 130 μm (i.e., working distance) apart from the lens that can be observed as a clear image. Therefore, our efforts have been focused on the development of a novel microscopic apparatus with an ultra-deep focusing range (UDF) by combining with an automatic Z-scanning system. Confocal microscopy [18, 20, 21] and deconvolution microscopy [17] should satisfy the requirement of deep focal distance in principle. However, it was difficult to modify these commercial models to fit for our specific purpose at reasonable cost. Thus, we intended to construct a novel fluorescent

microscopic system with a Z-scanning apparatus and an associated image-editing program.

Another point is the introduction of edible fluorescent probe for the detection of viable cells. For this purpose, the authors synthesized a fluorescent glucose, 2-[*N*-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino]-2-deoxyglucose (2-NBDG) [26]. 2-NBDG was found to be taken only by viable cells of various microorganisms [15, 16] as well as animal cells [28]. Based on these results, we decided to use this fluorescent glucose to visualize viable microbial cells on fabrics. This study reports the performance of the novel microscopic apparatus and thereby evaluation of EMR of cleanser using standard samples of cotton swatches.

Materials and methods

Microbial strains

As test microorganisms, 13 strains of 8 species were selected from ATCC cultures and environmental isolates [3]. They were *Pseudomonas aeruginosa* ATCC15442, *Pseuduomons aeruginosa* environmental isolate, *Burkhordelia cepacia* environmental isolate, *Pseudomonas* sp. environmental isolate (Zinc pyrithione resistant), *Pseudomonas fluorescence* environmental isolate, *Citrobacter freundii* environmental isolate, *Serratia marcescens* environmental isolate, *Staphylococcus aureus* ATCC6538, *Enterobacter gergoviae* environmental isolate, *Klebsiella pneumoniae* 2 environmental isolates, *Candida albicans* ATCC10231, and *Enterobacter cloacae* environmental isolate (HCHO resistant).

Each strain was revived from the frozen stock with MICROBANK kit (Pro-lab Diagnostics, Toronto, Canada) and precultured on Trypticase Soy Agar (TSA) plates (BBL Company, Franklin Lakes, NJ) for 24 h at 33°C. After that, the respective inocula were suspended in 0.9% saline solution as ca. 10^6 colony forming unit (cfu)/ml and used as seed cultures.

Chemicals

A commercially available reagent, Ariel Bleach Plus® (Procter & Gamble Far East, Inc.) was used to demonstrate EMR test. 2NBDG was prepared according to the protocol described in [16]. Other reagents were of commercially available analytical grade.

Fabric substrates and inoculation thereon

Test fabric was Kanakin No.3 described in JIS L0803 [7] as a standard cotton fabric to be used for a color fastness test after staining. The fabric swatches were prepared as 1.0 cm×1.0 cm square size, wrapped with aluminum foil, autoclaved at 121°C for 15 min, dried up under a sterilized condition. A 50 µl seed culture was inoculated onto each of fabric swatches. Then, the swatches were placed on TSA plates and incubated for 48 h at 33°C. Immediately after the incubation, each of these swatches was cut into two pieces (0.5 cm×1.0 cm each). One piece was used for the visualization experiment with 2NBDG and another was used for the colony count assay.

Treatment of fabric swatches with 2NBDG

2NBDG was used for the visualization of viable microbial cells on the inoculated fabric swatches. A 200 µl aliquot of 12 µM 2NBDG was placed on one fabric swatch (0.5 cm×1.0 cm) and incubated at 33°C for 10 min. After the incubation, the fabric swatch was treated with 50 µl of 30% HCHO solution for 1 min in order to fix the microorganisms. Immediately after the fixing, the fabric swatch was soaked into 0.9% saline solution for 5 min, 2 times, and centrifuged in an Ultrafree-MC centrifugal filter device (0.22 µm pore size, 0.5 ml size, Millipore Co.) for 30 s at 6,000 rpm to eliminate extracellular 2NBDG.

Construction of a novel UDF fluorescent microscope

The novel apparatus constructed in this study is shown in Fig. 1. The fundamental system of a novel UDF fluorescent microscope was ECLIPSE 80*i* system (NIKON Inc., Tokyo) with a *Z*-axis auto tuning system. As a fluorescence objective lens system, CFI Plan Apo VC60×oil (WD 130 µm) or VC100×oil (WD 130 µm) (NIKON Inc.) was used with a BV-2A fluorescence filter (excitation 400–440 nm, emission 470 nm). The image detection device was KRI-100K (KOGAKU Inc., Osaka). A Lumina Vision 2.20 Bio-imaging analysis program and a WinROOF automated macro program (Mitani Trading Inc., Fukui) were optimized for our purpose and installed on Windows XP system.

