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LR-White resin and tea: a quick and safe bacterial cells preparation method for transmission electron microscopy

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Abstract: [Objective] Oolong tea extract (OTE) was tested for its potential as an electron staining reagent to substitute for uranyl acetate (UA) in electron microscopy of bacterial cells. **[Methods]** Three electron microscopic staining methods (standard, 0.05% OTE, and 0.1% OTE) were compared for electron microscopic observation of ultrathin sections with or without lead citrate (Pb). First floating the sections on the compared staining solutions for 10−15 min, then if need to post-stained with lead citrate, after rinsing with deionized water three times, the sections were re-floated on lead citrate for 8−10 min. Electron microscopy was performed on sections from LR-White-embedded cells of a Gram-negative bacterium, *Escherichia coli*, and a Gram-positive bacterium, *Staphylococcus aureus*. **[Results]** Both concentrations of the OTE preparations showed the staining results were similar to that of standard method with UA and Pb in *E. coli* and *S. aureus* cells. **[Conclusion]** This study demonstrated that OTE could potentially be used as an alternative to UA in electron microscopy staining for certain structures.

Keywords: Oolong tea extract, Uranyl acetate, Bacterial cell, Transmission electron microscopy

伦敦白胶和茶：一种既快又安全的用于透射电子显微镜的细菌样品制备方法

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摘 要:【目的】检测乌龙茶提取物是否可作为电子染色剂取代醋酸双氧铀用于细菌细胞染色，使其能在透射电子显微镜下进行观察。【方法】利用伦敦白胶对细菌样品(大肠杆菌和金黄色葡萄球菌)进行胶块的制备，再在复染铅与不复染铅这两种情况下对超薄切片样品进行 3 种不同染色剂的电子染色，之后在透射电子显微镜下观察比较其不同之处。这 3 种不同的染色剂分别是醋酸双氧铀、0.05%乌龙茶提取物以及 0.1%乌龙茶提取物。首先将带有超薄切片样品的铜网悬浮于不同的待比较染液中 10−15 min，若需进一步用柠檬酸铅复染，则将经 3 次蒸馏水冲洗过后的铜网再次悬浮于柠檬酸铅染液中 8−10 min。【结果】复染铅的情况下，在透射电子显微镜下无论是大肠杆菌还是金黄色葡萄球菌，利用 3 种电子染色剂进行染色的结果均非常相似。【结论】实验结

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果表明，在观察细菌结构中，乌龙茶提取物可以替代醋酸双氧铀进行透射电子显微镜样品的电子染色。

关键词：乌龙茶提取物，醋酸双氧铀，细菌细胞，透射电子显微镜

1 Introduction

Uranyl acetate (UA) was first used by Watson as an electron microscopy stain in the 1950s[1] and has been widely applied in electron microscopies ever since. However, this staining solution, albeit effective, is unstable, highly toxic, and has become increasingly difficult to obtain due to strict regulations on internationally controlled nuclear materials[2-3]. Therefore, optimizing sample preparation and adjusting procedures to adhere to new health and safety regulations become a current challenge in electron microscopic examinations. To address these issues, sample preparation protocols are being developed for transmission electron microscopy (TEM) using polyphenolic compounds to substitute for the conventional UA method.

To resolve the previous mentioned problems, some researchers have done many tries. Kajikawa et al[4] used tannic acid as substitute, but tannic acid can't penetrate deeply into the tissue, they only used this method in thin sections. While Tanaka and Nakamura[5] compared four kinds of electron staining methods (tannic acid stain, orcein stain, palladium chloride stain, and iron-hematoxylin stain) for the observation of elastic fibers in ultrathin sections. They suggested that tannic acid staining was the best one. In 2003, Sato et al[6] got the results to show that OTE staining method proved to be a more useful than tannic acid staining method in observing lung tissue. And some years later, Carpentier et al[7] improved this method, they combined this method to microwaves in observing plants tissues. Even though OTE used as UA's substitute has been used in some areas, such as for observation animal tissues, plant tissues and microsporidia, they were still not let the sample preparation process become more time saving and easier. In the present work, we have optimized LR-White-assisted processing of different types of bacteria for ultrastructural observation. Oolong tea extract (OTE)[3,7-9], a mixture of polyphenolic compounds, is also explored as an alternative to UA for the staining of bacterial samples.

