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Ethanol limits *E. coli* growth kinetics and does not increase rChiC protein yield

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SUMMARY Chitin is an abundant polysaccharide which is an integral structural component in exoskeletons of plant-harming insects. Chitin can be degraded into its carbon and nitrogen-based components to be recycled by chitinase secreting organisms. *Pseudomonas aeruginosa* is a chitinolytic soil microbe known to express chitinase C (ChiC). ChiC has been a target for biopesticide design as ChiC has the potential to kill insects without the polluting effects of traditional chemical pesticides. Previous researchers have successfully cloned chiC from P. aeruginosa PAO1 into the pET-28a vector to generate a recombinant ChiC (rChiC) expression plasmid, pM3CRYY. This paper aims to optimize the yield of rChiC in *Escherichia coli* BL21(DE3) by inducing a heat shock response through ethanol supplementation. Growth curves generated with *E. coli* grown under 1%, 3% and 5% ethanol conditions revealed that ethanol decreases growth parameters. Then, SDS-PAGE analysis of pM3CRYY transformed *E. coli* BL21 (DE3) whole cell lysates showed increased rChiC expression under 1% ethanol conditions, but not under 3% or 5% ethanol conditions. Furthermore, western blot analysis probing for DnaK, which is a known heat-shock associated molecular chaperone, revealed that DnaK is only upregulated when induced with 5% ethanol. Although our findings suggest 1% ethanol increases rChiC yield, further research should be done on understanding its mechanism, optimizing growth conditions and harvesting times, and probing for other heat shock proteins. Altogether, this study offers insight into the use of ethanol as a heat-shock inducing agent in *E.coli* BL21 (DE3) that could potentially be used to optimize rChiC protein yield in large-scale production for industrial use.

INTRODUCTION

Chitin is a eukaryotic amino sugar polymer that is a major structural component of many organisms, including the exoskeleton of insects (1, 2). As a result, chitin is a promising target for pesticide design due to its integral role within plant-harming insects (1). Chitinases are enzymes in bacteria which are able to degrade chitin by hydrolyzing polymeric chitin into GlcNAc subunits (3, 4). In *Pseudomonas aeruginosa* PAO1, chitinolytic enzyme chitinase C (ChiC) has shown to exhibit chitinase activity (4). To use ChiC for broader commercial and industrial applications such as repurposing as a biopesticide, robust methods for ChiC isolation, expression, and activation need to be established. To this end, previous work has developed a plasmid, pM3CRYY, encoding a recombinant ChiC (rChiC), which has been successfully isolated and transformed into *Escherichia coli* BL21 (DE3) (5–8).

Proper protein folding of recombinant proteins is crucial for industrial applications, as protein solubility is an important factor in maximizing the yield of functional proteins in high-density culture microbial systems (9, 10). Saturating concentrations of recombinant proteins has been shown to have a deleterious impact on protein functionality, characterized by the occurrence of protein aggregation, misfolding, and precipitation (9, 10). Optimization studies have shown that a low isopropyl β-D-1-Thiogalactopyranoside (IPTG) concentration, a low incubation temperature, and longer incubation times optimize soluble rChiC expression (11). However, there may be additional parameters which increase rChiC yield in *E. coli*. A potential avenue for optimization is through supplementing growth media with chemical additives to enhance protein folding (12).

The heat shock response in bacteria has been documented to protect native proteins from misfolding and aggregation (13). Heat shock proteins, which include molecular chaperones, play a key role in this defense mechanism as they drive folding processes and prevent protein

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aggregation under osmotic or heat-induced cell stress (13). In bacteria, heat shock-associated upregulation of chaperone protein expression has been shown to increase recombinant protein solubility (13). In *E. coli*, ethanol has been documented to mimic a heat shock response characterized by the upregulation of chaperone proteins such as DnaK (14–16). Studies have determined that adding concentrations of ethanol between 2-3% to growth media elicits the highest recombinant protein production in *E. coli* (14, 15, 17).

In this study, we aim to validate these past findings by using recombinant *P. aeruginosa* chiC expression in *E.coli* BL21 (DE3) as a model to demonstrate the utility of ethanol supplementation as a strategy to increase recombinant protein production through the induction of heat-shock response. We hypothesize that the chaperone proteins associated with an ethanol induced heat shock response will increase the solubility of rChiC at concentrations within 1-3%. Specifically, we demonstrate the induction of a heat-shock response through the upregulation of DnaK chaperone protein. This study offers insight into the use of ethanol as a heat-shock inducing agent in *E.coli* BL21(DE3) which could be used to optimize recombinant protein yield in industrial high-density cultures.

METHODS AND MATERIALS

Growth curve assay. An overnight culture of *E. coli* BL21(DE3) cells transformed with pM3CRYY was used to inoculate 200 µl of LB medium containing kanamycin (25mg/mL) with ethanol concentrations ranging from 0% to 5% in a 96-well plate. A subset of cultures was supplemented with ampicillin (25mg/ml) as a negative control for *E. coli* growth. The 96-well plate cultures were incubated with orbital shaking at 37C for 24 hours, and A_{600} readings were measured at 30-minute intervals using a BioTek Epoch Microplate Spectrophotometer (Agilent). Five technical replicates were used for data analysis in R using the 'tidyverse' package (v2.0.0).

