

Chromocult Coliform agar and duplex PCR assays as methodologies for tracking *Escherichia coli* K12 in industrial biotechnological processes

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ABSTRACT

The objective of this work was to establish support for the validation of the microbiological method associated with the duplex PCR assay for *Escherichia coli* K12 in waste and intermediate products of an industrial biotechnological process. For this purpose, samples of *Escherichia coli* K12 from critical control points known as *Killing System* (KS) and *Germ Filtration* (GF) were analyzed. Experiments to determine the microbial load of *E. coli* K12 based on the direct plating and filter membrane methods revealed the absence of this microorganism in the KS and GF samples. The chromogenic medium was able to recover strains of *E. coli* inoculated intentionally in GF samples, but the same result was not possible in samples from KS. The robustness of the incubation time of the KS and GF samples were calculated by the ANOVA test and the results showed no statistically significant difference between them (*p-value* 0.181 and 0.733). Duplex PCR analysis was shown to be able to differentiate a standard strain of *Escherichia coli* ATCC 8739 from *Escherichia coli* K12. The results will be used as a guideline for validation of a method for *E. coli* K12 tracking in an industrial biotechnological process.

INTRODUCTION

The technology of manufacturing biopharmaceuticals and other biomolecules can be divided into upstream and downstream processes. Upstream processes range from the selection of production strains, culture media, growth parameters to the optimization of ideal conditions for biomolecule production. Downstream phases include all steps required for the purification of a biological product

from the culture medium to the final purified product. It involves multiple steps to obtain the target molecule and remove from the host cell impurities (host cell proteins, DNA, among others), process-related impurities (buffers, defoamers, leaching binders and others) and products (aggregates and fragments, among others). Each purification step must be able to remove one or more classes of impurities (Azevedo *et al.*, 2009; Rathore and Kapoor, 2015; Jozala *et al.*, 2016). Downstream processes include (1) initial recovery (extraction or isolation), (2) purification (removal of major contaminants), and (3) cleaning (removal of specified contaminants and undesired forms of the target biomolecule that may have been formed during isolation and purification) (Rosa *et al.*, 2010; Fields *et al.*, 2016; Jozala *et al.*, 2016).

The production of recombinant proteins in biotechnological industries can be carried out with genetically

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modified organisms (GMOs), including those derived from *Escherichia coli* K12 (Simões *et al.*, 2016; Gandra *et al.*, 2017). Different lineages of *Escherichia coli* K12 are used in research and development in the field of biotechnology, as well as in industrial production (Kuhnert *et al.*, 1995; Simões *et al.*, 2016; Azpiroz and Lavinã, 2017). In Brazil, the national biosafety policy with regard to genetically modified organisms (GMOs, which includes *Escherichia coli* K12) is regulated by the National Technical Commission on Biosafety (Conceição *et al.*, 2006; Simões *et al.*, 2016).

According to Brazilian legislation, the risks and effects of upstream and downstream biotechnological processes arising from the release of GMOs into the environment must be evaluated. In this sense, research institutions and industries active in the biotechnology field must develop methods for the detection of GMOs in developed processes, in order to identify them in case of accidental release to the environment (Miralles *et al.*, 2009; Costa *et al.*, 2011; Simões *et al.*, 2016).

The detection of *Escherichia coli* can be affected through microbiological methods, including those based on culture media using chromogenic substrates (Turner *et al.*, 2000; Finney *et al.*, 2003; Maheux *et al.*, 2014). Chromocult® Chromogenic Agar (CCA) is a simple medium containing two chromogenic substrates for the detection of total coliforms and *Escherichia coli*. The chromogenic substrates are X-GLUC (5-bromo-4-chloro-3-indoxyl- β -D-glucuronide), 96% specific for identification of *Escherichia coli* (Frampton and Restaino, 1993; Turner *et al.*, 2000) and Salmon-GAL (6-chloro-3-indoxyl- β -D-galactoside). Because *Escherichia coli* present a positive reaction to both substrates, their colonies in the CCA medium vary from dark blue to violet. Members of the family *Enterobacteriaceae* which do not encode β -Glucuronidase (*Citrobacter*, *Enterobacter* and *Klebsiella*), being positive for β -galactosidase, present colonies varying from salmon to red in this culture medium. Others which are β -Glucuronidase positive, but β -galactosidase negative, such as *Shigella*, *Salmonella* and *Yersinia* produce turquoise blue colonies. Gram-negative bacteria that do not encode the β -Glucuronidase and β -galactosidase enzymes produce clear colonies in the CCA medium (Turner *et al.*, 2000; Finney *et al.*, 2003). Gram-positive bacteria and other non-enteric bacteria are inhibited by the presence of Tergitol-7. Further confirmation of the *Escherichia coli* can be accomplished by detecting indole by adding tryptophan to the medium, thereby increasing the reliability of *Escherichia coli* detection (Finney *et al.*, 2003).