2 Materials and Methods

To evaluate the applicability of OTE as a substitute for UA staining, *Escherichia coli* (Gram-negative bacteria) and *Staphylococcus aureus* (Gram-positive bacteria) were selected as representatives.

2.1 Preparing the ultrathin sections for TEM

Bacterial cells were grown in Luria-Bertani (LB) broth overnight at 37 °C. The cells were collected by centrifuging at 3 000 r/min for 10 min, and then fixed in 2.5% glutaraldehyde in phosphate buffer (0.2 mol/L, pH 7.2) at 4 °C for 8 h. After rinsing with phosphate buffer for 5, 10, 15, 20, and 30 min, respectively, the specimens were post-fixed in 1% osmium tetroxide (in 0.2 mol/L phosphate buffer, pH 7.2) at 4 °C for 2 h. The samples were then rinsed again with phosphate buffer for 5, 10, 15, 20, and 30 min. The materials were dehydrated for 15 min in a series of ethanol solutions (30%, 50%, 70%, 80%, 90%, 100% (*V*/*V*)), and infiltrated overnight in a mixture of LR-White resin (London Resin Company, Reading, U.K.) and alcohol (1:1, *V*/*V*), followed by infiltration with pure LR-White resin twice (for 2 h and 1 h, respectively) at room temperature. Then pure LR-White resin was used for embedding and the samples were incubated at 60 °C for 48 h. Sections were obtained with a diamond knife on the Leica EM UC7 ultramicrotome (Leica, Nussloch, Germany), and picked up on the formvar coated grids.

2.2 Staining the ultrathin sections with different staining solutions

Sections on the grids were stained by floating them on single drops of the staining solution at room temperature following one of the protocols described below:

(1) 3% aqueous solution of UA for 10−15 min.

(2) 0.05% OTE (OTE powder; Suntory, Osaka, Japan) in 0.1 mol/L phosphate buffer (pH 7.2) for 30 min.

(3) 0.1% OTE in 0.1 mol/L phosphate buffer (pH 7.2) for 30 min.

After staining, the samples were rinsed with

deionized water and dried, and then divided each sample's sections into two groups. One group of the sections were directly subject to observation (single staining), while the remaining were post-stained with lead citrate (Pb) solution for 8−10 min (double staining).

2.3 Observing the ultrathin sections by TEM

All sections were examined under JEM-1230 transmission electron microscope (JEOL, Tokyo, Japan) at 80 kV, and all the images were recorded with side-inserted BioScan Camera Model 792 (Gatan, Pleasanton, Calif., USA).

3 Results and Analysis

3.1 Staining by different staining solutions

The ultrastructure of the bacterial cells were stained with standard TEM double staining (Figure 1a′ and 2a′), single staining (Figure 1a and 2a) and OTE-staining methods (Figure 1b, 1b′, 1c, 1c′, 2b, 2b′, 2c and 2c′). In this study, we focused on the contrast of the cell wall (outer membrane), peptidoglycan and the cytoplasmic membrane, which were most useful in detailed comparison of the contrast between the staining methods.

The results obtained with the OTE-staining methods at two concentrations were compared with those using obtained with UA (Figure 1a and 2a) or double staining with UA and Pb (Figure 1a′ and 2a′). The differences between these two bacteria could be observed in the sections by digital image acquisition without post-staining by Pb (i.e., single staining), but more markedly in the sections treated by Pb (i.e., double staining).

The surface structure of Gram-negative *E. coli* consisted of a thin peptidoglycan layer, and a clear dividing line was found in both the outer membrane and cytoplasmic membrane (Figure 1a and 1a′). Similarly, both concentrations of OTE have successfully distinguished the outer membrane, peptidoglycan layer and the cytoplasmic membrane (Figure 1b, 1b′, 1c and 1c′). However, the dividing lines appeared clearer with post-staining Pb treatment between outer membrane and peptidoglycan layer, or peptidoglycan layer and cytoplasmic membrane (Figure 1).

In contrary, the cell wall of Gram-positive *S. aureus* was composed of a thick peptidoglycan layer (Figure 2a and 2a′). Without post-staining Pb treatment, all ultrathin sections could not appear very clear boundary line. The components of *S. aureus* cell wall were barely distinguished. When post-stained with Pb, the components of *S. aureus* cell wall became distinguishable using both UA and OTE, though the septa was clearer in the image of section stained by UA+Pb double than OTE+Pb (Figure 1a′, 1b′ and 1c′). On the other hand, the OTE+Pb methods resulted in a better image quality compared to the UA+Pb protocol when the peptidoglycan component was concerned.