Bacterial Culture and Ethanol Treatment. One colony of *E. coli* BL21(DE3) transformed with pM3CRYY or pET28 was grown as a starter culture overnight (200 rpm, 37°C) in 6 mL LB-kanamycin (50ug/ml) in 50 mL flasks. Following overnight incubation, cultures were inoculated 1:100 in LB-kanamycin (200 rpm at 37°C), with 1 flask per colony. When each flask reached an OD_{600} of approximately 0.4, IPTG was added to a final concentration of 0.1mM and ethanol was added to a final concentration of 0-5%. Samples were incubated on the 37°C shaking incubator at 200 rpm for 24 hours.

Cell Lysis. Cultured cells were aliquoted into 250 mL oakridge tubes and spun down at 5000 rpm at 4°C for 8 minutes in a Beckman Coulter Avanti J-30I Centrifuge. Pelleted cells were then resuspended in 20 mL 1x TBS. Subsequently, the cell resuspension was aliquoted into 2 mL screw cap microtubes with 0.1g of 0.1mm glass beads (BioSpec Products, Inc). The bead beater homogenizer (MP FastPrep®-24) was used to lyse the samples at 4 m/s for 60 seconds, and the supernatants the whole cell lysates were stored at -20C.

SDS-PAGE. 2x Laemmli with 10% BME (Bio-Rad) was prepared by combining 100 uL of BME with 900 uL of 2x Laemmli Sample Buffer. Subsequently, protein in the whole cell lysates were denatured by heating at 100°C for 15 minutes. Aliquots of each sample were prepared at a 1:1 ratio in 2x Laemmli buffer with 10% BME to a final volume of 100 uL. Samples were loaded into a premade 4–20% Mini-PROTEAN® TGX Stain-Free™ Gels (Bio-Rad) and run at 150 V for 10 min and then 200 V for 20 - 25 min with 1X TRIS-base/Glycine/SDS running buffer. Loading volumes were calculated by normalizing total protein by OD_{600}. 10 µL of PageRuler Plus Pre-Stained Protein Ladder (ThermoFisher) and 10µL Precision Plus Protein™ Unstained Protein Standards Ladder (Bio-Rad) were ran on the gel. The SDS-PAGE gels were imaged using the ChemiDoc Imaging System (Bio-Rad).

Western Blot. The proteins from the SDS-PAGE gels were transferred to a nitrocellulose membrane using Trans-Blot Turbo transfer packs (BioRad, Cat#: 1704156) and the Trans-blot Turbo transfer system (BioRad) by running at 1.3A, 25V, for 7 min. Membranes were blocked with 1x TBS-T + 1% bovine serum albumin (Sigma-Aldrich, Cat#: A7906-500G) (1

hour, shaking at room temperature). Then, membranes were incubated (overnight, shaking at 4ºC) in 6-7 mL of a 1:1000 anti-DnaK antibody (Invitrogen, Cat#: MA1-21315) diluted in 1x TBS-T + 1% bovine serum albumin. After incubation, the membranes were washed for 3 rounds in 1x TBS-T for 5 min each. Membranes were further incubated in 15 mL goat-anti-mouse-IgG HRP-conjugated secondary antibody (ThermoFisher, Cat#: 31430) diluted 1:100000 in the blocking buffer (1 hour, shaking at room temperature). Three more washes in 1x TBS-T (5 min each) were performed. Immunoblots were visualized by incubating membranes in Clarity Western ECL substrate reagents (BioRad, Cat#: 170-5060) for 5 minutes. The Chemidoc MP Imager (BioRad) was used to visualize membranes.

Statistical analyses. Statistical tests were performed in R using the 'rstatix' package (v0.7.2). Statistical significance was determined by Tukey's test with Holm-Bonferroni adjustment after Kruskal-Wallis one-way ANOVA testing. Adjusted p-values less than 0.05 were considered statistically significant.

Code availability. All data analysis scripts are available here: https://github.com/felicialf/MICB471.

RESULTS

Differences between ethanol-treated *E. coli* BL21(DE3) culture densities decrease over time. When optimizing the efficiency of recombinant protein production in microbial systems, it is important to consider factors such as the host growth rates and maximal culture densities supported, as these parameters can greatly impact overall efficiency (5). We investigated the impact of ethanol on *E. coli* growth parameters by growing *E. coli* BL21(DE3) transformed with pM3CRYY under a range of titrating ethanol concentrations (0% to 5%) in a 96-well plate. We measured OD_{600} values at 30-minute intervals over 24 hours to generate a growth curve (n=5) (Fig. 1). We found that ethanol concentrations