Alternatively, the identification of *Escherichia coli* and other enteric bacteria can be obtained by molecular methods such as PCR (Polymerase Chain Reaction) for the detection of the *pal* gene which encodes a peptidoglycan-associated lipoprotein among the enterobacteria (Kuhnert *et al.*, 1995). Genetic identification of strains derived from *Escherichia coli* K12 can be obtained in a differentiated manner based on PCR detection of the presence of an IS5 insert in the gene cluster encoding Rhamnose transferase. The molecular method used presents a useful tool in the identification of *Escherichia coli* K12 for monitoring strains that are used as biologically safe vehicles in biotechnology research, development and production processes (Kuhnert *et al.*, 1995; Simões *et al.*, 2016). Simões *et al.* (2016) developed a method based on PCR for detection of *Escherichia coli* K12

carrying an ampicillin resistance gene in a pond of an effluent treatment station. The molecular method developed was preceded by a microbiological method, scattering in plates depending on cultivation, since the analysis is performed in order to monitor the presence of viable microorganisms transporting a gene marker for *E. coli* K12 according to recommendations of the National Biosafety technical Commission (Simões *et al.*, 2016).

The biotechnology industry where this work was conducted produces a recombinant protein on a large scale using *Escherichia coli* K12 as host. Any contaminated liquid or solid waste is decontaminated before being discarded, along with any material or equipment that has come into contact with the GMO, according to current legislation (BRASIL, 2006; Gandra *et al.*, 2017).

Microbiological methodologies associated with the molecular that allow the monitoring of the presence of the host *Escherichia coli* K12 in residues generated by industrial biotechnological processes are mandatory in Brazil. The objective of this study was to establish bases for validation of a microbiological methodology using the selective medium CCA associated with the duplex PCR assay for tracking the host *Escherichia coli* K12 in waste and intermediate product of an industrial biotechnological process.

MATERIALS AND METHODS

Bacterial lines and culture media

The strains used are *Escherichia coli* ATCC 8739 and *Escherichia coli* W3110 carrying genotyped plasmid pUC18, according to Simões *et al.* (2016). Pure cultures of the microorganisms used were previously quantified (approximately 5.0 of 10⁹ CFU/ml), preserved in 20% glycerol and stored at -80°C. The media Chromocult® Coliform Agar (Chromocult; Merck, UK) and Trypticase Soy Agar (TSA, BD-Difco™, USA) were prepared according to manufacturers' instructions. For selection of *Escherichia coli* W3110, with the presence of plasmid pUC18 the Chromocult® Coliform Agar medium was supplemented with ampicillin (Sigma-Aldrich, 200 μ g/ml).

Description and characterization of sample collection points

The samples analyzed in this study were composed of residual liquids and intermediate product obtained from a biotechnological process. The location and description of sample collection points are shown in Figure 1. Points 1 and 2 indicated in this figure correspond respectively to *Killing System* (physical system inactivator) and Sterilizing Filtration (stage for removal of the residual GMO from the final product). Samples were collected in triplicate from the *Killing System* (point 1) and Sterilizing Filtration (point 2) during the production of three different batches of a recombinant protein produced on an industrial scale.