3.2 Two different embedding mediums

In this study, we used LR-White resin to simplify the process (Epon 812 need 27 steps, while LR-White only need 22 steps) and cut the preparation time in half (the total time for Epon 812 was 153 h and 40 min, but LR-White only need 77 h and 10 min) (Table 1).

4 Discussion

The staining effect on ultrathin sections can be affected by many factors, including the nature of the staining solution, the vehicle in which it is dissolved, the concentration, temperature, and pH of the staining solution. Some technologic factors, such as fixatives and embedding medium, could also take effect[10]. Previous studies[2,8-9,11] have indicated that OTE polyphenols could not enter the cells and cause injury to cell membrane, but they reacted with peptide bonds of cell wall and enhanced staining in TEM. This was also observed in our studies (Figure 1b′, 1c′, 2b′ and 2c′). Researchers[8] also pointed out that electron-dense filaments 10−12 nm in diameter were clearly seen with OTE dissolved in PBS (pH 7.4), but OTE aqueous solutions (pH 5.0) resulted in faint staining of electron-dense filaments due to the pH buffering provided by PBS. So in our study, we chose OTE dissolved in PBS (pH 7.4). In order to check whether the concentration of the staining solutions could affect the results of the electron staining or not, we designed two concentrations in this research, as a result, we found that 0.1% OTE was better than 0.05% OTE.

Although better staining results were obtained with high OTE concentration, excessively concentrate OTE solutions could damage the formvar. In the previous studies, it was found that the formvar was solubilized by excessive staining time or by exceedingly strong staining solutions. In an