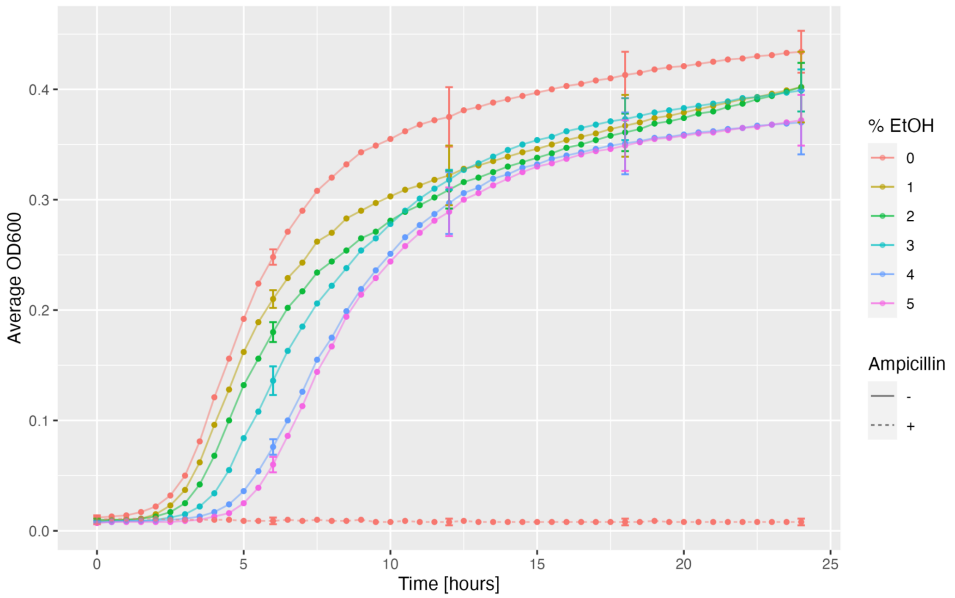

FIG. 1 Growth curves for *E. coli* BL21(DE3) under titrating ethanol concentrations. Line plots show average OD600 over 24 hours for *E. coli* BL21(DE3) cells transformed with pM3CRYY grown under titrating ethanol concentrations at 37°C. Individual points represent the mean OD600 across technical replicates taken at 30-minute intervals, n=5. Error bars express 95% confidence intervals (mean ± standard deviation). Continuous lines represent *E. coli* BL21(DE3) grown in media without ampicillin, whereas dashed lines represent cells grown in media supplemented with ampicillin (50ug/ml). Lines and individual points are colored based on ethanol concentration.

ranging from 1% to 5% significantly reduced the average culture densities of *E. coli* BL21(DE3) at 6-, 12-, and 18-hour post-incubation time points, compared to cultures grown without ethanol (0%) (Fig. 2). However, at the 24-hour post-incubation time point, only ethanol concentrations ranging from 4% to 5% significantly reduced the average culture densities of *E. coli* BL21(DE3) compared to cultures grown without ethanol (0%) (Fig. 2). Moreover, we observed a concentration-dependent decrease in the average culture densities of *E. coli* BL21(DE3) with increasing ethanol concentrations (Fig. 1). Overall, differences between average culture densities across the range of ethanol concentrations decreased over time (Fig. 1, 2).