Determination of the microbial load present in residuals and the intermediate product of an industrial biotechnological process

The amount of 100 μ L samples collected from point 1 (Figure 1) were inoculated in triplicate, using an automatic pipette directly on plates with the presence of CCA medium supplemented with ampicillin. The inoculation of 100 μ L of saline

solution used directly on plates containing the same medium was used as negative control. As a positive control, a suspension was used with a concentration of 100 CFU/100 μ l of *Escherichia coli* W3110 (Figure 2A). Plates were incubated at $32.5 \pm 2.5^\circ\text{C}$ for 24

hours. The whole procedure was carried out in a biological safety cabinet (Telstar, Bio II Advance) with due precautions against microorganisms from the air.

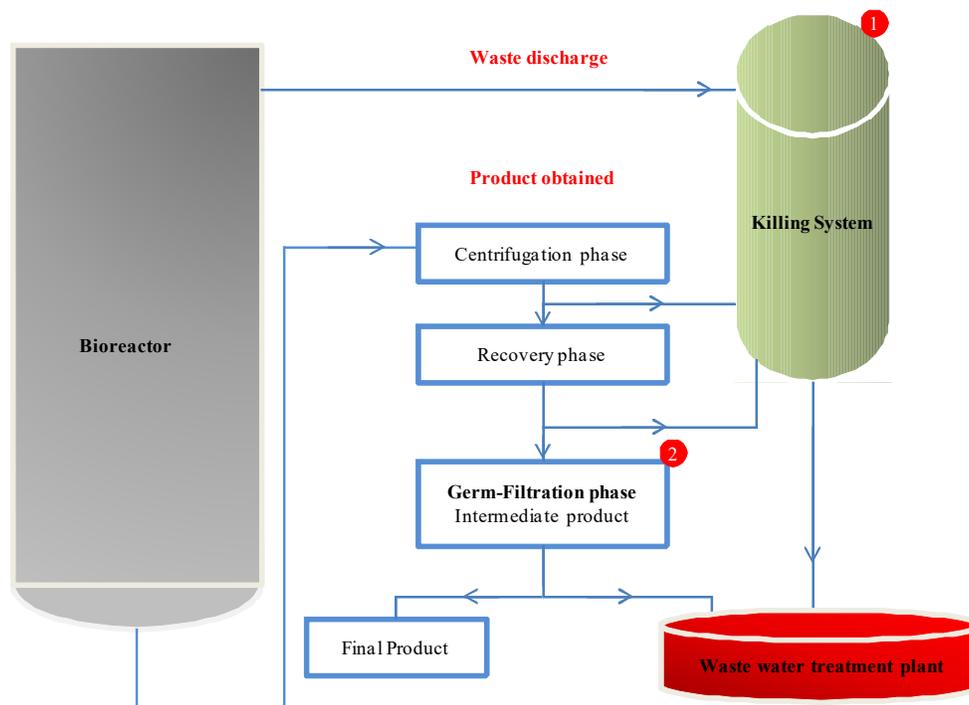


Fig. 1: Disposal flow and treatment of the wastes generated during and after the production of final recombinant product on an industrial scale. Point 1 corresponds to the *Killing System*, a system of thermal inactivation of residual liquids with a potential risk of the presence of genetically modified organisms. It is composed of tanks, which receive residual liquids from the industrial biotechnological process. Point 2 corresponds to the sterilizing filtration step (*Germ Filtration*) for removal of any microbial residue from the final product.

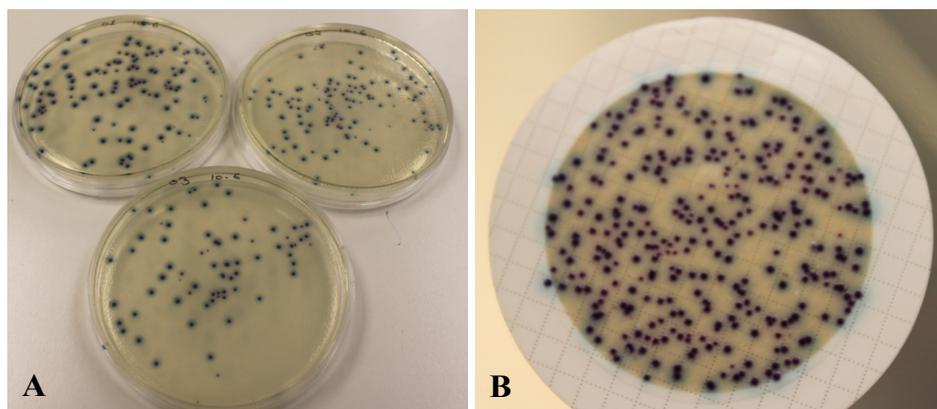


Fig. 2: Aspect of the growth of the colonies of *Escherichia coli* K12 in the medium Chromocult Cromogenic Agar (CCA). Panel A: Direct plating (scattering method) of the bacterial inoculum containing 100 CFU/100 μ l. Panel B: Filtration of the bacterial inoculum containing between 100 CFU/100 ml (filter membrane method).