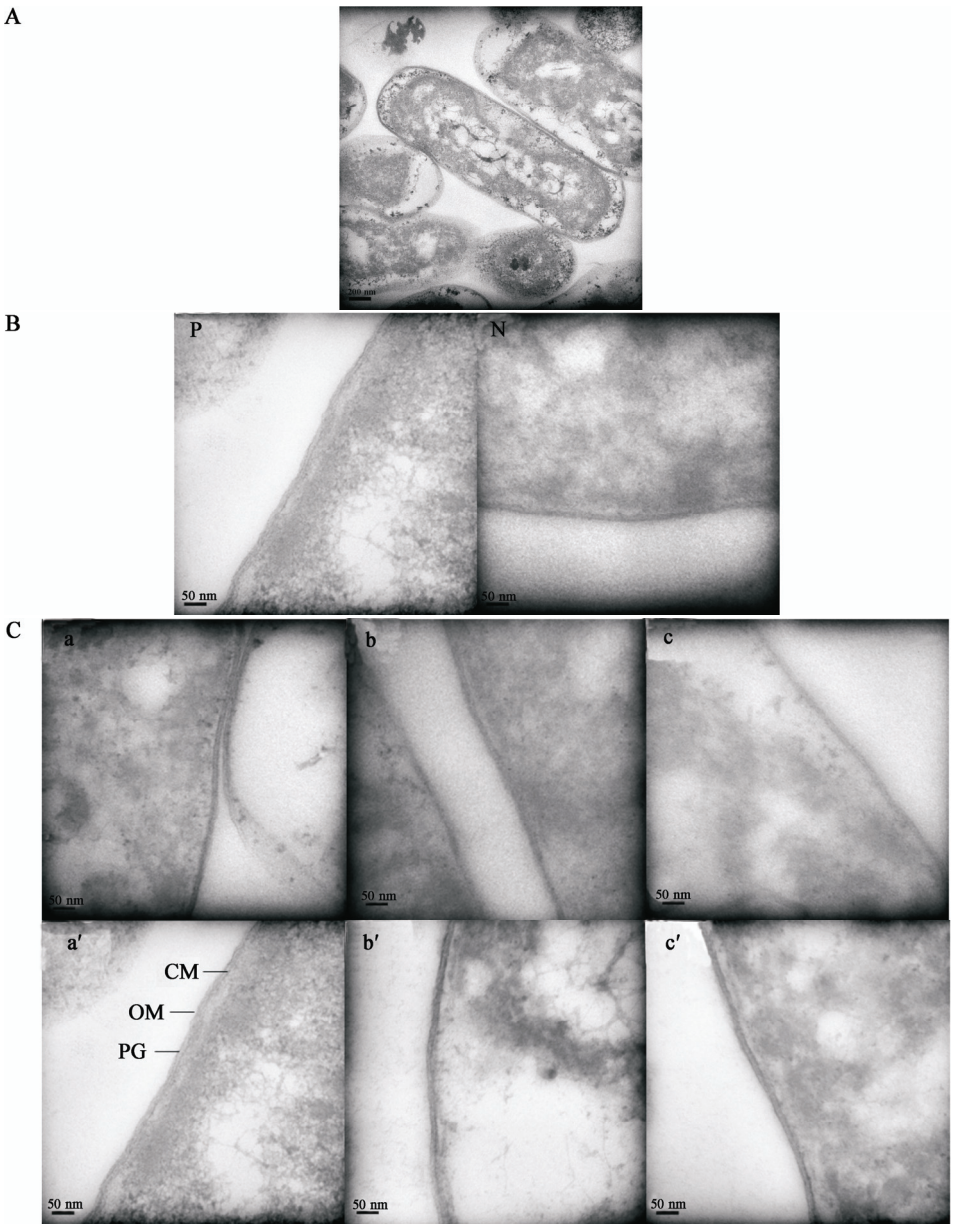

Figure 1 Ultrathin sections of *E. coli*

图 1 大肠杆菌的透射电镜图

Note: A: Ultrathin sections of *E. coli* stained with UA+Pb, which showed the over view of *E. coli*. The magnification was set at 50 000×. The scale bars represent 200 nm. B: Ultrathin sections of *E. coli* stained with UA+Pb (P, positive control), stained with nothing (N, negative control). The magnification was set at 200 000×. The scale bars represent 50 nm. C: Ultrathin sections of *E. coli* stained with UA (a), UA+Pb (a′), 0.05% OTE (b), 0.05% OTE+Pb (b′), 0.1% OTE (c), and 0.1% OTE+Pb (c′). Outer membrane (OM) components, including peptidoglycan (PG) and cytoplasmic membrane (CM), were clearly stained with all reagents in conjunction with post-staining Pb treatment, except that CM was not distinguishable with 0.05% OTE staining. The magnification was set at 200 000×. The scale bars represent 50 nm.

注：A：透射电镜下，传统方法染色的大肠杆菌整体图. 放大倍数：50 000×；标尺：200 nm. B：透射电镜下，传统方法染色的大肠杆菌图(P，阳性对照)，未染色的大肠杆菌图(N，阴性对照). 放大倍数：200 000×；标尺：50 nm. C：透射电镜下，醋酸双氧铀染色的大肠杆菌(a)、醋酸双氧铀及柠檬酸铅复染的大肠杆菌(a′)、0.05%乌龙茶提取物染色的大肠杆菌(b)、0.05%乌龙茶提取物及柠檬酸铅复染的大肠杆菌(b′)、0.1%乌龙茶提取物染色的大肠杆菌(c)以及 0.1%乌龙茶提取物及柠檬酸铅复染的大肠杆菌(c′). 除仅由 0.05%乌龙茶提取物单独染色不能很好区分质膜外，其他染料均可将外膜(OM)、肽聚糖层(PG)和质膜(CM)很好地区分开来. 放大倍数：200 000×；标尺：50 nm.