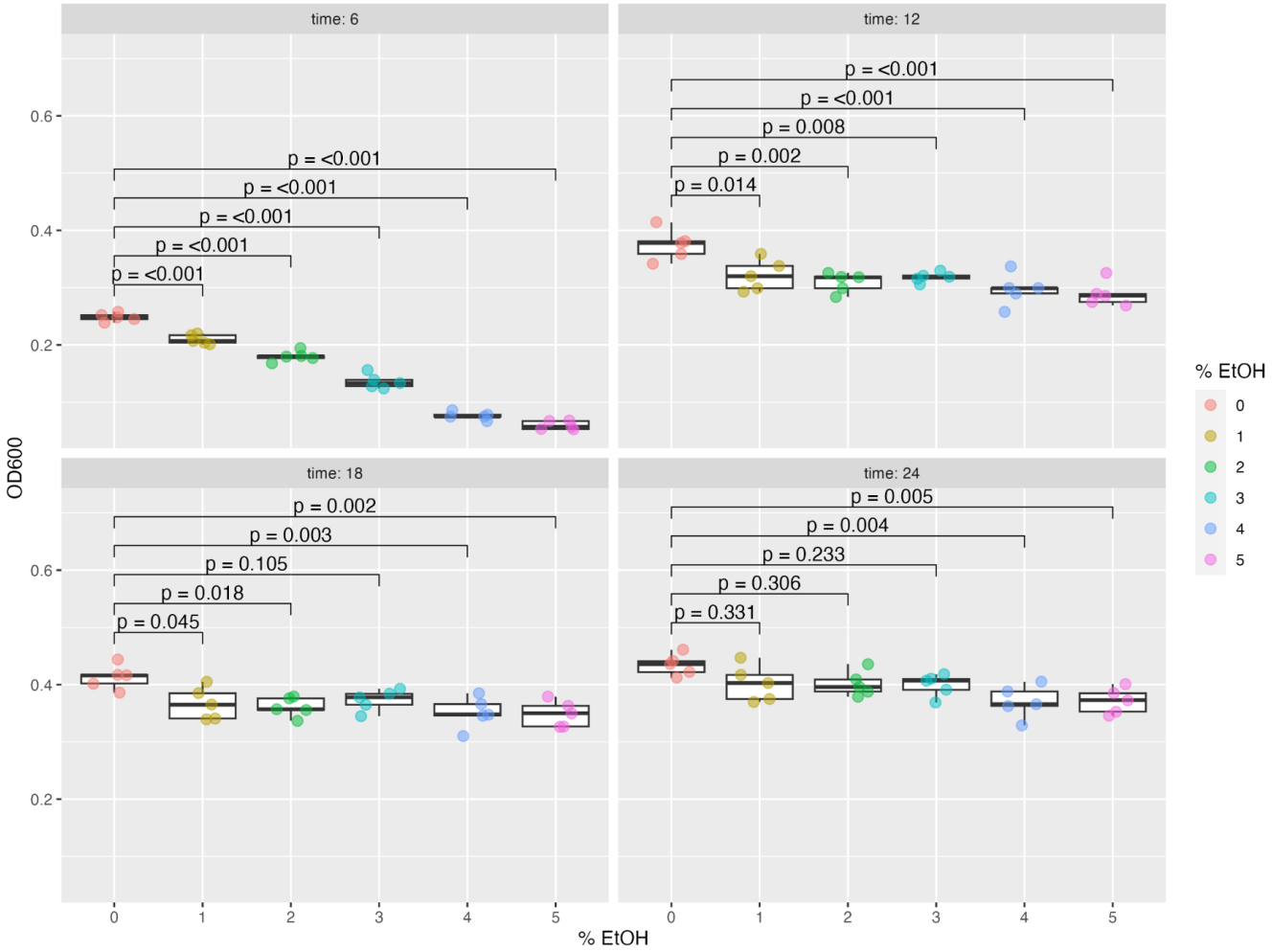

FIG. 2 Ethanol decreases the average culture densities of *E. coli* BL21(DE3). Box plots show the OD600 values for *E. coli* BL21(DE3) transformed with pM3CRYY grown under titrating ethanol concentrations at 6-, 12-, 18-, and 24- hour post-incubation time points. Comparisons show adjusted p-values from Tukey's test with Holm-Bonferroni adjustment after Kruskal-Wallis one-way ANOVA testing, n=5. Boxes denote the interquartile range (IQR), and the median is expressed as a bolded horizontal bar. Whiskers extend to 1.5X IQR, and raw values are shown as individual points colored based on ethanol concentration. Negative control ampicillin treated samples were excluded from this analysis.

We observed a decrease in the maximum growth rates of *E. coli* BL21(DE3) at ethanol concentrations ranging from 1% to 5% (Fig. S1). We also found that the growth rates of *E. coli* BL21(DE3) cultured in 0-5% ethanol, began to converge at approximately the 16-hour post-incubation time point (Fig. S1). Collectively, our findings indicate that ethanol exerts a detrimental effect on BL21(DE3) *E. coli* growth parameters within the 0- to 18-hour post-incubation time frame. However, at the 24-hour post-incubation endpoint, comparable culture densities and growth rates were observed in 0-3% ethanol concentrations.

1% ethanol treatment leads to increased rChiC production in *E. coli* BL21(DE3). Previous studies have determined that supplementing growth media with ethanol can increase recombinant protein production in *E. coli*, with 2-3% concentrations maximizing protein yield (15,16,19). To investigate whether varying ethanol concentrations in *E. coli* growth media could elicit differential yield of rChiC, we used IPTG to induce expression of rChiC through the T7 promoter of *E. coli* BL21(DE3) transformed with pM3CRYY. The supernatants taken from the whole cell lysates produced protein bands corresponding to rChiC at 55 kDa (Fig. 3A). No substantial band at 55kDa was observed in whole cell lysates from *E. coli* BL21(DE3) transformed with pET-28a (Fig. 3A). Interestingly, the 1% EtOH sample showed an increase in rChiC yield relative to the 0% EtOH sample, while the 3% and 5% treatments resulted in minor decreases in rChiC yield. However, differences in background signal amongst the lanes suggest uneven loading of the samples. After subtracting the background signal per sample to normalize for pixel densitometry (ImageJ), only the 1% lane showed approximately 2-fold increased yield of rChiC, while the 3% and 5% treatment groups showed no change relative to the 0% EtOH treatment group (n=2) (Fig. 3B).