In triplicate, 100 mL samples collected from point 2 (Figure 1) with the aid of a vacuum filtration system (EZ-STREAM vacuum pump and manifold EZ-FIT TRES, Merck Millipore) coupled to a membrane with 0.45 μ m pores. After filtration the membranes were added directly onto plates with the presence of CCA medium supplemented with ampicillin and incubated at $32.5 \pm 2.5^\circ\text{C}$ for 24 hours. The same methodology was followed for the negative control, where 100 mL of the saline solution was filtered. As a positive control, 100 mL of saline was intentionally

contaminated with a concentration of 100 CFU/100 ml of *Escherichia coli* W3110 (Figure 2B).

To determine the robustness of the incubation time the CFUs present in the plates with samples from points 1 and 2 were counted with the aid of the colony counter (CP600 plus, Phoenix) at times of 18, 21 and 24 hours. The results of the CFU count on the plates were recorded in an Excel spreadsheet and analyzed statistically.

Contamination and recuperation of *Escherichia coli* W3110 in samples of the *Killing System* and *Germ Filtration*

A standardized concentration of 100 CFU/100 ml of *Escherichia coli* W3110 was used in a contamination and recovery experiment on samples collected and previously sterilized from points 1 and 2 as indicated in Figure 1. Samples from point 1 after being intentionally contaminated with *Escherichia coli* W3110 were inoculated according to the direct plating method mentioned in the previous session. The reason for choosing the method used for the *Killing System* was because they have high viscosity, which makes filtration unfeasible. Samples from point 2 were subjected to the membrane filtration method according to the *United States Pharmacopoeia* (USP, 2016). Positive and negative controls were performed as mentioned in the previous session. To determine the robustness of the incubation time the CFUs present in the plates with samples from points 1 and 2 were counted with the aid of the colony counter (CP600 plus, Phoenix) at times of 18, 21 and 24 hours. The results of counting CFUs on the plates were recorded in Excel spreadsheet programmed to automatically calculate the recovery rate.

Calculation of Recovery rate and working range

The recovery rate (% R) of the microorganism intentionally inoculated in CCA culture medium was calculated by the following equation:

$$\% R = (\text{Mean of the test plate}) / (\text{Mean of control plate}) \times 100$$

The criterion was that the mean count of the microorganism should be in the range of 50 (lower limit) to 200% (upper limit) according to internal laboratory procedures and in accordance with the *United States Pharmacopoeia* (USP, 2016).

Duplex PCR assay

Colonies grown in TSA and CCA media supplemented with ampicillin were transferred directly to microtubes containing 100 µl of sterile saline (0.9% NaCl) and centrifuged at 8000 g for 5 min. The supernatant was discarded and the sediment was resuspended in 100 µl of sterile saline (0.9% NaCl). The suspension with microbial cells and nucleic acids was diluted 1:10 and 4 µl was used as template DNA for duplex PCR. The two distinct strains of *Escherichia coli* were submitted to the duplex PCR reaction with primers K12-L (TTCCCACGGACATGAAGACTACA), K12-R (ATCCTGCGCACCAATCAACAA), ECPAL-L (GGCAATTGCGGCATGTTCTTCC) and ECPAL-R (CCGCGTGACCTTCTACGGTGAC). The primers K12-L and K12-R generate fragments respectively of 1690 base pairs for *E. coli* K12 according to Kuhnert *et al.* (1995). A 280 base pairs fragment is expected with the amplification of ECPAL-L and ECPAL-R primers in any enterobacteriaceae (Kuhnert *et al.*, 1995). All primers were synthesized by *Integrated DNA Technology*, USA. Reactions were performed in duplex PCR with the 2x reagent *Gotaq Green Master Mix*® (Promega, Corporation, USA), MgCl₂ (2.5 mM), 10 µM of each primer and 4 µl of microbial extract in a final reaction volume of 50 µl. The amplification conditions were those indicated by Kuhnert *et al.* (1995) for each primer. The amplicons were visualized on 1.5% agarose gel stained with ethidium bromide and photodocumented. As a positive control of the PCR reaction, standard strains were

use of *E. coli* K12 W3110 and *E. coli* ATCC 8739, maintained in the laboratory. Water was used as a negative control.