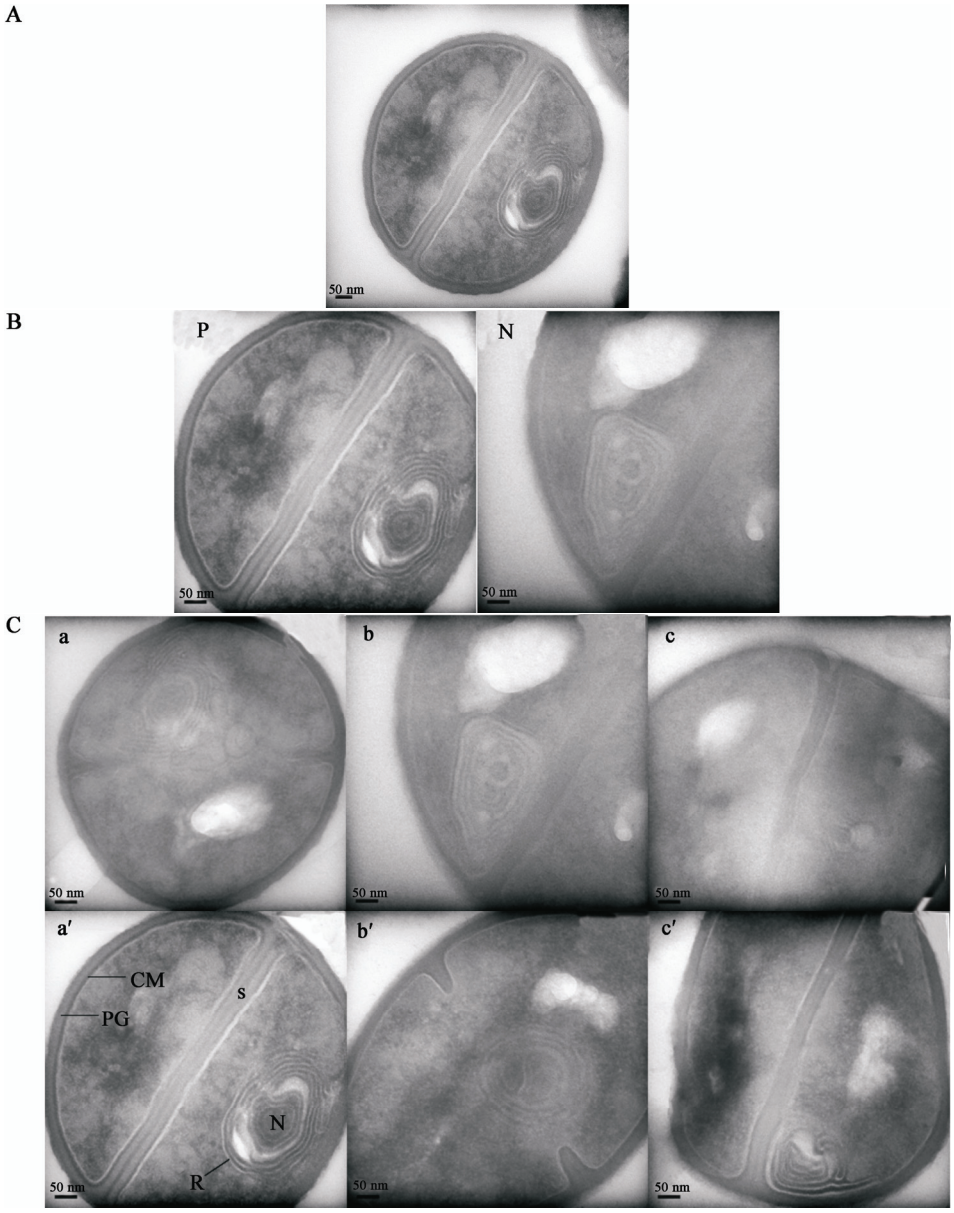

Figure 2 Ultrathin sections of *S. aureus*

图 2 金黄色葡萄球菌的透射电镜图

Note: A: Ultrathin sections of *S. aureus* stained with UA+Pb, which showed the over view of *S. aureus*. The magnification was set at 150 000×. The scale bars represent 50 nm. B: Ultrathin sections of *S. aureus* stained with UA+Pb (P, positive control), stained with nothing (N, negative control). The magnification was set at 200 000×. The scale bars represent 50 nm. C: Ultrathin sections of *S. aureus* stained with UA (a), UA+Pb (a′), 0.05% OTE (b), 0.05% OTE+Pb (b′), 0.1% OTE (c), and 0.1% OTE+Pb (c′). Peptidoglycan (PG), cytoplasmic membrane (CM), ribosome (R), nucleotide (N) and septa (S) were clearly stained with all reagents in conjunction with post-staining Pb treatment, except that CM was not easily distinguishable with OTE alone (at both concentrations). The magnification was at 200 000×, and scale bars were 50 nm.