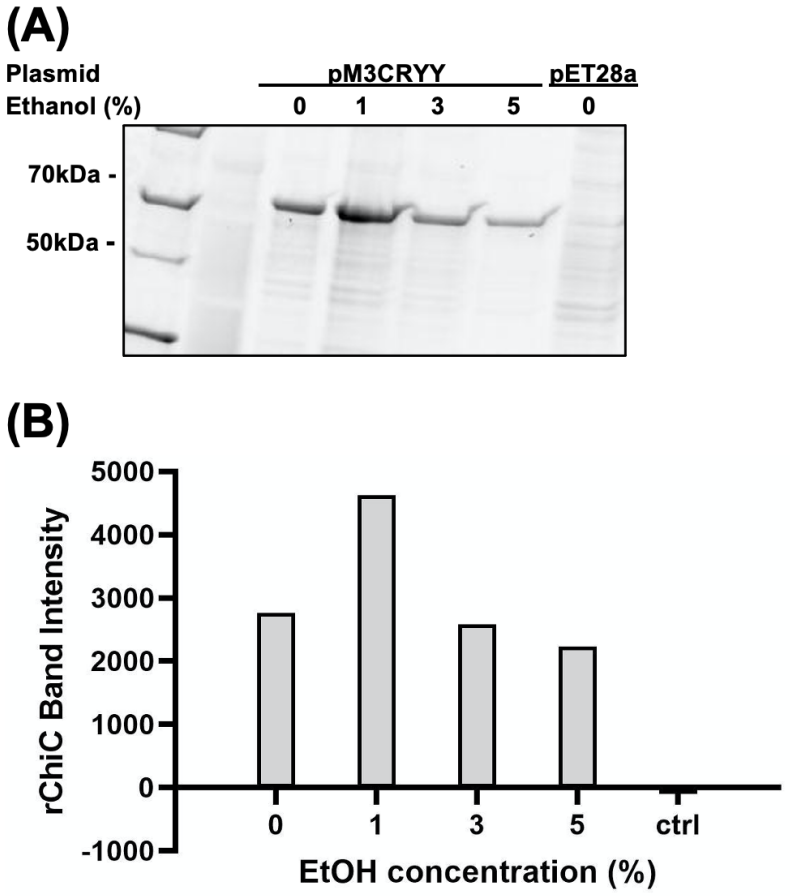

FIG. 3 1% ethanol treatment leads to increased rChiC production in *E. coli* BL21(DE3). (A) Representative SDS-PAGE gel showing whole cell lysates from *E. coli* BL21(DE3) transformed with pM3CRYY. Cells were treated with 0.1mM IPTG and concentrations of ethanol ranging from 0-5% for 24 hours (37°C, shaking at 200 rpm). Loading was controlled according to OD600 readings from each sample and protein bands were resolved on a 4–20% stain-free gel. Ethanol concentrations and plasmids are indicated above the gel image. Whole cell lysates from E. coli BL21(DE3) transformed with pET28a empty vector was used as a negative control for rChiC expression. (B) ImageJ quantification of Figure 3A protein bands. Background signal from each lane was subtracted from the band density quantification to normalize each band to the background. Band densities were averaged between samples, n=2.

5% ethanol in growth media increases DnaK expression to demonstrate a heat shock response in *E. coli* BL21(DE3). Moreover, ethanol has been documented to induce a heat shock response bacteria characterized by the upregulation of molecular chaperones such as DnaK (15, 16, 17). To determine whether ethanol concentrations between 1-5% could induce a detectable heat-shock response in *E. coli* BL21(DE3) relative to baseline (0% ethanol concentrations), we probed for DnaK expression in whole cell lysates extracted from *E.coli* BL21(DE3) transformed with pM3CRYY grown in 1-5% ethanol concentrations. Whole cell lysates were run on an SDS-PAGE gel, and proteins were transferred onto a nitrocellulose membrane. DnaK was detected by staining with an anti-DnaK primary antibody solution. Soluble fractions of the whole cell lysates extracted from all conditions produced bright bands corresponding to DnaK at ~65 kDa (Fig. 4A). Interestingly, bands corresponding to EtOH treatment at 1% and 3% showed decreased DnaK yield when compared to the 0% conditions.

After subtracting the background signal per sample to normalize for pixel densitometry (ImageJ), 1% and 3% EtOH showed similar DnaK expression to 0% (n=2). However, the 5% EtOH condition (n=2) showed higher DnaK expression relative to the 0% condition (Fig. 4B).