Statistical analysis

The means and standard deviations of the results of the CFU counts and recovery rate obtained in the experiments were calculated. For the analysis of robustness of the incubation time of the samples, the ANOVA statistical test with 95% significance was performed using Minitab.V16 software. Tables and charts were generated in Excel.

RESULTS AND DISCUSSION

A total of 36 samples, 18 of which were collected from the *Killing System* and 18 from the *Germ Filtration* (points 1 and 2 of Figure 1) were used as a source of microorganism isolation. In addition, CCA supplemented with ampicillin was used in experiments of intentional contamination with *E. coli* K12 and verification of recovery rate in medium.

Colony growth was not obtained in the samples isolated in points 1 and 2 shown in Figure 1. The results from the 18, 21 and 24-hour plate readings were 0 CFU. The positive and negative controls of the test presented results within that expected (growth and absence of growth, respectively). This result was expected and confirms the efficiency of the treatment of industrial liquid waste by the *Killing System* before being introduced into the Effluent Treatment Station.

Information on nutritional conditions, physical parameters for growth and thermal inactivation of bacteria, among which are those derived from *Escherichia coli* K12 are well established in the literature (Cornet *et al.*, 2010; Derlinden *et al.*, 2007; Fogolari *et al.*, 2012). According to internal procedures of the biotechnology industry where this study was carried out, the *Killing System* operates at a temperature of 121°C and among the residues received are those containing sodium hydroxide (from tank cleaning), which renders the environment alkaline and makes microbial growth difficult, and especially for *E. coli* K12.

Samples collected from *Germ Filtration* were those that underwent the sterilizing filtration process on a 0.22 µm filter capable of retaining bacteria which includes *E. coli* K12. The absence of growth of microorganisms in samples from the *Germ Filtration* also confirms the efficiency of the system in eliminating microorganisms from the final biotech product.

Lineages of *E. coli* are considered standard for measuring the quality of treated waste water from industrial waste (Bower *et al.*, 2005; Frigon *et al.*, 2013). The literature presents references on the microbial load found in waste sent to effluent treatment stations (Wang *et al.*, 2014; Li *et al.*, 2010; Martinez, 2009). Information about validations of industrial systems that use wet steam as a sterilization process are found in the literature (ISO11138-1, 2017; Mannermaa and Yliruusi, 1993) and the microorganism *Geobacillus stearothermophilus* is the main bioindicator of the efficiency of the sterilization process (Huesca-Espitia, 2016; Lundahl, 2003; McLeod, 2017). However, up to the present moment it has not been possible to verify information related to the determination of the surviving microbiota present in industrial scale systems for physical inactivation similar to the one used in this study. The literature revealed that this is the first work that uses a technique based on culture in selective medium

and genetic analysis by PCR for tracing *Escherichia coli* K12 in industrial waste before being sent to an effluent treatment plant. In previous work, a methodology for tracking *E. coli* K12 was standardized; however, the samples came from an effluent treatment plant pond, following upstream and downstream steps of a biotechnological process (Simões *et al.*, 2016).

In order to verify that the *Killing System* would be conducive to the growth of *E. coli* K12, samples taken from it were intentionally contaminated with a known concentration of this microorganism (100 CFU). Prior to contamination the samples were autoclaved and inoculated only after cooling to room temperature. Negative and positive controls were used. There was no *E. coli* K12 in the contaminated samples seeded in CCA medium and consequently the recovery rate was 0% compared to the positive control (Figure 3A). In alignment with the result obtained with the experiment for determination of the microbial load of the *Killing System* it was observed that the environment became inappropriate to the growth of the inoculated microorganism. PH measurements of the three sample batches of the *Killing System* confirmed an alkaline environment (pH \geq 9), in addition to the presence of detergent compounds potentially present in the waste disposal and treatment environment.