Observation with an UDF fluorescent microscope

A test swatch was attached on an Adhesive Durotak slide glass (Dermatologic Lab & Supply, Inc., Council

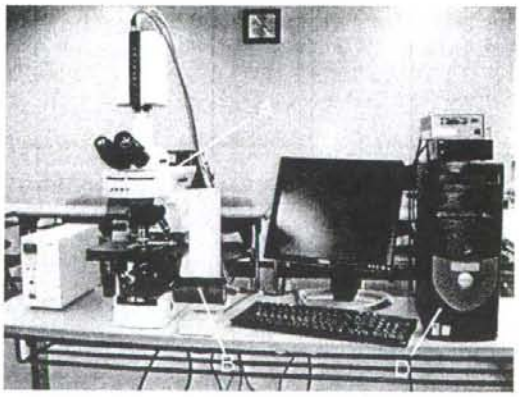

Fig. 1 UDF fluorescent microscope. *A* microscope, *B* *Z*-axis auto tuning system, *C* image detection device and *D* computer

Bluffs, IA) and observed with an UDF fluorescent microscope. The images of six square spots (each spot $78 \times 82\ \mu m^2$) were analyzed and total number of cells was estimated.

Cell collection from fabric swatches for colony count method

A test swatch was soaked in a 5 ml cell collection medium being composed of Modified Letheen Broth (MLB, BBL Co.), 1.5% Tween 80 (Wako Co., Tokyo), and 0.93% soybean lecithin (Wako Co.), which was prepared in a 15 mmϕ glass tube and autoclaved at 121°C for 15 min beforehand. The swatch in the tube was agitated for 5 min with a sonic device and for 5 min with a vortex device successively. We checked beforehand the influence of ultra sound on the cell viability and found it no lethal level (Data not shown). One milliliter aliquot of the cell collection medium was tenfold diluted with 9 ml 0.9% saline solution in glass test tubes. After the stepwise dilutions, the individual diluents were pour-plated on MLAT [(Modified Letheen Agar (MLA, BBL Co.) containing 1.5% Tween 80], which was autoclaved at 121°C for 15 min and maintained as molten condition at 47°C beforehand. Then, the MLAT plates were solidified, inverted, and incubated at 33°C for 72 h. After the incubation, the colonies that appeared on the plates were counted.

Washing treatment condition for microbial cell removal

The microbial cell removal from test swatches was performed according to the test method of ASTM E2274-03 [19]. Briefly, a strip of cotton swatch, Kanakin no. 3, was wound around the three horizontal extensions of a stainless steel spindle with sufficient tension. Then the test swatch was inserted in the laps of the wound cloth. The test swatches were washed with a detergent solution (1.0 g/l) in a 500 ml glass jar (8.1 cmϕ×12.8 cm) with agitation at 60 rpm/min. This washing condition was originally proposed for the color fastness test [8] and later confirmed to be a proper condition for bacteria removal test from various fabric samples (unpublished data). After the washing, the number of cells remaining on the test swatches was counted. Half the test swatches were applied to the 2-NBDG staining and its visualization process. The rest swatches were applied to the cell collection and successive colony count method as described above.

Comparison of the developed method and conventional agar plate colony count method

To demonstrate the feasibility of the developed method, test swatches inoculated with *C. albicans* ATCC 10231 were assayed by both methods. After the washing treatment with a detergent, each of test swatches was cut into two pieces (0.5 cm×1.0 cm each). One piece was used for the developed method involving 2NBDG staining and the other was used for the colony count method. As the control, test swatches without washing were assayed by both methods in the same manner. The EMR determined by both methods were compared.

Results

Fluorescent images of yeast cells obtained with the UDF fluorescent microscope and an ordinary microscope

Fabric swatches inoculated with *C. albicans* ATCC10231 were observed with an ordinary fluorescent microscope (Nikon X2-F) and the present UDF fluorescent microscope. The image obtained with an ordinary microscope could hardly give us a clear image of microbial cells (Fig. 2a). In contrast, the UDF fluorescent microscope could give us a bright and clear image of microbial cells being distributed on rough surface of the swatch (Fig. 2b). This indicates that the developed system is feasible for the direct detection of microbial cells with several micrometers in diameter being distributed in UDF (see Discussion about the detail).

The general non-specific fluorescence over the test swatches observed is speculated as a residue of extra cellular 2NBDG, and also trace level of florescence derived from the fabric components. Practically, however, the present fluorescent background is a permissible level,

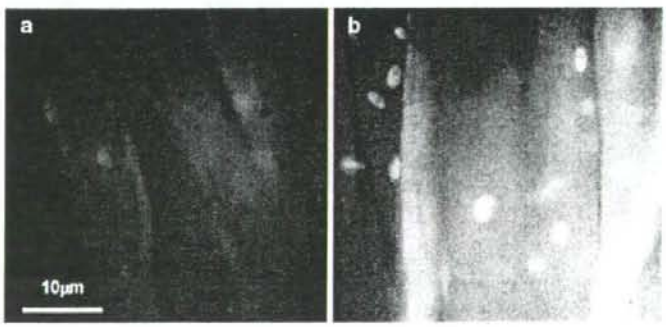

Fig. 2 Fluorescent images of a fabric swatch inoculated with *C. albicans* ATCC10231 taken with an ordinary microscope (**a**) and the UDF fluorescent microscope (**b**)