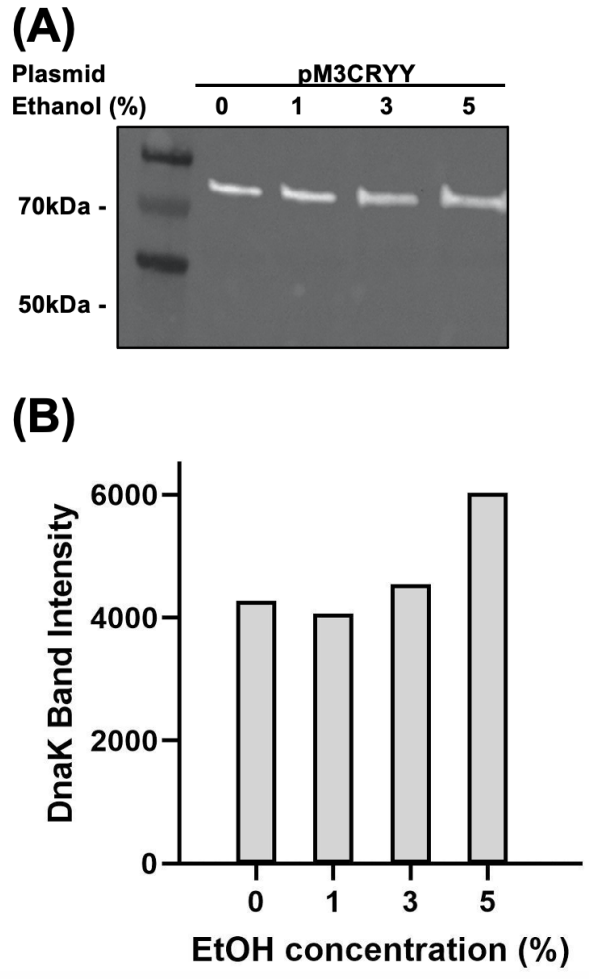

FIG. 4 Induction of heat-shock response using EtOH did not elicit differential DnaK expression using western blot. A) Representative western blot staining for DnaK protein in whole cell lysates extracted from IPTG-induced *E. coli* BL21(DE3) cells transformed with pM3CRYY. Cells were treated with 0.1mM IPTG and concentrations of ethanol ranging from 0-5% for 24 hours (37°C, shaking at 200 rpm). Loading was controlled according to OD600 readings from each sample following incubation. Lysates were separated on a 4–20% stain-free gel, transferred to nitrocellulose membrane and blotted with an antibody specific for DnaK. Ethanol concentrations are indicated above. (B) ImageJ quantification of Figure 4A protein bands. Background signal from each lane was subtracted from the band density quantification to normalize each band to the background. Band densities were averaged between samples, n=2.

DISCUSSION

Previous literature suggests that supplementation of *E. coli* growth media with ethanol can enhance protein yield. This effect can be attributed, in part, to the chaperone-mediated prevention of protein misfolding and aggregation (14, 17). In this study, we sought to demonstrate the effectiveness of ethanol supplementation in increasing recombinant protein production using P. aeruginosa rchiC expression in *E. coli* BL21(DE3) as a model system.

First, we found that 1-5% ethanol negatively affects the growth parameters of pM3CRYY transformed *E. coli* BL21(DE3) by reducing maximum cell culture density (Fig. 2) and delaying entry into log phase (Fig. 1). Then, we treated *E. coli* BL21(DE3) cells with ethanol for 24 hours and collected whole cell lysates to evaluate rChiC yield through SDS-PAGE analysis.

Interestingly, we only observed a darker band in the 1% ethanol condition, which we confirmed with ImageJ pixel densitometry. Furthermore, we investigated if rChiC yield was correlated to the heat shock response, as modeled by DnaK expression. Our results revealed that only 5% ethanol concentrations resulted in increased DnaK expression relative to the 0% ethanol concentration (positive control). Unexpectedly, pET-28a transformed *E. coli* BL21(DE3) showed greatest DnaK expression in whole cell lysates. Altogether, our results suggest that 1% ethanol concentrations support greatest rChiC yields relative to no supplementation. Although DnaK expression in whole cell lysates was not increased at 1%

ethanol concentrations, we observed increased DnaK expression at the 5% ethanol concentration.

1-5% ethanol inhibits *E. coli* BL21(DE3) growth parameters. Alcohols have long been described as toxic by-products of bacterial fermentation pathways when accumulated to high levels (18, 19). Ethanol-induced stress is hypothesized to occur through multiple mechanisms including damage to bacterial cell walls and membrane integrity, ultimately leading to reactive oxygen species production, hypoxia, and a diminished proton gradient (18). These changes drive cells to rely on fatty acid oxidation, anaerobic respiration, and fermentation pathways, which consequently inhibit cellular growth by impeding ATP generation. However, studies have indicated that ethanol concentrations up to 5% do not significantly alter *E. coli* growth in laboratory strains such as BL21(DE3), DH5α, Top10, and JM109 (20). As the capacity to support high-density cultures is a crucial determinant of recombinant protein yield in upscaled microbial systems (5), the impact of ethanol on *E. coli* growth is a significant practical consideration for its use as an enhancer for recombinant protein production. As such, our first experimental aim sought to characterize the growth curves of *E. coli* BL21(DE3) at titrating ethanol concentrations, ranging from 0% to 5%.

Interestingly, our results indicate that ethanol concentrations ranging from 1% to 5% negatively impact BL21(DE3) *E. coli* growth parameters during the 0- to 18-hour post-incubation time frame. These results suggest that using incubation growth media with ethanol for durations within the 0- to 18-hour timeframe, has a negative trade-off due to decreased culture densities. However, there were no significant differences in BL21(DE3) *E. coli* growth parameters observed at the 24-hour time point across ethanol concentrations ranging from 0% to 3%. Our findings show that using growth media supplementation with 1% to 3% ethanol in conjunction with 24-hour incubation periods can optimize recombinant protein production. This proposed approach we predict will lead to comparable culture densities supported with that of untreated cultures. Future studies would benefit from increasing the number of biological replicates used for this analysis to strengthen the reliability of these findings. Furthermore, we recommend that future studies replicate these results using larger culture volumes, as this approach would better model the *E. coli* growth kinetics observed in high-cell density cultures commonly utilized in industrial recombinant protein production (9, 10).