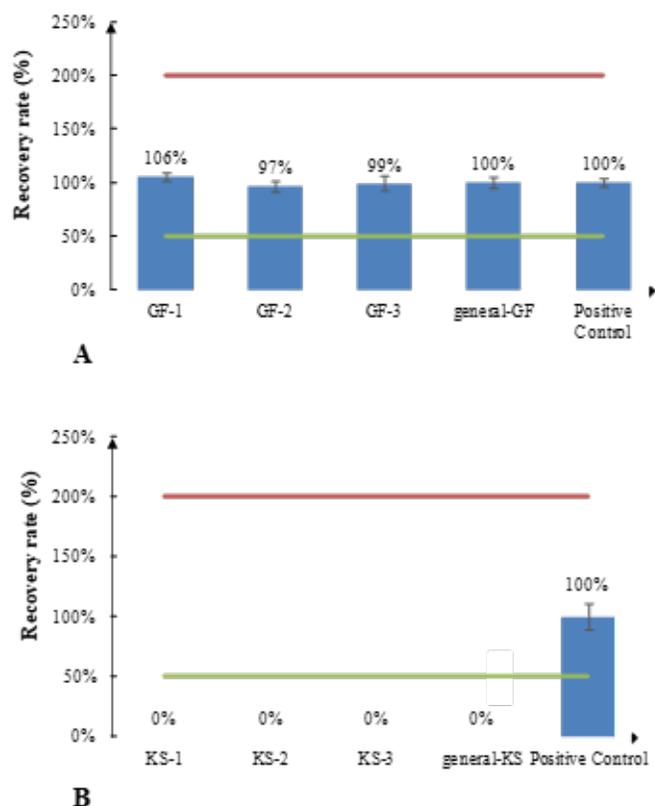


Fig. 3: Percentage of recovery rate of *Escherichia coli* K12 inoculated into samples from *Killing System* (KS) and *Germ Filtration* (GF). Panel A: Samples from 3 different batches of the contamination/recovery experiment (KS-1 to KS-3). The result of the overall mean between the batches is shown in the figure (general-KS), as well as the positive control (*E. coli* K12 inoculated in saline solution). Panel B: Samples from 3 different batches of the contamination/recovery experiment (GF-1 to GF-3). The result of the overall mean between the batches is shown in the figure (general-GF) as well as the positive control (*E. coli* K12 inoculated in saline solution).

Samples of the process of *Germ Filtration* were also contaminated with *E. coli* K12 and the results showed that it was possible to recover the amount of this microorganism inoculated within the expected range (Figure 3B). When comparing the test results with the positive control the CCA medium supplemented with ampicillin was able to recover *E. coli* K12 previously inoculated into samples from *Germ Filtration*. The results showed that the intermediate product has an environment favorable to microbial growth and especially the *E. coli* K12. However, the results of the microbial load experiments revealed that the sterilizing filtration step was efficient in eliminating microorganisms during the routine of the biotechnological process.

The robustness of the incubation times of the *Killing System* and *Germ Filtration* used in the contamination and recovery tests were statistically evaluated. Although there were slight variations between the 18, 21 and 24-hour counts, there were no statistically significant differences between the above samples. The *p*-value for samples of the *Killing System* was 0.181 and for *Germ Filtration* 0.733. Similar results were found in contamination and recovery of *E. coli* K12 when used in saline solution (Gandra *et al.*, 2017).