注：A：透射电镜下，传统方法染色的金黄色葡萄球菌整体图. 放大倍数：150 000×，标尺：50 nm. B：透射电镜下，传统方法染色的金黄色葡萄球菌(P，阳性对照)，未染色的金黄色葡萄球菌(N，阴性对照). 放大倍数：200 000×；标尺：50 nm. C：透射电镜下，醋酸双氧铀染色的金黄色葡萄球菌(a)、醋酸双氧铀及柠檬酸铅复染的金黄色葡萄球菌(a′)、0.05%乌龙茶提取物染色的金黄色葡萄球菌(b)、0.05%乌龙茶提取物及柠檬酸铅复染的金黄色葡萄球菌(b′)、0.1%乌龙茶提取物染色的金黄色葡萄球菌(c)以及 0.1%乌龙茶提取物及柠檬酸铅复染的金黄色葡萄球菌(c′)。除以乌龙茶提取物单独染色不能很好区分质膜外，其他染料均可将肽聚糖层(PG)、质膜(CM)、核糖体(R)、核苷酸链(N)和隔膜(S)很好地区分开来. 放大倍数：200 000×；标尺：50 nm.

Table 1 Comparison the time and preparation process of samples embedded by LR-White and Epon 812 resins
表 1 LR-White 与 Epon 812 树脂包埋样品时间和制备过程的比较

Preparation process	Step number	LR-White resin		Epon 812 resin	
		Time	Reagent	Time	Reagent
Prefixation	1	8 h	2.5% Glutaraldehyde	8 h	2.5% Glutaraldehyde
Rinse	2	5 min	Phosphate buffer	5 min	Phosphate buffer
	3	10 min	Phosphate buffer	10 min	Phosphate buffer
	4	15 min	Phosphate buffer	15 min	Phosphate buffer
	5	20 min	Phosphate buffer	20 min	Phosphate buffer
	6	30 min	Phosphate buffer	30 min	Phosphate buffer
Postfixation	7	2 h	1% Osmium tetroxide	2 h	1% Osmium tetroxide
Rinse	8	5 min	Phosphate buffer	5 min	Phosphate buffer
	9	10 min	Phosphate buffer	10 min	Phosphate buffer
	10	15 min	Phosphate buffer	15 min	Phosphate buffer
	11	20 min	Phosphate buffer	20 min	Phosphate buffer
	12	30 min	Phosphate buffer	30 min	Phosphate buffer
Dehydration	13	15 min	30% Ethanol	15 min	30% Ethanol
	14	15 min	50% Ethanol	15 min	50% Ethanol
	15	15 min	70% Ethanol	15 min	70% Ethanol
	16	15 min	80% Ethanol	15 min	80% Ethanol
	17	15 min	90% Ethanol	15 min	90% Ethanol
	18	15 min	100% Ethanol	15 min	100% Ethanol
	19			15 min	Acetone
	20			15 min	Acetone
Infiltration	21	12 h	LR-White: Ethanol (1:1)	2 h	Epon 812: acetone (1:3)
	22	2 h	LR-White	5 h	Epon 812: acetone (1:1)
	23	1 h	LR-White	12 h	Epon 812: acetone (3:1)
	24			24 h	Epon 812
	25			24 h	Epon 812
Polymerization	26	48 h	60 °C	24 h	30 °C
	27			48 h	60 °C
Total time		77 h and 10 min		153 h and 40 min	

approximately 120% (*W*/*V*) methanol solution of hydrated UA, all the formvar was dissolved off the immersed grids within 15 min. In a 60% (*W*/*V*) solution, researchers found most of the formvar intact the immersed grids within same time, but tending toward fragility under the TEM beat by beam[12]. In light of these findings, we lowered the concentration of OTE and shorted the staining time (0.1% or 0.05% for 30 min), compared to 0.2% for 60 min used by Yamaguchi et al[10].

LR-White resin is thinner with a higher permeability than Epon 812 resin, which is frequently used for preparing ultrathin samples. However, LR-White could not fix the samples in good positions in the mold, due to a limitation in the mold design. In the future, more advanced mold design may serve to resolve this issue.

5 Conclusions

We have probed for optimal concentrations of OTE extract as an alternative staining reagent to UA in bacterial observation. The staining effect of OTE was also compared with that of UA in both single and double staining protocols. The results indicated although single and double staining with OTE could fully display all bacterial cell structures, some differences in the details could still be observed. For

example, with double staining, the OTE protocols resulted in more successful staining of peptidoglycan than the UA protocol. In the cytoplasm, the granularities were prominent in the images obtained after UA staining, whereas OTE staining provided a higher contrast. Moreover, considering the safety factor, OTE is much safer than UA. Therefore, OTE is a very promising substitute for UA in electron staining for TEM. In the meantime, substituting LR-White resin for Epon 812 resin provided simpler sample preparation within a relatively short time.

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