1% ethanol conditions increase rChiC yield. Many previous studies have had success increasing both recombinant and native protein yield with 3% ethanol (Chhetri, Kusano & Thomas). Conversely, our results revealed greater rChiC yield in 1% ethanol conditions, and no major differences between pM3CRYY transformed *E. coli* BL21(DE3) cells grown in 0%, 3% and 5% ethanol (**Fig. 3**). This suggests that only a 1% ethanol concentration increases rChiC yield. However, the mechanism underlying this observation is not correlated with DnaK expression and remains to be elucidated. One potential explanation for discrepancies between our results and prior literature is a difference in induction duration and procedure. We induced pM3PCRYY transformed *E. coli* BL21(DE3) cells at OD_{600} = 0.4 with IPTG and ethanol for 24 hours. Upon harvesting after this 24 hour period, OD_{600} was > 2.0, indicating cells were in the death phase, which decreases cell viability. This is comparable to results reported by Choi et al., who found that rChiC yield was higher after harvesting at 6 hours post-induction (with 0.1 mM IPTG) compared to 24 hours post-induction (21). Choi et al. postulated that rChiC yield after 24 hours was lower compared to 6 hours despite longer incubation due to lower cell viability and nutrient depletion (21). However, considering that our growth curve analyses in 96-well plate cultures showed that a 24-hour incubation period did not lead to a reduction in the maximum culture densities supported, we chose to collect cell lysates following the 24-hour incubation period. Nevertheless, the findings presented by Choi et al. offer a strategy for future investigations to replicate our findings while avoiding cell entry into the death phase by collecting cells at earlier time points. Future research will need to confirm this hypothesis and determine the optimal harvesting time and ethanol concentration for rChiC yield.

Another potential reason accounting for why only 1% ethanol increased rChiC protein yield while 3 and 5% ethanol did not, is the possibility that ethanol-induced heat-shock response had no effect on rChiC expression. This is supported by literature determining that only some recombinant proteins increase with ethanol induction (14). In this study, it was

observed that when ethanol was administered to growth media, P450scc expression increased, while there was no change in P450c27 (14).

A heat-shock response was observed in 5% ethanol conditions as modeled by DnaK expression. DnaK is a heat-shock associated molecular chaperone in *E. coli* which functions by supporting proper protein folding or by targeting damaged proteins for degradation (22). In this study, we used DnaK as a surrogate measure for a heat shock response. Our results suggest that ethanol concentrations below 5% does not increase DnaK expression in pM3CRYY transformed *E. coli* BL21(DE3) range of 0% to 5% ethanol concentrations (**Fig. 5**). These results are contradictory to previous literature, which claim that E. coli growth under ethanol concentrations ranging from 1% to 10% are sufficient to induce a heat shock response which improves both recombinant and native protein yield (14, 15, 17).

The low levels of DnaK expression in the ethanol-treated conditions could be due to poor experimental design. As aforementioned, we grew all pM3CRYY transformed *E. coli* BL21(DE3) cells to OD_{600} = 0.4 in the same flask before separating to grow in 0, 1, 3 and 5% ethanol. The stock flask was left on ice momentarily to prevent exponential cell growth beyond the log-phase, leading to a potential confounding cold shock effect (23). Unfortunately, previous literature has shown that a cold shock response can block high temperature induction of heat shock proteins (24). Moreover, our negative control (pET-28 transformed *E. coli*) was put on ice for a substantially shorter amount of time, as we were made aware of our possible introduction of a confounding variable; future experiments repeating similar methods should be cautious to not stress cells with a sudden cold temperature change.

Given that the greatest DnaK levels were found in lysates from cells grown in the highest ethanol concentrations tested (5%), we expected that increased DnaK expression would correlate with increased rChiC yield. A plausible explanation for why our results conflicted with this model, may be due to our low concentrations of IPTG used during recombinant protein induction (0.1 mM). Although previous papers have recommended that 0.1 mM IPTG should be used to achieve high levels of rChiC yield in the soluble fractions of *E. coli* cell lysates, we predict that 0.1 mM IPTG is not sufficient to drive protein expression to saturating concentrations. We hypothesize that IPTG induction at greater concentrations would lead to a buildup of insoluble proteins due to misfolding or aggregation events, which would otherwise not be detectable in the soluble fractions of cell lysates from previous studies.

Since our model predicts that heat-shock chaperone proteins support the folding of insoluble proteins, it would be beneficial to saturate rChiC expression, regardless of potential higher incidences of misfolded rChiC. Moreover, a recent paper found that DnaK expression is dependent on host protein synthesis rates and stability (25). This suggests that a higher rate of rChiC synthesis in conjunction with poor rChiC stability would upregulate DnaK expression. The paper could also explain why we only saw increased DnaK in 5% ethanol, as a higher ethanol concentration could destabilize proteins which induces more DnaK expression (25, 26).

Whereas past papers sought to maximize soluble rChiC to better model industrial high-density cultures, we aimed to supplement ethanol in culture to saturating rChiC levels, taking into account both soluble and insoluble fractions. We anticipate that highest chaperone protein activity will be observed under these saturating conditions.