Qualitative and quantitative methods for screening for GMOs based on the detection of nucleic acids are described in the literature (Kuhnert *et al.*, 1995; Simões *et al.*, 2016; Cardoso *et al.*, 2017). Although the observed results show the efficiency of the *Killing System* and *Germ Filtration* to eliminate or prevent the growth of *E. coli* K12 in the tested samples, the development of methods for identifying GMOs is a mandatory activity in biotechnological industries (Simões *et al.*, 2016). The need to develop methods capable of differentiating wild *E. coli* (commonly found in industrial waste) from those derived from *E. coli* K12 is salutary in biotechnology industries. A duplex PCR assay was therefore standardized in samples of *E. coli* K12 grown in CCA medium, since it is the culture medium that will be used for screening and tracking of *E. coli* in discard samples and the final product of the biotechnological process. Figure 4 shows the genetic identification of the strain *E. coli* K12 where the expected profile for this microorganism was found (Kuhnert *et al.*, 1995; Simões *et al.*, 2016). The lineages *E. coli* K12 W3110 and *E. coli* ATCC 8739 presented distinct profiles that could be differentiated from each other by the duplex PCR technique standardized in this study. The 1690 base pair PCR product related to the insertion of the IS5 sequence into the gene encoding for Rhamnose Transferase in *Escherichia coli* K12 (Kuhnert *et al.*, 1995) was only visualized in the strains of *E. coli* K12 W3110 (Figure 4). In both strains a fragment of 280 base pairs corresponding to the gene *pal*, expected marker of the family to which these bacteria belong (Kuhnert *et al.*, 1995).

CONCLUSION

The method of analysis based on microbiological and molecular techniques for tracking *E. coli* K12 in the biotechnological process was developed in this study. The results confirmed the efficiency of the *Killing System* and *Germ Filtration* in ensuring that residuals and final product derived from recombinant DNA technology are free of GMOs. The microbiological method used based on direct plating or filter membrane in CCA culture medium was able to promote the

growth of the strain *E. coli* K12. The duplex PCR methodology allowed the distinction between a species of *E. coli* K12 and one of *E. coli* ATCC. To our knowledge, this is the first time that a microbiological methodology has been described in the literature using the selective medium CCA associated with the duplex PCR assay for monitoring the presence of the host

Escherichia coli K12 in the intermediate product and wastes of an industrial biotechnological process. The observed results will serve as a guideline for the validation of a *E. coli* K12 in routine biotechnological process, as well as in case of accidental disposal of this GMO.

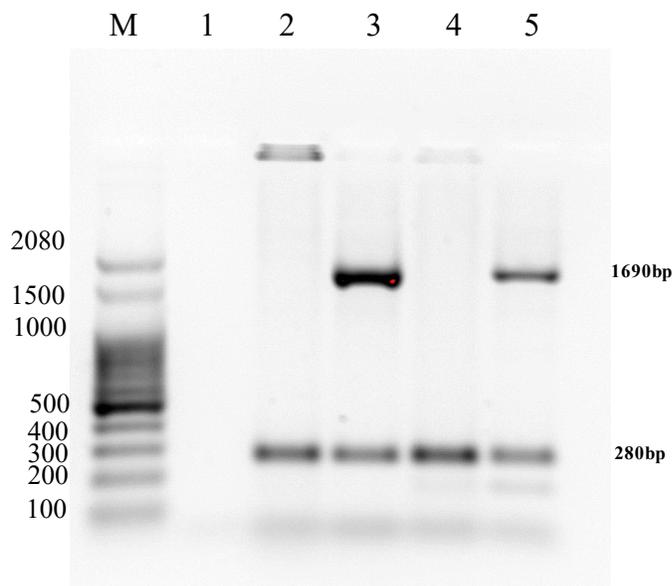


Fig. 4. PCR duplex for identification of *E. coli* K12 W3110 maintained in the laboratory and subcultured on CCA medium. M: 100 bp molecular weight marker (Ludwig). Line 1: negative control (primers K12L/K12R; ECPAL-L/ECPAL-R, no DNA). Lines 2 and 3: positive controls (*Escherichia coli* ATCC8739 and *Escherichia coli* W3110 respectively maintained in TSA medium amplified with primers K12L/K12R; ECPAL-L/ECPAL-R). Lines 4 and 5: *Escherichia coli* ATCC8739 and *Escherichia coli* W3110 maintained in CCA medium amplified with primers K12L/K12R; ECPAL-L/ECPAL-R. The sizes of the expected fragments amplified with these primers are shown to the right of the 1.5% electrophoresis gel.

AUTHORS CONTRIBUTIONS

Gisele d'Angelis Antunes, Janine Aparecida Correia Durães Gandra, and Alessandra Rejane Ericsson de Oliveira Xavier contributed equally to this work.

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CONFLICT OF INTEREST

There are no conflicts of interest to declare.

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