While DnaK expression increased in 5% ethanol conditions, we did not see corresponding rChiC increase. Misfolded rChiC may lack internal hydrophobic residues which DnaK preferentially binds to, or conversely, is rich in negatively charged peptides, resulting in poor affinity to DnaK (27). However, we cannot completely rule out the possibility of DnaK being able to bind rChiC, and it remains unclear if other heat-shock related chaperones can bind rChiC. Therefore, future experiments should perform *in vitro* assays testing the binding of DnaK and other chaperones to rChiC.

Interestingly, we observed the greatest levels of DnaK expression in pET-28a transformed *E. coli* BL21(DE3), which does not express rChiC (Fig. 5). This observation suggests that the rChiC insert had a negative impact on DnaK expression, potentially by siphoning molecular resources to rChiC protein production as opposed to DnaK.

Conclusions In conclusion, 1-5% ethanol induction lowered pM3CRYY transformed *E. coli* BL21 (DE3) growth parameters and only increased rChiC (from *P. aeruginosa* PAO1) yield when grown in 1% ethanol. Additionally, only 5% ethanol slightly increased DnaK expression, a surrogate measure for an *E. coli* BL21(DE3) heat shock response. Overall, while the exact mechanism of increased rChiC yield in 1% ethanol growth condition remains to be elucidated, it demonstrates potential as a cost-effective method of increasing rChiC yield and more generally, an effective method of increasing recombinant protein yield. Future studies can explore if rChiC functionality is not reduced by ethanol, which can lead to useful applications of rChiC as a bioinsecticide against various agricultural pests, like *Helicoverpa armigera* and *Holotrichia parallela* (28, 29).

Future Directions Our findings demonstrate the utility of using 1% ethanol supplementation to enhance recombinant protein yield. However, more research is needed to elucidate the precise mechanisms underlying the heat shock response that contribute to increasing recombinant protein yield. In the short term, we suggest conducting experiments which confirm that ethanol does not negatively impact chitinase functionality, as previous studies have shown that ethanol treatment decreases the enzymatic activity of periplasmic proteins such as AP (11, 30). To do this, an in vitro pull-down assay using chitin resin beads can be performed to verify the chitin-binding capacity rChiC under ethanol-treated conditions. Additionally, we suggest using β-actin or GAPDH as a loading control for western blots to better control for protein loading. Specific staining for a housekeeping protein to control for protein loading would better adjust for total protein loading through semi-quantification with ImageJ when compared to strategies such as adjusting loading volumes based on OD600 readings at the time of protein extraction (31). This would allow more confident and quantifiable conclusions to be drawn regarding increased or decreased rChiC and DnaK expression as a function of ethanol treatment. Although we found that only 5% ethanol induces detectable increases in DnaK expression, future investigations should explore alternative heat shock proteins as a readout for heat-shock response induction, as the literature describes chaperone proteins such as GroEL and GroES as canonical markers of the heat-shock response (30).

We also recommend that future studies utilize 1.0 mM IPTG concentrations as many similar studies cited in the literature use 1mM IPTG when evaluating the effects of ethanol stress on recombinant protein production. Specifically, we predict that this higher limit of IPTG induction would drive rChiC expression to saturating concentrations where protein misfolding and aggregation becomes prevalent (32). As the ethanol induced heat shock response has been documented to upregulate chaperone proteins which support protein folding under these saturating conditions, we hypothesize that the greatest effect size for chaperone-mediated protein production enhancement will be observed under saturating protein concentrations (14, 33).

Furthermore, we recommend performing a more granular characterization of E. coli BL21(DE3) growth characteristics under ethanol-treated conditions, by testing the upper limits of E. coli growth. Such results would better inform future experimental design for experiments that seek to balance recombinant protein yield, with the toxic effects on growth parameters in E. coli.

Lastly, in our current experiment, we preserved sterile conditions by covering Erlenmeyer flasks containing viable cells with foil. While this prevents contamination, the gas exchange and aeration supported by this strategy is suboptimal. We recommend that future experiments should allocate resources to obtain baffled flasks and coverings with porous membranes or sterile cotton plugs which would better facilitate the optimized culture aeration conditions utilized in industrial high-density cultures (9, 10, 34, 35). As our ultimate goal was to optimize rChiC expression for industry applications, we recommend that future studies reproduce these data in larger culture volumes, as such conditions would better model high-cell density cultures used in industrial recombinant protein production (9, 10).

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CONTRIBUTIONS

Laboratory Work. The team contributed equally to the experimental design and derived protocols. LZ took the lead on bacterial culture growth and ethanol induction. AF took the lead on the growth curve and subsequent analysis. LU took the lead on the SDS-PAGE. FL took the lead on the Western Blot.

Manuscript. LZ wrote the abstract, discussion, and future directions, and edited all parts of the manuscript. LU wrote the abstract, introduction, and results, and edited all parts of the manuscript. AF wrote the abstract, methods, results and discussion, and edited all parts of the manuscript. FL wrote the abstract, introduction, methods, discussion, and edited all parts of the manuscript. AF and LU generated figures and corresponding figure legends.

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